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Allelic variations of *LcyE* and *crtRB1* genes in tropical adapted yellow endosperm maize inbred lines

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Abstract

Maize is an important staple cereal crop in sub-Saharan Africa. Tropical-adapted yellow endosperm maize varieties in Africa have low amounts of pro-vitamin A. Use of molecular markers that detect alleles associated with high level of pro-vitamin A can help to improve the pro-vitamin A content in maize through conventional breeding. In this study polymerase chain reaction (PCR) based markers were used to characterize two sets of tropical-adapted yellow endosperm maize inbred lines for allelic variants, present in two carotenoid biosynthesis genes encoding lycopene epsilon cyclase (*LcyE*) and -carotene hydroxylase 1 (*crtRB1*). Frequency of the favourable allele at *LcyE* 3'TE polymorphism is 29% in Set 1 and 67% in Set 2, and the 5TE was present at frequencies of 24% in S1 and 3.3% in 2. The frequency of the favourable allele at the *LcyE* SNP216 polymorphism was more than 95% in both the sets of inbred lines. None of the lines included in Set 1 had favourable alleles for *crtRB1*. On the other hand, in Set 2 the favourable allele in the *crtRB1* 5TE insertion, had a frequency of 14% whereas the favourable allele-frequency was 11% for *crtRB1* 3TE and was 1% for InDel4. Twelve maize inbred lines (9.8%) had favourable alleles for *crtRB1* 3'TE and 5TE in Set 2. None of the maize inbred lines carried favourable alleles for all three *crtRB1* polymorphisms. Favourable haplotypes of *LcyE* and *crtRB1* were found together in fifteen (12%) inbred lines.

Key words: Maize inbred lines, allele specific markers, -carotene, pro-vitamin A

Introduction

Vitamin A is an important micronutrient required for maintaining human health. It can be ingested in diets in the form of pro-vitamin A carotenoids including -carotene, -cryptoxanthin or -carotene. These carotenoids are present at very low concentrations in major staple food crops including maize. Millions of people that depend on maize as a staple food suffer from vitamin A deficiency (VAD), which constitutes a major public health problem affecting 100 million children [1]. VAD leads to night blindness and depressed immune system, which increases susceptibility to childhood diseases including measles and diarrhoea or death [2-4]. About 6.2 million women are also affected by xerophthalmia [5]. Increasing the concentrations of pro-vitamin A in maize through breeding may contribute to the reduction of VAD among people consuming maize in sub-Saharan Africa. Therefore, -carotene content can be considered as a quality trait that can be improved through breeding [6].

Carotenoids in maize are produced through carotenoid biosynthesis pathway. Many genes of the carotenoid biosynthesis pathway in maize have been mapped, cloned and characterized [7-9]. These genes are directly involved in the endosperm color variation of the maize grain encoding various enzymes including phytoene synthase (PSY), 6.01 bin [7], phytoene desaturase (PDS), 1.02 bin [8], zeta carotene desaturase (ZDS), 7.02 bin [9] and lycopene beta cyclase (*LcyB*), 5.04 bin [10]. The lycopene epsilon cyclase (*LcyE* locus, 8 bin 5 has recently been cloned and found to be responsible for variation in concentrations of precursors of vitamin A in maize grains [11]. In other genetic and transcript profiling studies, -carotene hydroxylase 1 gene (*crtRB1*) was found to have significant and large effect on -carotene accumulation in the endosperm [12, 13].

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Further, characterisation identified favourable and unfavourable alleles of the genes involved in observed differences in ß-carotene content. *lcyE* has four principal functional polymorphic sites that can affect the two branches of the carotenoid biosynthesis [11]. Rare genetic variations were also found in *crtRB1* (also known as *HYD3*) that affect accumulation of ß-carotene [12]. Three polymorphic sites of *crtRB1* that significantly affect the conversion of ß-carotene to zeaxanthin have also been identified. Alleles in the same gene (*crtRB1*) were found to have significant effect on ß-carotene content in maize endosperm [12, 13]. Both *LcyE* and *crtRB1* have been fully sequenced using selected maize inbred lines, which led to detection of allelic differences between inbred lines due to single nucleotide polymorphisms (SNPs) and insertions/deletions (inDels) [14]. Polymerase Chain Reaction (PCR) marker sets have been designed to amplify and score SNPs and INS/DEL in *LcyE* and *crtRB1* in maize inbred lines [11-13].

