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# Comparative Assessment of Effectiveness of Alternative Genotyping Assays for Characterizing Carotenoids Accumulation in Tropical Maize Inbred Lines

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**Abstract:** The development of maize varieties with increased concentration of Provitamin A (PVA) is an effective and affordable strategy to combat vitamin A deficiency in developing nations. However, the considerably high cost of carotene analysis poses a major challenge for maize PVA biofortification, prompting the use of marker-assisted selection. Presently, two types of genotyping with PVA trait-linked functional markers have been developed and extensively used in breeding programs. The two systems are low throughput gel-based genotyping and genotyping with Kompetitive Allele-Specific PCR (KASP) single nucleotide polymorphism (SNPs) markers. Although the KASP SNPs genotyping was developed to replace the gel-based genotyping, studies have not been conducted to compare the effectiveness of the KASP SNPs markers with the gel-based markers. This study was conducted to assess the carotenoid content of 64 tropical PVA biofortified maize inbred lines containing temperate germplasm in their genetic backgrounds and screen them with both gel-based and KASP markers of *PSY1*, *LCYE* and *crtRB1* genes. Many of the 64 inbred lines had PVA concentrations surpassing the 15 μg/g provitamin A breeding target set by the HarvestPlus Challenge Program. Favorable alleles of *crtRB1*, *crtRB1* and the KASP SNPs markers were detected in 25 inbred lines with high PVA concentrations. Inbred lines with the favorable alleles of *LCYE* had the highest concentrations of non-PVA carotenoids, whereas those with the favorable alleles of *crtRB1* had high levels of PVA carotenoids. Data from the sequenced region of *LCYE* revealed one SNP in the first intron that clearly differentiated the high and low β-carotene maize inbred lines. The results of our study demonstrate that the automated KASP SNPs markers can replace the gel-based genotyping for screening a large number of early generation maize inbred lines for PVA content.

**Keywords:** provitamin A; carotenoids; biofortification; marker-assisted selection; tropical maize inbred lines

## 1. Introduction

Vitamin A deficiency (VAD) is a major health concern in sub-Saharan Africa (SSA) and many developing countries. Over 190 million pre-school children and 19 million pregnant women in Africa and South Asia are affected by VAD [1]. In addition, VAD is a risk factor for blindness and mortality from measles and diarrhoea in children aged 6–59 months [2]. The primary sources of vitamin A are animal-based foods, fresh fruits and

vegetables [3] that are not readily available to poor people who constitute 41% of the population in developing countries [4], many of whom are rural families. Increasing the concentration of provitamin A carotenoids (PVA) in staple crops, such as maize, is an affordable and durable solution to the problem of VAD. HarvestPlus has developed an online Biofortification Priority Index (BPI) tool that shows that enriching maize with PVA can reduce the prevalence of VAD in developing nations where maize is consumed as staple crop (www.harvestplus.org/knowledgemarket/BPI, accessed on 9 September 2020).

Considering the loss of up to 50% of carotenoids during storage and processing [5] and the conversion factor of maize β-carotene to retinol [6], HarvestPlus has set a biofortification breeding target of 15 μg/g PVA in maize. Pixley et al. [7] reported maize breeding lines with up to 30 μg/g PVA. However, the most common maize varieties cultivated and consumed globally accumulate less than 2 μg/g PVA [8]. Considerable efforts have been made to increase PVA concentration in maize cultivars grown in SSA with more than 40 PVA varieties released [9]. However, the PVA concentrations in these varieties fall short of the target 15 μg/g. There is, therefore, a need to develop hybrid and synthetic varieties with a higher level of PVA concentrations and desirable agronomic performance.

Maize breeders working on PVA biofortification confronted with the challenge of high cost of carotenoid quantification in maize endosperm. High-performance liquid chromatography (HPLC) can cost up to $100 per sample. Ultra-performance liquid chromatography (UPLC) could be used as an alternative, but this technique is also not affordable given the thousands of samples breeding programs may need to analyse each year. The use of visible yellow to orange kernel color to select genotypes with a high concentration of total carotenoids is limited by the weak correlation with PVA concentration [8]. DNA markers linked to target loci are now affordable and could accurately screen a large number of genotypes in breeding programs.

The PVA carotenoids accumulated in maize are $\alpha$-carotene, β-carotene, and β-cryptoxanthin. Biofortified maize varieties also contain non-provitamin A carotenoids such as lutein and zeaxanthin, which are also beneficial to human health [10]. The carotenoid biosynthesis pathway in maize kernels is well elaborated, and the genes controlling each step have been identified [11]. Harjes et al. [8], Yan et al. [12] and Fu et al. [13] identified three genes (*LCYE crtRB1* and *PSY1)* underlying the critical steps in carotenoid biosynthesis. Gel-based markers associated with both the favorable and unfavorable alleles of the three genes have been developed and their effects on accumulation of PVA and non-PVA carotenoids were validated in tropical maize [14,15]. The markers were linked to insertions/deletions (InDels) and single nucleotide polymorphism (SNPs) in different regions of the genes. Sequence analysis of 3′-untranslated region (UTR) of *crtRB1* [16] and 5′-UTR of *LCYE* gene [17] also detected SNPs and InDels associated with PVA accumulation in maize. Although the gel-based markers have been used for developing maize genotypes with high levels of PVA [18,19], the assay is slow and amenable to genotyping a limited number of samples at a time. Furthermore, it is often difficult to visualize the difference between DNA fragments with very small differences in weight. This may require repeating genotyping several times, resulting in increases in the assay cost and delays in the selection process.

To reduce the cost of genotyping and accelerate the rate of genetic gain in carotenoid concentrations in maize, seven Kompetitive Allele-Specific PCR (KASP) SNPs markers associated with the favorable alleles of *crtRB1* gene on chromosome 10 were developed at the International Maize and Wheat Improvement Center (CIMMYT) to select maize with high PVA [20]. The KASP genotyping is easy to run, accurate and offers flexibility in terms of number of SNPs markers and samples for screening [21]. Though the KASP genotyping assay was developed for replacing the gel-based genotyping to breed maize for increased carotenoids levels, there are no published reports about the effectiveness of the seven *crtRB1* KASP SNPs markers relative to the gel-based markers to screen maize germplasm for PVA content. Obeng-Bio et al. [22] used only one of the seven PVA KASP SNPs markers along with *crtRB1* to characterize PVA content in early maturing maize inbred lines.

Assessing the effectiveness of the seven *crtRB1* KASP SNPs markers relative to the gel-based markers can validate their usefulness for optimizing selection for high PVA carotenoids in maize. This study was therefore conducted to (i) investigate the comparative effectiveness of PVA KASP SNPs markers relative to the gel-based functional markers for selecting lines with high PVA content and (ii) sequence the PCR products of *LCYE 5′TE* and *crtRB1 3′TE* to identify sequence variations separating inbred lines with high and low PVA content.

## 2. Materials and Methods

### *2.1. Plant Materials*

Sixty-four tropical-adapted maize inbred lines with yellow to orange kernel color developed at IITA were used in this study (Table S1). The inbreds were developed from tropical-adapted lines containing temperate germplasm as donors of high levels of β-carotene. The inbred lines were derived from both bi-parental crosses as well as backcrosses involving tropical-adapted inbred lines with intermediate levels of PVA as recurrent parents and exotic lines as donors of high PVA.

### *2.2. Field Evaluation*

The 64 inbred lines were planted at the IITA research field, Ibadan (7°29′11.99″ N, 3°54′2.88″ E, altitude 190 m), Nigeria in 2020. The experimental design was a 16 × 4 alpha-lattice with two replications. Plots consisted of single rows, each 5 m long, with a plant-to-plant spacing of 0.25 m within rows, and 0.75 m distance between rows. One plant was maintained per hill to give a population density of 53,333 plants $ha^{-1}$. The fertilizer NPK 15:15:15 was applied at the rate of 60 kg N $ha^{-1}$, 60 kg P $ha^{-1}$ and 60 kg K $ha^{-1}$ at planting; additional 30 kg N $ha^{-1}$ was applied 4 weeks after planting. Herbicides (Primextra and Gramazone) were used to control weeds as recommended for optimum maize production. All plants in each plot were self-pollinated for the production of kernel samples for carotenoid analysis. Self-pollinated ears in each row were harvested, dried with minimal exposure to direct sunlight, and shelled immediately to minimize loss of carotenoids due to degradation. One hundred kernels were drawn from each sample (replication) after shelling for carotenoid analysis.

### *2.3. Carotenoid Analysis*

The extraction protocol for carotenoid analysis used was the method of Howe and Tanumihardjo [23]. Kernels of each line were finely ground and 0.6 g from each of the two replications was transferred into a 50 mL glass centrifuge tube; 6 mL of ethanol and 0.1% butylated hydroxyl toluene were added into the tube. The tubes were then vortexed for 15 s, and incubated at 85 °C in a water bath for 5 min. Each sample was mixed with 500 µL of 80% potassium hydroxide (*w*/*v*), vortexed for 15 s and again incubated in a water bath at 85 °C for 10 min, with vortexing at intervals of 5 min. Thereafter, each sample was placed on ice and mixed with 3 mL ice cold deionized water, 200 µL internal standard β-Apo-8′-carotenal and 4 mL hexane. After vortexing and centrifugation, the top hexane layer formed was transferred into a new test tube. The hexane extraction was repeated thrice, adding 3 mL hexane each time. A concentrator (Organomation Associates, Inc., Berlin, MA, USA) was used to dry the samples under nitrogen gas. The samples were then reconstituted in 1 mL of 50:50 Methanol:Dichloroethane and vortexed for 10 s. For each sample, 50 µL aliquot of each extract was injected into the HPLC (Water Corporation, Milford, MA, USA) system and run for major carotenoids based on the calibration of the standard of each carotenoid. Carotenoids were separated by a C30 Column (4.6 × 250 mm; 3 µm) eluted by a mobile phase using methanol/water (92: 8 *v*/*v*) as solvent A and 100% Methyl Tertiary Butyl Ether (MTBE) as solvent B. The flow rate of solvent was 1 mL/min, and absorbance was measured at 450 nm for carotenoid detection. Chromatograms were extracted after the runs and major carotenoids were identified.

Total carotenoid (μg $g^{-1}$ dry weight) was calculated as the sum of concentrations of α-carotene, lutein, β-carotene, β-cryptoxanthine and zeaxanthine. Provitamin A was calculated as the sum of β-carotene and half of each of β-cryptoxanthin and α-carotene concentrations [24].

### *2.4. PCR and Gel-Based Genotyping*

Leaf samples were collected from 15 randomly selected plants of each line at 30 days after planting in the field. The samples were freeze-dried and genomic DNA was extracted using modified Cetyl-trimethyl ammonium bromide (CTAB) protocol as described by Azmach et al. [14]. The 64 lines were genotyped with PCR based functional markers of three genes, namely *LCYE*, *crtRB1* and *PSY1*. Primers, PCR conditions and thermal cycling profiles used were described by Harjes et al. [8] for *LCYE*, Yan et al. [12] for *crtRB1* and Fu et al. [13] for *PSY1*. However, primers *crtRB1*-3′TE and *LCYE*-5′TE associated with transposable element (TE) insertions/deletions in the 3′UTR and 5′UTR of *crtRB1* and *LCYE* genes, respectively, were used to amplify the same target regions following the protocols of Babalola et al. [25]. The primers used to amplify *crtRB1*-3′TE marker were forward *CTCACCGAAACTTCTGTAGC* and reverse *AATCCTAGCGATAAGAACAGC*, whereas those used to amplify the *LCYE*-5′TE marker were forward *TAACAGCCGAGCCCAATG* and reverse *CCAAACGGGCAAACTATGTC* [25]. PCR products were resolved using 2% agarose gel. For the markers, *crtRB1*-inDel4 and *LCYE*-3′indel 2% *w*/*v* super fine resolution (SFR) agarose gel was used. The recorded polymorphisms of the three genes are summarized in Table 1.

**Table 1.** Nomenclature of functional DNA markers and their allelic series.

| Gene | Polymorphic Site/Marker Gene Name-Polymorphism) | Nature of Polymorphism | Allelic Series and Notations * |
|---|---|---|---|
| *PSY1* [12] | *PSY*-SNP7 | A-C substitution SNP | **A**, C |
| | *PSY1*-InDel 1 | 378 bp indel | 0, **378** |
| *LCYE* [8] | *LCYE*-5′TE | 285 indel | **1**, 2, 3, **4** |
| | *LCYE*-SNP (216) | G-C SNP | **G**, T |
| | *LCYE*-3′indel | 8 bp indel | 8, **0** |
| *crtRB1* [11] | *crtRB1*-5′TE | 397/206 bp indel | 1, **2**, 3 |
| | *crtRB1*-InDel4 | 12 bp indel | **12**, 0 |
| | *crtRB1*-3′TE | 325/1250 bp indel | **1**, 2, 3 |

* Allelic variants denoted in bold and underlined are the best favourable alleles [14].

### *2.5. KASP Genotyping*

Genomic DNA of the 64 PVA inbred lines was extracted as described for the gel-based genotyping. The DNA samples were diluted to 30 ng/μL as required for KASP genotyping (Table 2). KASP reaction was performed in a 96-well plate in a reaction volume of 10 μL consisting of 5 μL template DNA and 5 μL of the prepared genotyping mix (2× KASP master mix and primer mix). Protocols for the preparation and running of KASP reactions are provided in the KASP manual (https://www.biosearchtech.com/, accessed on 28 September 2020). KASP assay kit was purchased from LGC Genomics (LGC Group). All amplification reactions were performed using the Roche LightCycler 480 II (LC480 II) System (Roche Life Science) at the Bioscience Center of IITA Ibadan, Nigeria. The amplification condition was as follows: 1 cycle of KASP special Taq activation at 94 °C for 15 min, followed by 36 cycles of denaturation at 94 °C for 20 s and annealing and elongation at 60 °C (dropping 0.6 °C per cycle) for 1 min. Endpoint detection of the fluorescence signal was acquired for 1 min at 30 °C using the same instrument.

**Table 2.** *crtRB1* KASP SNPs markers used to genotype the provitamin A Inbred lines.

| SNP ID | Owner | Intertek ID | Trait Category | Chromosome Position | Favorable Allele | Unfavorable Allele |
|---|---|---|---|---|---|---|
| S10_134583972 | CIMMYT | snpZM0013 | PVA | 10 | GG | CC |
| S10_134655704 | CIMMYT | snpZM0014 | PVA | 10 | CC | TT |
| SYN11355 | CIMMYT | snpZM0015 | PVA | 10 | AA | GG |
| PZE-110083653 | CIMMYT | snpZM0016 | PVA | 10 | GG | AA |
| S10_136072513 | CIMMYT | snpZM0017 | PVA | 10 | TT | GG |
| S10_136840485 | CIMMYT | snpZM0018 | PVA | 10 | CC | TT |
| S10_137904716 | CIMMYT | snpZM0019 | PVA | 10 | CC | TT |

### *2.6. Sequencing and SNP Discovery*

PCR products of *LCYE* 5′TE and *crtRB1 3′TE* from 14 selected inbred lines with high and low β-carotene content were purified and sent to the office of biotechnology of Iowa State University for sequencing (https://www.biotech.iastate.edu/biotechnology-service-facilities/dna-facility/, accessed on 9 June 2021). The sequenced regions of the genes are indicated in Figure 1. We sequenced the 3′-UTR of *crtRB1* and 5′-UTR of *LCYE* considering the success of previous studies in identifying PVA-associated sequence variations in the same regions [16,17]. The sequencing was carried out in both directions using forward and reverse primers. The presence of SNPs and InDels was analysed by aligning the sequences using CodonCode Aligner (LI-COR, Inc., CodonCode Corporation, Massachusetts, USA).