These markers can be used for marker assisted selection or introgression to develop maize inbred lines with favourable *LcyE* and *crtRB1* alleles. The potential usefulness of these markers to identify inbred lines with favourable alleles need to be validated using early or adequate generation lines derived from crosses with diverse genetic backgrounds that are available in a breeding program. This study was therefore, conducted to assess the presence of the key allelic variants of *LcyE* and *crtRB1* using gene specific PCR-based markers in tropical-adapted yellow endosperm maize inbred lines with varying levels of carotenoids.

Materials and methods

Genetic materials

Two sets of yellow endosperm maize inbred lines with varying carotenoid content developed at the International Institute of Tropical Agriculture (IITA) were taken for the study. The first set (S1) comprised 38 inbred lines selected from existing tropical-adapted yellow endosperm maize inbred lines (Table 1). The second set (S2) consisted of 122 new maize inbred lines derived from different bi-parental crosses and backcrosses involving some of the inbred lines included in the first set as parents and temperate inbred lines as sources of high pro-vitamin A. The founder inbred lines chosen in the generation of S2 genotypes were A619, NC298, KVI3, DE3, NC298, SC55, NC350, NC358, M162W, NC350 and NC354. The two sets of inbred lines were grown in two independent nurseries

Table 1. Pedigree for the 38 yellow endosperm tropical-adapted maize inbred lines

Inbred	Pedigree
PVL01	9450xKI 21-7-3-1-1-1-B-B-B
PVL02	9450xKI 21-7-3-1-1-3-B-B-B
PVL03	9450xKI 21-7-3-1-2-4-B-B-B
PVL04	9450xKI 21-7-3-1-2-5-B-B-B
PVL05	(9450xCM 116x9450)-5-2-2-2-B-B-B
PVL06	(9450 x KI 28)-1-2-1-2-B-B-B
PVL07	9450xKI 21-7-2-1-1-B-B-B
PVL08	9450xKI 21-7-2-1-2-B-B-B
PVL09	4001 x B73LPA x 4001-33-2-1-B-B-B
PVL10	1368 x GT-MAS-Gk-10-3-1-2-B-B-B
PVL11	(9450xCM 116x9450)-3-3-1-2-1-B-B-B-B
PVL12	9450xKI 21-3-2-2-1-3-B-B-B-B
PVL13	9450xKI 21-1-5-3-2-2-B-B-B-B
PVL14	9450xKI 21-1-5-3-2-1-B-B-B-B
PVL15	SYN-Y-STR-34-1-1-1-1-2-1-B-B-B-B-B-B-B-B
PVL16	9450xKI 21-1-4-1-1-2-B-B-B-B
PVL17	9450xKI 21-5-2-3-1-B-B-B
PVL18	ACR97TZL-CCOMP1-Y-S3-12-2-B-B-B-B-B
PVL19	ACR97TZL-CCOMP1-Y-S3-33-6-B-B-B-B-B
PVL20	ACR97TZL-CCOMP1-Y-S3-40-3-B-B-B-B-B
PVL21	KU1414-SR/NC350-4-1-B-B-B
PVL22	KU1414-SR/NC350-1-1-B-B-B
PVL23	(9450 x KI 28)-1-2-1-1-B-B-B-B
PVL24	KU1414-SR/KVI43-6-4-B-B-B
PVL25	KU1414-SR/KVI43-6-1-B-B-B
PVL26	KU1414-SR/KVI11-7-2-B-B-B
PVL27	KU1414-SR/KVI11-7-1-B-B-B
PVL28	(9450xCM 116x9450)-5-1-3-3-1-B-B-B-B
PVL29	9450xKI 21-4-2-3-1-1-B-B-B-B
PVL30	Taraba-14-2-2-4-2-B-B-B-B-B
PVL31	Z.Diplo.BC4-467-4-1-2-1-1-B-1-B-B-B-B-B-B-B
PVL32	TZE-COMP5-Y-C7-S3-61-B-B-B-B-B-B-B
PVL33	(9450x KI 21)-8-2-1-1-B-B-B
PVL34	(9450 x KI 28)-5-1-2-1-1-B-B-B
PVL35	9450xKI 21-7-2-2-1-1-B-B-B
PVL36	9450xKI 21-7-2-4-2-1-B-B-B
PVL37	9450
PVL38	KU1414-SR

at Ibadan in single rows of 5 m length spaced 0.75 m apart with 0.25 m spacing between plants within a row. Two seeds were planted in each hole and later thinned to one plant per hill after emergence. Fertilizer was applied at the rate of 60 kg/ha N, 60 kg/ha P, and 60 kg/ha K two weeks after planting. An additional 60 kg/ha N was applied four weeks after planting. The nurseries were kept weed free using pre-emergence and post-emergence herbicides complemented with manual weeding. All plants in each line were self-pollinated and harvested after maturity.

Molecular Characterization at LcyE and crtRB1

Maize inbred lines were characterized for the three important *lcyE* functional polymorphisms: the single nucleotide polymorphism (SNP) 216, the 5′ transposable element (TE) and the 3' Untranslated Region (UTR) InDel identified by Harjes *et al*. [11]. The inbred lines were subsequently characterized for allelic variants of crtRB1: 5′TE, the 3′TE and InDel4 identified by Yan *et al*. [13]. Additionally, three different crtRB1 variants (denoted as A, B and C alleles) found in a ~ 40 bp region adjacent to the transcript start site identified by Vallabhaneni *et al*. [12]. The 4-primer cocktail for LcyE 5′TE and 3′TE failed to amplify. Amplification was obtained when primers were used in pairs (uniplex) in these situations (Table 2). Similarly, the multiplex PCR assay for some amplification of *crtRB1*/*HYD3* alleles could not amplify but worked for some (Table 3). Primer information obtained from published supporting online material was synthesized by Integrated DNA Technologies Leuven, Belgium (IDT) Primer Company.

DNA was extracted from fresh leaf tissue of 5-6 seedlings of each line using the modified method of Dellaporta [15]. The quality and quantity of DNA was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and agarose gel electrophoresis. PCR conditions and cycling profile for amplification of *LcyE* and *crtRB1* polymorphic sites followed that of [11-13]. Primer and $MgCl_2$ concentrations were adjusted for some primer pairs to produce clear-cut PCR products under the PCR conditions used. PCR products were separated by electrophoresis for 4 h in a 2% agarose gel run in 0.5X TBE buffer. Gels were later stained with ethidium bromide, followed by distaining in deionized water and exposed to ultra violet light (UV) to visualize DNA amplicons. Photographs were taken using a gel documentation system (GDS-8000 System UVP Bioimaging System, UVP Upland CA 91786, USA). Size of each amplicon was estimated using a 50 bp DNA ladder. Scoring of amplicons was based on the allele sizes and classes (haplotypes) of target polymorphisms for each maize inbred line in each set.

Analysis of frequency of favourable and unfavourable alleles detected for each class of marker or primer pairs was computed.

Table 2. PCR Primers used for genotyping polymorphisms in Lycopene Epsilon Cyclase (*LcyE*) gene and expected allele size (Source, Harjes *et al*. [11])

Primer sets	Sequence 5′ to 3′	Expected allele size (bp)	*Plex condition used
LcyE-*Exn-216*			
S216-L1 S216-R1	F:GCGGCAGTGGGCGTGGAT R:TGAAGTACGGCTGCAGGACAACG	G (best), T (worst), Null	Uniplex
LcyE 3′ InDel			
3pINDL-L1 3pINDL-R1 3pINDL-L2 3pINDL-R2	F:GTACGTCGTTCATCTCCCGTACCC R:CTTGGTGAACGCATTTCTGTTGG F:GGACCGGAACAGCCAACTG R:GGCGAAATGGGTACGGCC	993 (best), 150 + 280 (2nd best) 250 (3rd best), 250 + 380 (worst)	Uniplex
LcyE 5′ InDel /TE			
TE103PF F-1 TE103PR R-1 TE105PR R-1 ZGT111204-976R(1) F1	F:CGCTAGCAAGCCCATTATTTTTA R:CGGTATGGTTTTTGGTATACGG F:GAGAGGGAGACGACGAGACAC R:AGCATCCGACCAAAATAACAGA	144 + 502 (best), 399 + 502 (worst), Null	Uniplex