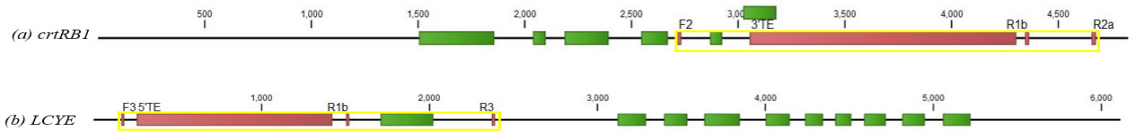


**Figure 1.** Schematic diagram of *crtRB1* and *LCYE* genes. The sequenced regions are framed yellow; Green-filled boxes represent Exons while transposable element insertions are represented by red-filled boxes; The 3′ insertion (1250 bp) is labelled 3′TE in *crtRB1* while the 5′ transposable element insertion (1156, 1166 or 1173 bp) is labelled 5′TE in the *LCYE* sequence; locus of primers used for sequencing region of interest are tagged F2, R1b and R2a for crtRB1 and F3, R1b and R3 for lcyE. Details of the primers are listed in Table S4.

### *2.7. Statistical Analysis*

PROC MIXED procedure of SAS version 9.4 [26] was used to analyse the carotenoid data. Lines were treated as fixed effects, while blocks and replications were considered as random effects. Proc FREQ and Proc GLM in SAS were used to obtain descriptive statistics and conduct analysis of variance. Association between the favorable alleles of each marker with mean concentration of each carotenoid was analysed using a two-tailed independent samples t-test with equal pooled variance in SAS [26]. To conduct the t-test, the favorable allele of each marker was coded as "1" while the unfavorable allele was coded as "0". The heterozygotes were represented by "." For each marker, the mean value of the lines carrying the favorable allele was compared with the mean value of the lines carrying the unfavorable alleles using the t-values. The KASP genotyping results were analysed using KlusterCaller software (LGC Group), and genotyping data were visualized as cluster plots and downloaded using SNPviewer software (LGC Group).

## 3. Results

### *3.1. Analysis of Variance for Provitamin A Carotenoids*

The distribution of the carotenoid concentrations for the 64 inbred lines is presented in Figure 2. The predominant carotenoids identified were β-carotene, Zeaxantine and lutein, with mean values of 21.1 (μg/g), 20.5 (μg/g) and 15.6 (μg/g), respectively (Figure 2).

The $\alpha$-carotene concentration was lowest in each of the lines. Differences among the lines for all carotenoids were significant ($p < 0.0001$) (Table 3) and the repeatability estimates ranged from 79 to 95%, indicating that a high proportion of the total variation observed for the traits was due to genetic effects.

**Table 3.** Mean squares from the analysis of variance for carotenoid content of 64 inbred lines evaluated in 2020.

| Source | df | Mean Squares of Carotenoids | | | | | |
|---|---|---|---|---|---|---|---|
| | | Lutein | Zeaxantine | β-Cryptoxanthine | α-Carotene | β-Carotene | Total Provitamin A |
| Rep | 1 | 0.02 | 94.56 * | 5.07 | 0.02 | 111.43 * | 87.60 * |
| Inbred | 63 | 119.62 ** | 232.34 ** | 26.57 ** | 0.88 ** | 230.10 ** | 199.51 ** |
| Error | 63 | 25.50 | 10.95 | 3.21 | 0.15 | 11.93 | 12.74 |
| r | | 0.79 | 0.95 | 0.88 | 0.83 | 0.95 | 0.94 |

df degrees of freedom, r repeatability. *, **Corresponding mean squares significant at $p < 0.01$, and $p < 0.0001$ respectively

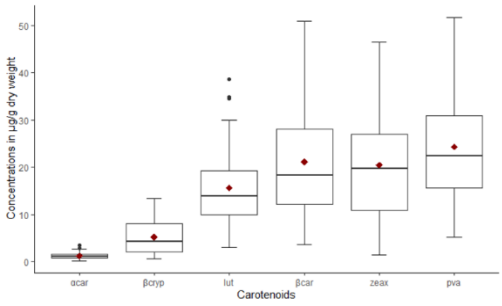


**Figure 2. Distribution of mean concentrations of carotenoids for 64 inbred lines.** Box plots; Whiskers represent standard error of least squared means of the respective carotenoid concentration; endpoints of upper and lower whiskers represent maximum and minimum concentrations, respectively; upper and lower edges of boxes represent third and first quartiles, respectively; line inside box represent median; symbol ◆ represent mean. Carotenoids are abbreviated as lut Lutein, zeax Zeaxantine, βcry β-cryptoxanthine, αcar α-carotene, βcar β-carotene, pva total provitamin A.

### *3.2. Effects of LCYE and crtRB1 Functional Markers on Provitamin A Carotenoids*

The PCR markers of *PSY1* were monomorphic across all the 64 inbred lines, whereas those of the gel-based markers viz. *crtRB1-3′TE, crtRB1-InDel4, crtRB1-5′TE, LCYE-3′indel, LCYE-5′TE, LCYE-SNP (216)* and *crtRB1*-KASP SNP markers were polymorphic. The results of the marker-trait association analysis indicated that the gel-based markers *crtRB1*-5′TE and *crtRB1*-3′TE were associated with significant reduction in zeaxanthine, β-cryptoxanthine and $\alpha$-carotene, but significant increases in β-carotene and PVA content (Table 4). In contrast, *LCYE*-5′TE was associated with significant increases in zeaxanthine, β-cryptoxanthine and $\alpha$-carotene, but significant decreases in β-carotene and PVA content. The remaining markers were not significantly associated with each of the carotenoids, except *crtRB1-InDel4*, which was associated with a significant increase in β-cryptoxanthine. The t-test also showed that all the KASP SNPs markers were associated with significant reductions in zeaxanthine, β-cryptoxanthine and $\alpha$-carotene, but with significant increases in β-carotene and PVA content (Table 4).

**Table 4.** Association of the presence of favorable alleles of gel-based and KASP markers with a mean concentration of each carotenoid in maize inbred lines.

| Markers | t-Values of Carotenoids | | | | | |
|---|---|---|---|---|---|---|
| | **Lutein** | **Zeaxantine** | **β-cryptoxanthine** | **α-carotene** | **β-carotene** | **Provitamin A** |
| Gel based markers | | | | | | |
| *crtRB1-3′TE* | −1.35 | −4.54 † | −2.91 ** | −2.31 * | 5.39 † | 4.86 † |
| *crtRB1-InDel4* | −1.32 | 1.50 | 2.30 * | 1.76 | −0.94 | −0.54 |
| *crtRB1-5′TE* | −1.35 | −5.17 † | −4.39 † | −3.25 ** | 6.26 † | 5.34 † |
| *LCYE-3′indel* | 1.85 | 1.02 | 0.26 | 0.86 | 0.37 | 0.47 |
| *LCYE-5′TE* | −0.22 | 4.46 † | 3.22 ** | 2.31 * | −3.11 ** | −2.65 * |
| *LCYE-SNP (216)* | 1.06 | 1.5 | 0.55 | 0.73 | −0.02 | 0.11 |
| KASP SNP markers | | | | | | |
| snpZM0013 | −1.63 | −4.84 † | −4.89 † | −4.73 † | 8.26 † | 6.99 † |
| snpZM0014 | −1.09 | −4.91 † | −4.86 † | −4.41 † | 8.18 † | 6.84 † |
| snpZM0015 | −1.00 | −6.30 † | −5.65 † | −6.15 † | 9.11 † | 7.38 † |
| snpZM0016 | −1.15 | −2.14 | −3.88 ** | −3.27 ** | 2.97 * | 2.72 * |
| snpZM0017 | −1.17 | −6.49 † | −5.56 † | −4.82 † | 9.62 † | 7.41 † |
| snpZM0019 | −0.60 | −4.17 *** | −3.56 *** | −3.03 ** | 4.34 † | 3.75 *** |

*, **, ***, † Corresponding t-values significant at $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively; The t value for a carotenoid is positive if the mean value of the lines carrying the favorable allele is higher than the mean value of the lines carrying the unfavorable alleles, whereas the t value for a carotenoid is negative if the mean value of the lines carrying the favorable allele is less than the mean value of the lines carrying the unfavorable alleles.