InDel/TE Insertion deletion/Transposable element, Exn Exon, SNP single nucleotide polymorphism, *Plex condition; used in this paper. β-carotene is considered favourable allele (best) and allele not associated with higher β-carotene is considered unfavourable allele (worst) in previous study. Favourable allele is denoted in bold. Expected allele sizes were reported in previous study and observed allele sizes were amplified in this study

Table 3. PCR primers used for genotyping polymorphisms in ß-carotene hydroxylase 1 *CrtRB1* (HYD3) and expected allele size (Source: [12, 13])

Primer sets	Sequence 5′ to 3′	Expected allele size (bp)	*Plex condition used
*CrtRB1*5'TE			
*CrtRB1*H1UF	F:TTAGAGCCTCGACCCTCTGTG	600 (best), 400 (2nd best),	Uniplex
*CrtRB1*H1UR	R:AATCCCTTTCCATGTTACGC	800 (worst)	
*CrtRB1*D4			
*CrtRB1*D4F	F:ACCGTCACGTGCTTCGTGCC	129 (best)	Uniplex
*CrtRB1*D4R	R:CTTCCGCGCCTCCTTCTC	117 (worst)	
CrtRB13′ InDel /TE			
*CrtRB1*65F	F:ACACCACATGGACAAGTTCG	543 (best), 296 +875 (worst)	Multiplex
*CrtRB1*62R	R:ACACTCTGGCCCATGAACAC	296 + 1221 + 1800 (2nd best)	
*CrtRB1*66R	R:ACAGCAATACAGGGGACCAG		
HYD3-Duplicated TSS			
HYD3(Cc) F	F:GACTTGTGAGCAAGGGGAAG	608 + 473 (best) / 608 + 476 (best)	Uniplex
HYD3 (Cc) R	R:GACGTGACTCCGAGGCTAGA	608 + 163 (worst)	
HYD3(C) F	F:AACACTCCCGCTCCCGCGGCTCG		
HYD3(A) R	R:TTATATGGATAGTTCACATACCTC		
HYD3(B) F	F:AACACTCACGCTCCCGCG		

InDel/TE Insertion deletion/Transposable element, TSS transcript start site, *Plex condition; used in this paper. Allele associated with higher ß-carotene is considered favourable allele (best) and allele not associated with higher ß-carotene is considered unfavourable allele (worst) in previous studies. Favourable allele is denoted in bold. Expected allele sizes were reported in previous study and observed allele sizes were amplified in this study

Results and discussion

Two important candidate genes, lycopene epsilon cyclase (*LcyE*) and ß-carotene hydroxylase 1 (*crtRB1*) encoding enzymes in carotenoid biosynthesis pathway in maize endosperm had large effects on ß-carotene and pro-vitamin A accumulation in a diverse set of yellow maize inbred lines with different genetic backgrounds [11-13, 16]. Gene-specific markers for amplification of allelic variations of the two genes have also been designed from polymorphic regions to facilitate marker assisted selection (MAS) for enhanced pro-vitamin A content in maize [17]. In the present study, we assayed the presence of allelic variations in completely different sets of tropically-adapted yellow endosperm maize inbred lines using those gene-specific markers (Tables 2 and 3).

The markers amplified all expected classes of alleles (favourable and unfavourable alleles) for the three *LcyE* polymorphisms in two sets of inbred lines. SNP-based PCR assay detected the G/T polymorphism allele in the lines analysed. At the 3′TE and 5′TE, PCR products gave expected classes of favourable and unfavourable alleles in the two sets of inbred lines (Table 2). The frequencies of the favourable alleles for the three *LcyE* polymorphisms were variable in the two sets. Frequencies were equal for the best (favorable) allele (G) at the SNP216 polymorphism in the two sets, 97% of the maize inbred lines in S1 and 98% in S2. The unfavourable allele T was present in S1 but absent in S2. The most favourable class (1700 and 150 + 280 bp) at the 5′TE was present at frequencies of 24% in S1 and 3.3% in 2 (Table 4). The two favourable classes (the first and second best classes) occurred in the same lines as they are functionally similar. These two classes collapsed together for many of the haplotype statistical analysis [11]. The seven lines with the favourable allele at the *LcyE*5′TE have total beta carotene (TBC) ranging from 3.07 to 4.58 µg g^{-1} and 2 lines have TBC

from 0.76 to 1.03 µg g^{-1} among S1 inbred lines. All the 4 lines with favourable alleles at the *LcyE*5′TE polymorphism among S2 inbred lines have 3.47-3.96 µg g^{-1} TBC content.