The inbred lines were grouped based on their PVA and non-PVA carotenoid content (Table 5). A total of 11 inbreds with the highest PVA carotenoid concentrations had the lowest levels of Lutein and Zeaxanthin (Table 5). All the 11 inbreds had the favorable alleles of *crtRB1* with three of them carrying the favorable alleles of both *crtRB1* and *LCYE* (Table 5). The best 18 inbred lines combined high levels of PVA carotenoids with high concentrations of Lutein and Zeaxanthin. The favorable alleles of *LCYE* were present in 12 of them (Table 5). The group of inbreds with high levels of PVA carotenoids and low levels of Lutein and Zeaxanthin also had the highest number of favorable alleles of the seven *crtRB1*-KASP SNP markers (Table 6). All inbreds in this group had the favorable alleles of snpZM0015, snpZM0016 and snpZM0017. In contrast, only very few inbred lines in this group having the favorable alleles of the *crtRB1*-KASP SNP markers combined high PVA with high non-PVA carotenoids (Table 6). A similar observation was made for the group of inbred lines with less than 15 μg/g PVA (Table 6). The carotenoid levels and *crtRB1* and *LCYE* genotypes of five inbred lines with the highest concentration of total PVA carotenoids from each group are presented in Table 7.

**Table 5.** Number of inbred lines harbouring the favorable alleles of PVA functional genes and summary of descriptive statistics of carotenoids for 64 maize inbred lines.

| Carotenoids | No. of Inbred Lines | Minimum | Maximum | Mean (±Standard Error) | Number of Lines with Favorable Alleles of PVA Functional Genes | | |
|---|---|---|---|---|---|---|---|
| | | | | | *LCYE* | *crtRB1* | *LCYE* & *crtRB1* |
| Lines with high PVA but lowest levels of Lutein, Zeaxanthin and β-cryptoxanthin | | | | | | | |
| Lutein (μg/g) | 11 | 3 | 12.3 | 7.98 ± 0.81 | 3 | 11 | 3 |
| Zeaxanthin (μg/g) | | 1.4 | 13 | 7.19 ± 1.09 | | | |
| β-cryptoxanthin (μg/g) | | 0.65 | 3 | 1.45 ± 0.22 | | | |
| α-carotene (μg/g) | | 0.23 | 0.97 | 0.54 ± 0.07 | | | |
| β-carotene (μg/g) | | 21.52 | 51 | 34.08 ± 2.60 | | | |
| Provitamin A (μg/g) | | 22.92 | 51.65 | 35.07 ± 2.55 | | | |
| Lines with high PVA and high levels of Lutein or Zeaxanthin | | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Lutein (µg/g) | 21 | 7.75 | 38.57 | 19.80 ± 1.75 | 13 | 10 | 7 |
| Zeaxanthin (µg/g) | | 8.55 | 39.37 | 18.97 ± 2.05 | | | |
| β-cryptoxanthin (µg/g) | | 1.13 | 5.96 | 3.33 ± 0.35 | | | |
| *α*-carotene (µg/g) | | 0.38 | 1.6 | 0.99 ± 0.09 | | | |
| β-carotene (µg/g) | | 12.26 | 42.39 | 24.74 ± 1.96 | | | |
| Provitamin A (µg/g) | | 15.11 | 43.37 | 26.90 ± 1.85 | | | |
| Lines with high PVA and moderate to high levels of Lutein and high levels of Zeaxanthin, and β-cryptoxanthin | | | | | | | |
| Lutein (µg/g) | 18 | 5.56 | 34.76 | 16.76 ± 1.75 | 12 | 5 | 2 |
| Zeaxanthin (µg/g) | | 17.48 | 46.5 | 30.29 ± 1.80 | | | |
| β-cryptoxanthin (µg/g) | | 7.39 | 13.34 | 10.15 ± 0.48 | | | |
| *α*-carotene (µg/g) | | 0.91 | 3.48 | 1.98 ± 0.16 | | | |
| β-carotene (µg/g) | | 10.82 | 32.14 | 18.77 ± 1.54 | | | |
| Provitamin A (µg/g) | | 15.53 | 38.48 | 24.83 ± 1.54 | | | |
| **Lines with less than 15 µg/g PVA** | | | | | | | |
| Lutein (µg/g) | 14 | 6.49 | 25.52 | 13.98 ± 1.37 | 10 | 3 | 1 |
| Zeaxanthin (µg/g) | | 10.57 | 34.13 | 20.44 ± 1.62 | | | |
| β-cryptoxanthin (µg/g) | | 2.05 | 8.93 | 4.56 ± 0.56 | | | |
| *α*-carotene (µg/g) | | 0.5 | 1.62 | 1.1 ± 0.09 | | | |
| β-carotene (µg/g) | | 3.69 | 12.74 | 8.54 ± 0.77 | | | |
| Provitamin A (µg/g) | | 5.27 | 14.57 | 11.37 ± 0.88 | | | |

**Table 6.** Number of inbred lines harbouring the favorable alleles of KASP SNPs markers and summary of descriptive statistics of carotenoids for 64 studied maize inbred lines.

| **Carotenoids** | **No. of Inbred Lines** | **Minimum** | **Maximum** | **Mean (±Standard Error)** | **Number of Lines with Favorable Alleles of *crtRB1*-KASP SNP Markers *** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | **zm13** | **zm14** | **zm15** | **zm16** | **zm17** | **zm18** | **zm19** |
| Lines with high PVA but lowest levels of Lutein, Zeaxanthin and β-cryptoxanthin | | | | | | | | | | | |
| Lutein (µg/g) | **11** | 3 | 12.3 | 7.98 ± 0.81 | 9 | 9 | 11 | 11 | 11 | 9 | 6 |
| Zeaxanthin (µg/g) | | 1.4 | 13 | 7.19 ± 1.09 | | | | | | | |
| β-cryptoxanthin (µg/g) | | 0.65 | 3 | 1.45 ± 0.22 | | | | | | | |
| *α*-carotene (µg/g) | | 0.23 | 0.97 | 0.54 ± 0.07 | | | | | | | |
| β-carotene (µg/g) | | 21.52 | 51 | 34.08 ± 2.60 | | | | | | | |
| Provitamin A (µg/g) | | 22.92 | 51.65 | 35.07 ± 2.55 | | | | | | | |
| Lines with high PVA and high levels of Lutein or Zeaxanthin | | | | | | | | | | | |
| Lutein (µg/g) | 21 | 7.75 | 38.57 | 19.80 ± 1.75 | 12 | 11 | 15 | 19 | 14 | 13 | 11 |
| Zeaxanthin (µg/g) | | 8.55 | 39.37 | 18.97 ± 2.05 | | | | | | | |
| β-cryptoxanthin (µg/g) | | 1.13 | 5.96 | 3.33 ± 0.35 | | | | | | | |
| *α*-carotene (µg/g) | | 0.38 | 1.6 | 0.99 ± 0.09 | | | | | | | |
| β-carotene (µg/g) | | 12.26 | 42.39 | 24.74 ± 1.96 | | | | | | | |
| Provitamin A (µg/g) | | 15.11 | 43.37 | 26.90 ± 1.85 | | | | | | | |
| Lines with high PVA and moderate to high levels of Lutein, Zeaxanthin and β-cryptoxanthin | | | | | | | | | | | |
| Lutein (µg/g) | 18 | 5.56 | 34.76 | 16.76 ± 1.75 | 3 | 3 | 3 | 18 | 3 | 3 | 1 |
| Zeaxanthin (µg/g) | | 17.48 | 46.5 | 30.29 ± 1.80 | | | | | | | |
| β-cryptoxanthin (µg/g) | | 7.39 | 13.34 | 10.15 ± 0.48 | | | | | | | |
| *α*-carotene (µg/g) | | 0.91 | 3.48 | 1.98 ± 0.16 | | | | | | | |
| β-carotene (µg/g) | | 10.82 | 32.14 | 18.77 ± 1.54 | | | | | | | |
| Provitamin A (µg/g) | | 15.53 | 38.48 | 24.83 ± 1.54 | | | | | | | |
| Lines with less than 15 µg/g PVA | | | | | | | | | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Lutein (μg/g) | | 6.49 | 25.52 | 13.98 ± 1.37 | | | | | | | |
| Zeaxanthin (μg/g) | | 10.57 | 34.13 | 20.44 ± 1.62 | | | | | | | |
| β-cryptoxanthin (μg/g) | 14 | 2.05 | 8.93 | 4.56 ± 0.56 | 3 | 0 | 1 | 6 | 0 | 1 | 0 |
| $\alpha$-carotene (μg/g) | | 0.5 | 1.62 | 1.1 ± 0.09 | | | | | | | |
| β-carotene (μg/g) | | 3.69 | 12.74 | 8.54 ± 0.77 | | | | | | | |
| Provitamin A (μg/g) | | 5.27 | 14.57 | 11.37 ± 0.88 | | | | | | | |

* KASP SNP markers are abbreviated as *zm13* snpZM0013; *zm14* snpZM0014; *zm15* snpZM0015; *zm16* snpZM0016; *zm17* snpZM0017; *zm18* snpZM0018; *zm19* snpZM0019.

**Table 7.** Carotenoid levels and *crtRB1* and *LCYE* genotypes of 5 inbreds with the highest PVA content selected from four groups of inbred lines.

| Inbred | Carotenoids (μg/g Dry Weight) | | | | | | Genotype * | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *crtRB1* | | | *LCYE* | | |
| | lut | zeax | βcry | $\alpha$car | βcar | pva | 3′TE | InDel4 | 5′TE | 3′indel | 5′TE | SNP (216) |
| Lines with high PVA but lowest levels of Lutein, Zeaxanthin and β-cryptoxanthin | | | | | | | | | | | | |
| IITATZI1653 | 3.0 | 1.4 | 0.9 | 0.6 | 51.0 | 51.7 | **1**/3 | 0 | **2**/1 | **0** | 2 | 2 |
| IITATZI1715 | 6.8 | 4.6 | 0.7 | 0.4 | 45.3 | 45.8 | **1** | 0 | **2** | 8 | 2 | 2 |
| IITATZI2117 | 8.7 | 10.3 | 1.2 | 0.5 | 37.7 | 38.5 | **1** | 0 | **2** | **0** | 2 | 2 |
| IITATZI2116-1 | 9.4 | 11.0 | 3.0 | 1.0 | 35.7 | 37.6 | **1** | 0 | **2** | 8 | 2 | 2 |
| IITATZI2182 | 4.4 | 1.7 | 1.0 | 0.3 | 36.4 | 37.0 | **1** | 0 | **2** | 8 | 2 | 2 |
| Lines with high PVA and high levels of Lutein or Zeaxanthin | | | | | | | | | | | | |
| IITATZI2066 | 16.3 | 10.6 | 1.9 | 0.8 | 42.0 | 43.4 | **1** | 0 | **2** | **0** | 2 | 2 |
| IITATZI2071 | 26.0 | 10.2 | 1.3 | 0.7 | 42.4 | 43.3 | **1** | 0 | **2** | 8 | 2 | 2 |
| IITATZI2065 | 17.1 | 15.4 | 3.6 | 1.3 | 34.1 | 36.5 | **1** | 0 | **2** | **0** | 2 | 2 |
| IITATZI2065 | 16.0 | 8.6 | 1.1 | 0.4 | 35.5 | 36.2 | 3 | 0 | **2** | **0** | 2 | 2 |
| IITATZI1310-2 | 9.7 | 33.3 | 5.1 | 1.2 | 31.9 | 35.1 | 3 | 0 | 1 | 8 | 2 | **1** |
| Lines with high PVA and moderate to high levels of Lutein and high levels of Zeaxanthin and β-cryptoxanthin | | | | | | | | | | | | |
| IITATZI2116-2 | 14.7 | 26.2 | 10.1 | 2.6 | 32.1 | 38.5 | **1** | 0 | **2** | 8 | 2 | 2 |
| IITATZI2142-1 | 10.0 | 24.2 | 8.0 | 1.4 | 31.0 | 35.7 | 3 | 0 | 1 | 8 | **4** | **1** |
| IITATZI2142-2 | 19.2 | 21.4 | 10.6 | 2.8 | 25.4 | 32.1 | **1** | 0 | **2**/1 | **0** | 2/**4** | **1** |
| IITATZI2161 | 23.2 | 35.9 | 11.9 | 2.3 | 23.8 | 30.9 | 3 | 0 | 1 | 8 | 2 | 2 |
| IITATZI2005-3 | 22.1 | 17.5 | 12.1 | 0.9 | 24.0 | 30.5 | **1** | 0 | **2**/1 | 8 | 2 | 2 |
| Lines with less than 15 μg/g PVA | | | | | | | | | | | | |
| IITATZI2019 | 17.9 | 10.6 | 4.3 | 1.4 | 11.8 | 14.6 | 3 | 0 | 1 | 8 | 2 | 2 |
| IITATZI2028-1 | 15.2 | 20.1 | 4.9 | 1.2 | 11.5 | 14.6 | 3 | 0 | 1 | 8 | 2 | **1** |
| Tester2 | 13.4 | 17.4 | 8.7 | 1.6 | 9.3 | 14.4 | 3 | 0 | 1 | 8 | **4** | 2 |
| IITATZI1276 | 10.1 | 21.4 | 2.0 | 0.7 | 12.7 | 14.1 | 3 | 0 | 1 | **0** | **4** | 2 |
| IITATZI1278 | 10.1 | 24.5 | 3.7 | 1.0 | 11.0 | 13.4 | 3 | 0 | 1 | **0** | **4** | 2 |

* Heterozygous alleles are separated by "/". Favorable alleles are bolded and underlined. For *LCYE*-SNP (216), "1" stands for allele "G" while "2" stands for allele "T". Abbreviations of carotenoids described under Figure 2.

The inbred lines were also grouped according to the total number of favorable alleles of *LCYE* and *crtRB1*genes and their combinations. For *LCYE*, inbreds with more favorable alleles had the highest level of non-PVA carotenoids (lutein + zeaxanthin) (Table S2). As the number of *LCYE* favorable alleles increased, the level of non-PVA carotenoids also increased. The concentration of β-carotene and PVA carotenoids was consistently lower than the level of non-PVA carotenoids in the inbreds with more favorable alleles of *LCYE* (Table S2).

The inbreds with the highest number of favorable alleles of *crtRB1* had the highest level of β-carotene and PVA carotenoids (Table S2). Overall, the inbreds harboring one or

two favorable alleles of *crtRB1* had higher levels of PVA carotenoids compared with the genotypes without any of the favorable alleles (Table S2).

Inbreds with or without favorable alleles of *LCYE* and *crtRB1* genes had high levels of non-PVA carotenoids. However, the inbreds with three to five favorable alleles of *crtRB1* genes had higher levels of PVA carotenoids (Table S2) than inbreds with no or one favorable allele. Only one genotype, IITATZI2142-2, had the maximum number of favorable alleles (5), and had 32.1 μg/g of PVA. Among the inbred lines studied, the genotype (IITATZI1653) with the highest PVA concentration (51 μg/g β-carotene) had favorable alleles at three markers viz. *crtRB1*-3′TE, *crtRB1*-5′TE and *LCYE*-3′indel.