Frequency of the lines containing favourable allele (144 + 502 bp haplotype class) at the 3′TE is 29% in the S1 and 67% in S2. At the *LcyE* 3′TE polymorphism, 11 lines in S1 inbred lines have the TBC varying from 0.60 to 3.77 µg g^{-1}. The inbred lines in S2 having favourable alleles at *LcyE*3′TE polymorphism have the TBC content varied from 2.65 to 8.99 µg g^{-1}. The allelic frequencies for all the unfavourable alleles in both sets of maize inbred lines are shown in Table 4.

The favourable allele G for *LcyE* SNP216 polymorphism was most frequent in lines of both sets. This demonstrates that this marker is not discriminating the inbred lines and might not be useful for MAS for β-carotene and pro-vitamin A in maize. In addition, the lines with high β-carotene and pro-vitamin A may not be due to *LcyE* SNP216. Although line (9450×KI 21-1-4-1-1-2-B-B-B-B) had an unfavourable allele T for *LcyE* SNP216, it had a favourable allele for *LcyE* 5′TE, indicating that this allele may be responsible for the total β-carotene content in the grain of this line as reported by Menkir *et al*. [18]. The inbred lines with promoter transposon element insertion of 5'TE was few in S1 and fewer in S2 while more inbred lines had favourable allele for *LcyE* 3'TE in S2 than in S1. The frequency of *LcyE* 3'TE and *LcyE* 5′TE in tropical adapted maize inbreds in both sets (S1 and S2) may be due to differences in the genetic backgrounds. The presence of allele 144 bp for *LcyE* 3′TE in all the 122 lines indicates that this allele is most likely to be provided by the different exotic lines with high pro-vitamin A used as donors in the breeding of these lines.

In the present study, additional classes of alleles were also amplified among tropical maize inbred lines. Additional classes of alleles; 280 + 250 + 1700 + 150 and 280 bp were present in S2 but not in S1. Null alleles (29 in S1 and 95 in S2) were detected at the 5′TE polymorphism. The additional allelic class at the 3′TE polymorphism, 399 + 502 + 144 bp, were present in S1 and S2 whereas an additional allele class (502 bp) was present only in S1. These additional classes of alleles were not reported by [11]. The results from gel-separated PCR fragments for the three polymorphisms at *LcyE* loci are shown in Figs. 1-4.

Table 4. Comparison of allele frequencies of Lycopene Epsilon Cyclase (*LcyE*) polymorphism in the 38 (Set 1) and 122 (Set 2) tropical adapted yellow endosperm maize inbred lines

Gene polymorphism/ Classes of alleles (bp)	Set 1 No. of lines	Set 2 No. of lines
LcyE 5′InDel/TE		
150 + 280 +1700	9	4
Null	29	95
250 + 380	-	18
280 + 250 + 1700 + 150	-	1
280	-	4
LcyE 3′InDel/TE		
144 + 502	11	82
399 + 502	18	-
502	7	-
399 + 502 + 144	2	40
LcyE -Exn- SNP216		
G	37	120
T	1	-
Null	-	2

InDel/TE Insertion deletion/Transposable element, Exn Exon, SNP single nucleotide polymorphism

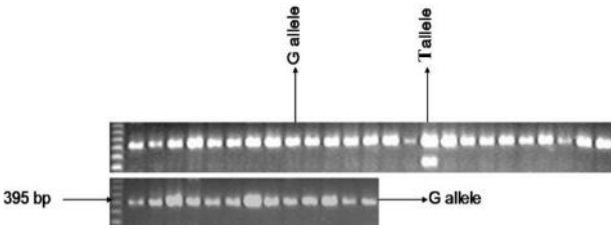

Fig. 1. Amplicons generated by *LcyE* PCR assay SNP216 of 38 tropical yellow maize inbred lines and separated on 2% agarose gel.The first lanes are standard marker. The best allele (G) and worst allele (T) were pointed by arrows

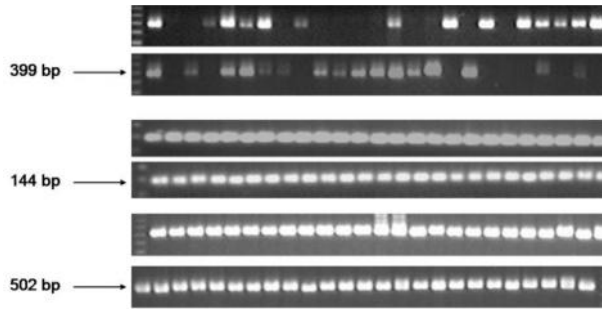

Fig. 2. Amplicons generated by *LcyE* PCR assay 3′ InDel of some 122 tropical yellow maize inbred lines and separated on 2% agarose gel.The first lanes are standard markers.The best allele (144 + 502 bp) and worst allele (399 + 144 + 502 bp) were pointed by arrows

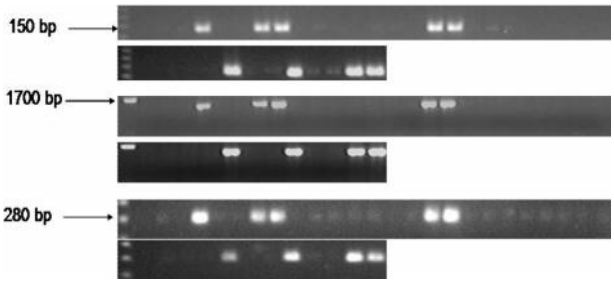

Fig. 3. Amplicons generated by *LcyE* PCR assay 5′InDel of 38 tropical yellow maize inbred lines and separated on 2% agarose gel. The first lanes are standard marker. The best allele (150 + 280 + 1700 bp) was pointed by arrows and worst allele (null)

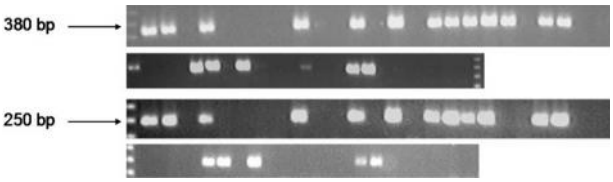

Fig. 4. Amplicons generated by *LcyE* PCR assay 5′InDel of some 122 tropical yellow maize inbred lines and separated on 2% agarose gel. The first lanes are standard marker. The worst allele (250 + 380 bp) was pointed by arrows

β-carotene hydroxylase 1 gene (*crtRB1*) specific to the α-ring and β-ring branches catalyzes the double hydroxylation of α-carotene and β-carotene resulting in the formation of lutein and zeaxanthin, respectively at high concentrations [11, 13]. Diminished hydroxylation of β-carotene has large effect on concentration of β-carotene using favourable *crtRB1* alleles [13]. The PCR assay on the three functional polymorphisms in *crtRB1* enabled the screening of the gene polymorphisms in the lines. At 5′TE, inbred lines with 397 and 206 bp insertions were identified, while lines with no amplicons were also observed in some S2 inbred lines. The three alleles identified by [13], 543, 296 + 875 and 296 + 1221 + 1800 bp were amplified at the 3'TE polymorphism. For InDel4 polymorphism, the expected allele classes were amplified in this study (Table 4).

For *crtRB1* 5′TE, 3′TE and InDel4 polymorphisms, the lines with favourable allele were found in S2. At the *crtRB1*5′TE polymorphism favourable allele (600 bp) was found in 17 inbreds from S2 and none for S1 (Table 5). Twenty eight inbreds in S2 have null alleles, while we also detected unfavourable allele in both sets, 65 inbreds in S2 and in all inbreds in S1 at the *crtRB1* 5′TE polymorphism (Table 5). The number of favourable alleles detected for *crtRB13′*TE polymorphism was 14 for S2 and none for S1 (Table 4). The PCR assay at indel4 polymorphism among the inbred lines contained favourable allele, one in S2 and none for S1 (Table 5). The 17 inbred lines in S2 with favourable allele at *crtRB1*5′TE had high TBC (4.54-9.09 μg g^{-1}) concentration. The 14 lines possessing favourable allele at *crtRB13′TE* polymorphism had high TBC ranging from 4.92 to 9.09 μg g^{-1}. The only one line with *crtRB1* indel4 favourable allele has TBC of 8.67 μg g^{-1}. P1 and P2 primer pair of HYD3 failed to produce the 608 bp allele in all genotypes. All inbred lines (S1 and S2) had the 168 bp at the transcript which is the unfavourable allele A and no favourable allele of "C" (473 bp) or "B" (476 bp) in the transcript start site (duplicated sequence variant polymorphism).