### *3.3. Favorable Alleles of LCYE and crtRB1 Genes Associated with Inbred Carotenoid Content*

Alleles 1 and 3 of the 5′TE polymorphic site of *LCYE*, allele 3 of *crtRB1*-5′TE and allele 2 of *crtRB1*-3′TE (Table 8) were not detected among the lines used in the present study. The favorable allele frequencies varied from 14 to 39% for *LCYE*, 2 to 36% for *crtRB1* and 21 to 57% for *crtRB1*-KASP SNP markers (Table 8). Favorable alleles of the most reliable markers, *crtRB1*-5′TE and *crtRB1*-3′TE, were detected in 26 inbred lines, with 23 of them having the favorable alleles of both markers (Table 9). The 26 inbred lines also had the highest β-carotene concentrations (Table 9). It is, however, noteworthy that inbred IITATZI2068 carried the favorable alleles of both *crtRB1* and *LCYE* but still had very low PVA concentration (5.4 μg/g). The KASP SNPs markers also successfully separated the 64 inbred lines with the favorable and unfavorable alleles of the *crtRB1* gene (Figures 3 and S1).

**Table 8.** Observed alleles and frequencies of the favorable allelic class of *PSY1*, *LCYE* and *crtRB1* markers.

| Marker | Expected Allelic Series | Allelic Variants Observed * | Favorable Allele | Frequency of the Favorable Allele (%) |
|---|---|---|---|---|
| | | Gel based markers | | |
| *PSY* -SNP7 | A, C | A | A | 100 |
| *PSY1*-InDel 1 | 0, 378 | 378 | 378 | 100 |
| *LCYE*-5′TE | 1, 2, 3, 4 | 2, 4 | 4 | 29 |
| *LCYE*-SNP (216) | G, T | 1, 2 | 1 | 14 |
| *LCYE*-3′InDel | 8, 0 | 8, 0 | 0 | 39 |
| *crtRB1*-5′TE | 1, 2, 3 | 2, 1 | 2 | 35 |
| *crtRB1*-InDel4 | 12, 0 | 12, 0 | 12 | 2 |
| *crtRB1*-3′TE | 1, 2, 3 | 1, 3 | 1 | 36 |
| | | KASP SNP markers | | |
| snpZM0013 | G, C | G, C | GG | 30 |
| snpZM0014 | C,T | C,T | CC | 28 |
| snpZM0015 | A, G | A, G | AA | 40 |
| snpZM0016 | G, A | G, A | GG | 57 |
| snpZM0017 | T, G | T, G | TT | 31 |
| snpZM0018 | C, T | C, T | CC | 29 |
| snpZM0019 | C, T | C, T | CC | 21 |

* Some individuals were heterozygous for some markers. For *LCYE*-SNP (216), "1" stands for allele "G" while "2" stands for allele "T".

**Table 9.** Carotenoid levels and *crtRB1* and *LCYE* genotypes of 26 inbred lines with the best favorable alleles of *crtRB1*-3′TE and *crtRB1*-5′TE.

| Inbred | Carotenoids (μg/g Dry Weight) | | | | | | Genotype * | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *crtRB1* | | | | *LCYE* | |
| | lut | zeax | βcry | αcar | βcar | pva | 3′TE | InDel4 | 5′TE | 3′indel | 5′TE | SNP (216) |
| IITATZI1653 | 3.0 | 1.4 | 0.9 | 0.6 | 51.0 | 51.7 | **1**/3 | 0 | **2**/1 | **0** | 2 | 2 |
| IITATZI1715 | 6.8 | 4.6 | 0.7 | 0.4 | 45.3 | 45.8 | **1** | 0 | **2** | 8 | 2 | 2 |
| IITATZI2071 | 26.0 | 10.2 | 1.3 | 0.7 | 42.4 | 43.3 | **1** | 0 | **2** | 8 | 2 | 2 |
| IITATZI2066-2 | 16.3 | 10.6 | 1.9 | 0.8 | 42.0 | 43.4 | **1** | 0 | **2** | **0** | 2 | 2 |
| IITATZI2117 | 8.7 | 10.3 | 1.2 | 0.5 | 37.7 | 38.5 | **1** | 0 | **2** | **0** | 2 | 2 |
| IITATZI2182 | 4.4 | 1.7 | 1.0 | 0.3 | 36.4 | 37.0 | **1** | 0 | **2** | 8 | 2 | 2 |
| IITATZI2116-1 | 9.4 | 11.0 | 3.0 | 1.0 | 35.7 | 37.6 | **1** | 0 | **2** | 8 | 2 | 2 |
| IITATZI2065-1 | 16.0 | 8.6 | 1.1 | 0.4 | 35.5 | 36.2 | 3 | 0 | **2** | **0** | 2 | 2 |
| IITATZI2116-3 | 6.3 | 7.2 | 1.3 | 0.5 | 35.4 | 36.3 | **1** | 0 | **2** | 8 | 2 | 2 |
| IITATZI2065-2 | 17.1 | 15.4 | 3.6 | 1.3 | 34.1 | 36.5 | **1** | 0 | **2** | **0** | 2 | 2 |
| IITATZI2116-2 | 14.7 | 26.2 | 10.1 | 2.6 | 32.1 | 38.5 | **1** | 0 | **2** | 8 | 2 | 2 |
| IITATZI2142-1 | 10.0 | 24.2 | 8.0 | 1.4 | 31.0 | 35.7 | **1** | 0 | **2**/3 | **0** | 2/**4** | **1** |
| IITATZI2066-1 | 16.5 | 8.6 | 1.2 | 0.5 | 30.2 | 31.1 | **1** | 0 | **2** | **0** | 2 | 2 |
| IITATZI2006 | 10.9 | 6.4 | 2.0 | 0.8 | 29.9 | 31.4 | **1** | 0 | - | 8 | 2 | **1** |
| IITATZI2037 | 12.3 | 7.0 | 0.9 | 0.2 | 28.4 | 29.0 | **1** | 0 | **2** | 8 | 2 | 2 |
| IITATZI2163-1 | 8.7 | 8.7 | 1.1 | 0.3 | 27.1 | 27.8 | **1** | 0 | **2** | 8 | 2 | 2 |
| IITATZI2163-2 | 8.6 | 7.9 | 1.9 | 0.8 | 26.7 | 28.0 | **1** | 0 | **2** | 8 | 2 | 2 |
| IITATZI2012-1 | 23.5 | 16.4 | 1.5 | 0.9 | 26.1 | 27.3 | **1**/3 | 0 | **2**/1 | **0** | 2 | **1** |
| IITATZI2005 | 19.6 | 16.2 | 3.1 | 1.5 | 24.8 | 27.0 | **1** | 0 | **2**/3 | 8 | 2 | 2 |
| IITATZI2130 | 34.5 | 9.3 | 3.7 | 1.4 | 24.3 | 26.8 | **1** | 0 | **2** | **0** | 2 | 2 |
| IITATZI2004 | 8.8 | 13.0 | 2.2 | 0.6 | 21.5 | 22.9 | **1**/3 | 0 | **2**/1 | 8 | 2 | 2 |
| IITATZI2012-1 | 27.4 | 21.0 | 2.1 | 0.9 | 20.8 | 22.3 | **1**/3 | 0 | **2**/1 | **0** | 2 | **1** |
| IITATZI2015 | 9.4 | 15.0 | 6.0 | 1.5 | 17.0 | 20.7 | **1** | 0 | **2**/3 | 8 | 2 | 2 |
| IITATZI2024 | 12.2 | 21.3 | 2.9 | 0.5 | 16.0 | 17.7 | **1** | 0 | - | 8 | 2 | 2 |
| IITATZI2025 | 13.2 | 38.7 | 8.4 | 2.0 | 15.5 | 20.7 | **1** | 0 | **2** | **0** | **4** | 2 |
| IITATZI2068 | 12.9 | 18.2 | 2.5 | 0.9 | 3.7 | 5.4 | **1** | 0 | **2** | **0** | 2 | 2 |
| **Max** | 38.6 | 46.5 | 13.3 | 3.5 | 51.0 | 51.7 | | | | | | |
| **Min** | 3.0 | 1.4 | 0.7 | 0.2 | 3.7 | 5.3 | | | | | | |
| **GrandMean** | 15.6 | 20.5 | 5.2 | 1.2 | 21.1 | 24.3 | | | | | | |
| **CV** | 33 | 17 | 35 | 31 | 17 | 16 | | | | | | |
| **SED** | 5.0 | 3.4 | 1.8 | 0.4 | 3.7 | 3.9 | | | | | | |
| **LSD** | 10.1 | 6.8 | 3.7 | 0.8 | 7.4 | 7.7 | | | | | | |

* Heterozygous alleles are separated by "/". Favorable alleles are bolded and underlined. For *LCYE*-SNP (216), "1" stands for allele "G" while "2" stands for allele "T". Abbreviations of carotenoids described under Figure 2.