The occurrence of favourable alleles of the *crtRB1* gene was not high in the two sets of inbred lines supporting the findings of other studies to the effect that genetic variation in *crtRB1* gene is rare [13]. The best allele for all the *crtRB1* polymorphisms was not found among the inbred lines included in S1. The presence of these alleles has been associated with the accumulation of more β-carotene in maize endosperm because they have significant reduction in zeaxanthin [13]. The absence of *crtRB1* functional alleles might be responsible for the low accumulation of β-carotene in maize lines adapted to the tropics. There is absence of favourable allele for *HYD3* (*crtRB1*) promoter variation (B, C) among the lines analysed in this study.

In this study the additional allele class, 600 + 800 bp was present in S2 lines but not in S1 at the 5′TE polymorphism. Furthermore, high allelic variation was observed at the 3′TE, as seven different classes of amplicon patterns were found in addition to the expected classes at this polymorphic site in S2 inbred lines in our study. The highly variable allele classes (296 + 1221, 543 + 296, 296 + 875 + 1221, 543 + 296 + 1221, 543 + 1221, 296 bp) were found among the S2 inbred lines and 296 + 1800 in S1 at the 3′TE in this study (Table 5). Although, the occurrence of some of the additional classes (543 + 296, 296 + 875 + 1221, 543 + 296 + 1221, 543 + 1221, 296 bp) at 3′TE polymorphism is low. This may be due to the genetic recombination in the *crtRB1* gene in maize inbreds where they were found.

Table 5. Comparison of allele frequencies of β-carotene hydroxylase 1 *CrtRB1* (*HYD3*) polymorphism in the 38 (Set 1) and 122 (Set 2) tropical adapted yellow endosperm maize inbred lines

Gene polymorphism/ Classes of alleles (bp)	Set 1 No. of lines	Set 2 No. of lines
*CrtRB*15′ InDel /TE		
800	38	75
600	-	17
Null	-	28
800 + 600	-	2
CrtRB13′ InDel /TE		
543	-	14
296 + 1221 + 1800	-	33
296 + 1221	-	40
296 + 1800	38	43
543 + 296	-	6
296 + 875 + 1221	-	1
543 + 296 + 1221	-	4
543 + 1221	-	1
296	-	19
*CrtRB1*D4		
129	-	1
117	38	121
HYD3*TSS*		
163	38	102
163 + 608	-	20
473/476 + 608	-	-

InDel/TE Insertion deletion/Transposable element, D4 deletion 4,TSS transcript start site

The results from gel-separated PCR fragments for the three polymorphisms at HYDB1/crtRB1 loci are shown in Figs. 5-8.

Favourable haplotypes of *LcyE* and *crtRB1* occurred together in 15 inbred lines included in the present study (Table 6). The 5 inbred lines having favourable alleles at LcyE (SNP216, 3′TE) and *crtRB1* (3′TE, 5′TE) polymorphisms had TBC varying from 5.52 to 8.99 μg g^{-1}, while the 7 maize inbred lines

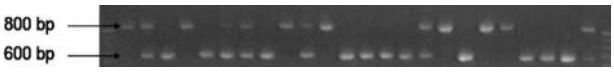

Fig. 5. Amplicons generated by *HYDB1/crtRB1* PCR assay 5'InDel of some 122 tropical yellow maize inbred lines and separated on 2% agarose gel. The first lane is standard marker. The top amplicons are worst allele (800 bp) and bottom amplicons are best allele (600 bp)

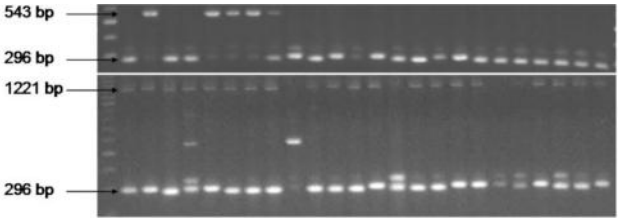

Fig. 6. Amplicons generated by *HYDB1/crtRB1* PCR assay 3′TE/InDel some of 122 tropical yellow maize inbred lines and separated on 2% agarose gel. The first lanes are standard marker. The top amplicons are best allele (543 bp), worst allele (296 bp) and bottom amplicons are (296 + 1221 bp)

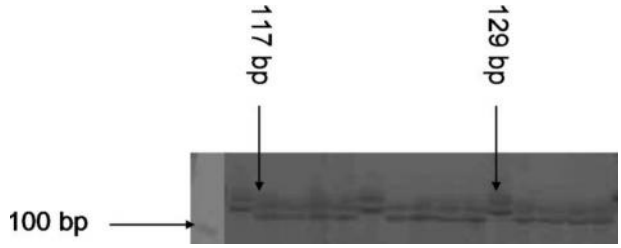

Fig. 7. Amplicons generated by *HYDB1*/*crtRB1*Del4 PCR assay some of 122 tropical yellow maize inbred lines and separated on 6% denaturing polyacrylamide gel. The first lane is standard marker. The worst allele (117 bp) and best allele (129 bp) were pointed by arrows

Fig. 8. Amplicons generated by *HYD3* PCR assay of some 122 tropical yellow maize inbred lines and separated on 2% agarose gel. The first lanes are standard markers. The worst allele (163 bp) and a common allele (608 bp) were pointed by arrows

possessing the *LcyE* (SNP216) and *crtRB1* (3′TE, 5'TE) favourable alleles had TBC varying from 4.93 to 9.09 μg g^{-1}. Line PVL 105 had favourable alleles at *LcyE* (SNP216) and *crtRB1* (3′TE, indel4) and its TBC was 8.67 μg g^{-1}. The two maize inbred lines combining favourable alleles at the *LcyE* (SNP216, 3′TE) and *crtRB1* (3′TE) polymorphisms have TBC ranging from 6.43-6.99 μg g^{-1}. The combined effects of favourable functional allelic variants at *LcyE* and *crtRB1* can contribute to the accumulation of high β-carotene in the maize inbred lines. Such lines possessing more than one favourable allele are desirable base materials for breeding. The *crtRB1* polymorphic sites may

Table 6. Maize inbreds with *LCYE* and *crtRB1* haplotypes for favourable alleles in set 2

Inbred lines
(KU1409/KU1414-SR/A619)-S2-2-B-B, (KU1409/DE3/KU1409) S2-7-B-B-B, (KU1409/DE3/KU1409)S2-15-B-B-B, (KU1409/DE3/ KU1409)S2-35-B-B-B, (KU1409/DE3/KU1409)S2-2-B-B-B, (KU1409/DE3/KU1409)S2-31-B-B-B, (KU1409/DE3/KU1409)S2-4-B-B-B, (KU1409/DE3/KU1409)S2-11-B-B-B, (KU1409/DE3/ KU1409)S2-13-B-B-B, (KU1409/DE3/KU1409)S2-14-B-B-B, (KU1409/DE3/KU1409)S2-26-B-B-B, (KU1409/DE3/KU1409)S2-18-B-B-B, KU1409/SC55/KU1409-4-B-B, (KU1409/DE3/ KU1409)S2-18-2-B-B, (KU1409/DE3/KU1409)S2-32-B-B-B

increase ß-carotenes concentration and its effects may be additive with that of *LcyE* [13].

This study also shows that polymerase Chain reaction (PCR) products from uniplex primer pairs had strong and visible bands which allowed for correct characterisation of the the *LcyE* and *crtRB1* polymorphisms identified [11, 13] in the two sets of maize inbred lines for increased pro-vitamin A. In conclusion, the functional markers were effective in distinguishing the inbred lines for naturally occurring allelic variants of the two genes and are appropriate for MAS.

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