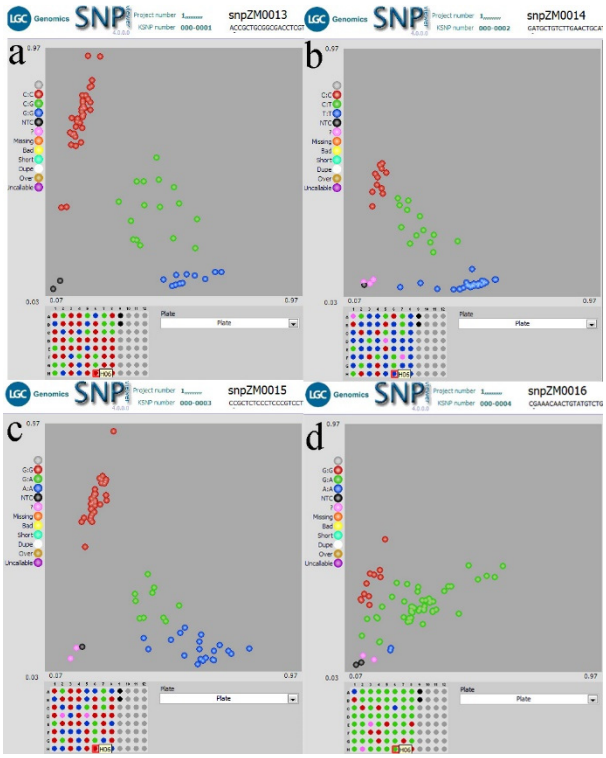


**Figure 3.** Genotype Plot for 64 Provitamin A Maize inbred lines genotyped using *crtRB1*-KASP SNP markers. (**a**) snpZM0013 G:G (blue) = Favorable alleles, C:C (red) = Unfavorable alleles, C:G (green): Heterozygous, (pink) = No amplification, NTC (black) = no template controls; (**b**) snpZM0014 C:C (red) = Favorable alleles, T:T (blue) = Unfavorable alleles, C:T (green): Heterozygous, (pink) = No amplification, NTC (black) = no template controls; (**c**) snpZM0015 A:A (blue) = Favorable alleles, G:G (red) = Unfavorable alleles, G:A (green): Heterozygous, (pink) = No amplification, NTC (black) = no template controls; (**d**) snpZM0016 A:A (blue) = Favorable alleles; G:G (red) = Unfavorable alleles; G:A (green): Heterozygous; (pink) = No amplification; NTC (black) = no template controls.

Of the 26 inbred lines with the favorable alleles of *crtRB1*-3′TE and *crtRB1*-5′TE gel-based markers, 25 also had the favorable allele of the KASP SNP snpZM0016 (Table 10). The favorable alleles of most of the 7 KASP SNPs markers were also found in the 25 inbreds (Table 10). Inbreds IITATZI2163, IITATZI2071, IITATZI2006 and IITATZI1715 were homozygous for the favorable alleles of all the 7 KASP SNPs markers (Table 10). Both snpZM0016 and gel-based *crtRB1*-5′TE markers identified the inbreds IITATZI2142, IITATZI2004 and IITATZI2012 as heterozygous for *crtRB1* alleles. However, three inbreds, namely IITATZI2015, IITATZI2068 and IITATZI2025, had the unfavorable alleles of the KASP SNP marker snpZM0015 but had the favorable alleles of the gel-based *crtRB1*-3′TE and *crtRB1*-5′TE markers. The clustering of the non-template controls (NTC) away from the inbred samples validated the amplification and efficiency of the KASP genotyping (Figures 3 and S1).

**Table 10.** Genotypes of 26 Provitamin A maize inbred lines with the favorable alleles of *crtRB1*-KASP SNP markers.

| Sample ID | PEDIGREE | snpZM0013 | snpZM0014 | snpZM0015 | snpZM0016 | snpZM0017 | snpZM0018 | snpZM0019 |
|---|---|---|---|---|---|---|---|---|
| 11 | IITATZI1653 | G:G | C:C | A:A | G:A | T:T | C:T | C:C |
| 10 | IITATZI1715 | G:G | C:C | A:A | G:G | T:T | C:C | C:C |
| 25 | IITATZI2071 | G:G | C:C | A:A | G:G | T:T | C:C | C:C |
| 33 | IITATZI2066 | G:G | C:C | A:A | G:G | G:T | C:C | T:T |
| 29 | IITATZI2117 | G:G | C:C | A:A | G:G | T:T | C:C | T:T |
| 9 | IITATZI2182 | C:C | T:T | A:A | G:A | T:T | T:T | T:T |
| 26 | IITATZI2116-1 | C:G | C:T | G:A | G:A | G:T | C:T | T:T |
| 28 | IITATZI2116-3 | G:G | C:T | A:A | G:G | G:T | C:T | T:T |
| 31 | IITATZI2065 | C:G | C:T | A:A | G:A | G:T | C:T | C:T |
| 27 | IITATZI2116-2 | C:G | C:T | G:A | G:A | G:T | C:T | T:T |
| 32 | IITATZI2066 | G:G | C:C | A:A | G:G | G:T | C:C | T:T |
| 16 | IITATZI2006 | G:G | C:C | A:A | G:G | T:T | C:C | C:C |
| 41 | IITATZI2037 | C:C | T:T | A:A | G:A | T:T | T:T | T:T |
| 44 | IITATZI2163 | G:G | C:C | A:A | G:G | T:T | C:C | C:C |
| 45 | IITATZI2163 | G:G | C:C | A:A | G:A | T:T | C:T | C:C |
| 15 | IITATZI2012-2 | C:C | - | - | G:A | - | T:T | T:T |
| 18 | IITATZI2142-2 | C:C | T:T | G:G | G:A | G:G | T:T | T:T |
| 19 | IITATZI2130 | C:C | T:T | A:A | G:G | T:T | C:C | C:C |
| 24 | IITATZI2005-3 | C:G | C:T | A:A | G:A | G:T | C:T | C:T |
| 21 | IITATZI2004 | C:G | C:T | G:A | G:A | G:T | C:T | C:C |
| 14 | IITATZI2012-1 | C:C | T:T | G:A | G:A | G:T | T:T | T:T |
| 13 | IITATZI2015 | C:C | T:T | G:G | G:A | G:G | T:T | T:T |
| 60 | IITATZI2024 | C:G | C:T | G:A | G:A | G:T | C:C | C:T |
| 46 | IITATZI2025 | C:C | T:T | G:G | G:A | G:G | T:T | T:T |
| 34 | IITATZI2068 | C:C | T:T | G:G | G:A | G:G | T:T | T:T |

Genotypes highlighted GREEN, RED and YELLOW have the favorable, unfavorable and heterozygous alleles, respectively.

### *3.4. Sequence Variation in 5'TE of LCYE*

The *crtRB1* region sequenced is 1976 bases long, while the sequenced region on the *LCYE* gene includes a total of 2277 bases. The multiple sequence alignment indicated no clear sequence variation in the 5'UTR of *LCYE* and 3'UTR of *crtRB1* separating inbred lines with high β-carotene from those with low β-carotene. The sequence variations observed in the two regions were similar for the two groups of inbred lines (data not shown). However, one SNP named *SNP1,* located in the first intron of *LCYE* 5'TE at position 1875 bp (C/T transitional mutation), clearly differentiated the low and high β-carotene lines (Figure 4). A short sequence of 21 bp flanking *SNP1* (*ATTAGATTGC**C**AACACTAATT*) was used as a query sequence to execute a BLAST against the sequence database of the maize representative genome, B73, version 5 (Zm-B73-REFERENCE-NAM-5.0) using blastn program at maizeGDB (https://www.maizegdb.org, accessed on 16 August 2021) to find its position on the reference genome. The SNP was located at position 142588003 on chromosome 8.

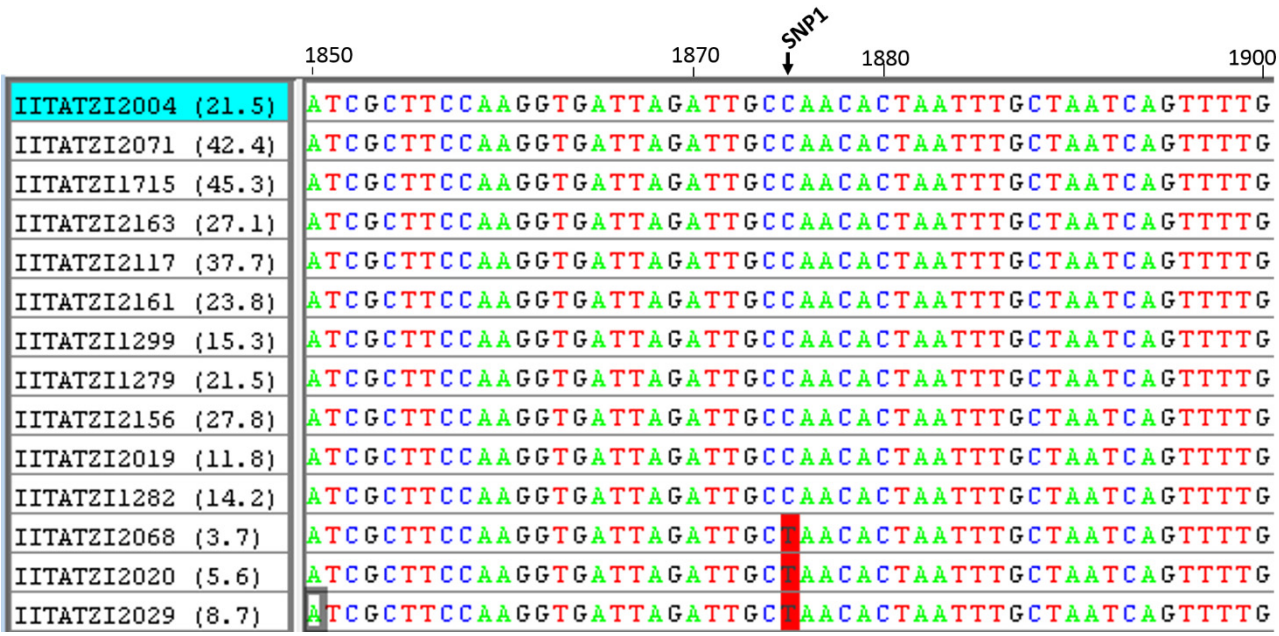


**Figure 4.** Multiple Sequence Alignment using nucleotide sequence of *LcyE* 5′TE indicating the position of a SNP from 14 selected maize inbred lines with high and low *β*-carotene concentrations. Numbers in parentheses are mean β-carotene concentrations in μg/g.

## 4. Discussion

The wide ranges in concentrations of the PVA and non-PVA carotenoids detected among inbred lines in our study indicate the suitability of the lines to compare the two types of marker assays. The high repeatability values (0.78 to 0.95) obtained for all carotenoids indicate the high level of accuracy and reliability of the results obtained from carotenoid analyses. These findings are consistent with the results of Egesel et al. [27], Kurilich and Juvik [28], Menkir and Maziya-Dixon [29] and Menkir et al. [30] reported on maize.

Lutein and zeaxanthine were the predominant non-PVA carotenoids while β-carotene was the dominant one among the PVA carotenoids. The inbred line with the highest PVA concentration (IITATZI1653, 51 μg/g β-carotene) and many other inbreds identified in this study had considerably higher PVA content than those reported in other studies involving tropical inbred lines [14,22,31]. The present study has also identified several inbred lines that have high levels of lutein and zeaxanthin, in addition to high PVA carotenoid content. These inbred lines can be used as promising parents for increasing the concentrations of all beneficial carotenoids for human health.

Of the eight functional gel-based markers of *LCYE* [8], *crtRB1* [12], *PSY1* [13], and seven *crtRB1*-KASP SNPs markers used to investigate the effect of favorable alleles on carotenoids, only the markers of *LCYE* and *crtRB1* were polymorphic while the *PSY* markers of were monomorphic in the 64 inbred lines. There are reports of fixation of the *PSY1* gene within and across species [13,14]. We found 26 inbred lines carrying the favorable alleles of *crtRB1* that also had high concentrations of β-carotene, consistent with the results in other studies [14,22]. These favorable alleles have been found to be the major contributors to high PVA content in maize [12,15]. The results obtained using the KASP SNPs markers assay were similar to the results obtained from the gel-based *crtRB1* markers in identifying inbred lines with favorable alleles of this gene. Amongst the seven KASP SNP markers, marker snpZM0016 was found to be the most reliable in identifying the largest number of inbred lines carrying favorable alleles. All the inbred lines carrying the favorable alleles of the gel-based *crtRB1*-3′TE and *crtRB1*-5′TE markers also harboured the favorable allele of snpZM0016. However, three inbred lines, namely IITATZI2015,

IITATZI2068 and IITATZI2025, did not carry favorable alleles of almost all the KASP markers while they had favorable alleles for the gel-based *crtRB1*-3′TE and *crtRB1*-5′TE markers. This contradicts the findings of Obeng-Bio et al. [22] who reported an agreement between the results obtained using snpZM0015 and the gel-based *crtRB1*-3′TE and *crtRB1*-5′TE markers. Studies involving a large number of inbred lines with diverse carotenoid composition and content need to be conducted for better understanding of concordance of the gel-based and KASP assays.

The similarity of association of the gel-based and KASP SNPs markers with individual and total carotenoids indicates the effectiveness of the two assays in identifying inbreds with high levels of PVA carotenoids. The favorable alleles of *LCYE* gene significantly increased the level of non-PVA carotenoids in the inbred lines included in our study, consistent with the results obtained by Gebremeskel et al. [31]. In general, the combination of several favorable alleles of *crtRB1* and *LCYE* resulted in higher levels of PVA carotenoids. It is reasonable to assume that the favorable allele of *LCYE*-3′indel having a significant effect on β-branch carotenoids [8], in combination with the favorable alleles of *crtRB1*, can have a beneficial effect on the accumulation of PVA carotenoid. Yan et al. [12] evaluated the independent effect of 3′TE alleles on *crtRB1* expression in the endosperm and found that lines with favorable *crtRB1* alleles (1250 bp deletion) had the lowest expression while lines with unfavorable alleles (1250 bp insertion) had the highest expression. The deletion of the last 124 base pairs in exon 6 of the crtRB1 allele present in high beta-carotene maize genotypes could have led to a functional loss of the gene. Moreover, the expression profiling experiment by Harjes et al. [8] also revealed that lines with insertion of the transposon near the *LCYE* transcription start site had a much lower expression of the gene leading to alteration in the ratio of an α- to β-branch carotenoid.

It is noteworthy that some inbred lines that did not carry any of the favorable alleles of *LCYE* and *crtRB1* had relatively high PVA carotenoids. These results indicate that genes other than *LCYE* and *crtRB1* such as *zep1* and *lut1* [32] could be associated with the accumulation of PVA carotenoids in these inbred lines. Another possibility is that *SNPs*/*InDels* present in the 5′- and 3′-UTR of *LCYE* and *crtRB1* may play a regulatory role in the expression of the genes [33,34]. We attempted to find sequence variations in the *LCYE*-5′TE and *crtRB1*-3′TE genes from 14 maize inbreds having contrasting levels of β-carotene. The sequence variations found in the *LCYE*-5′UTR and *crtRB1*-3′UTR could not be correlated with the β-carotene accumulation while the *SNP1* found in the intronic region of *LCYE* clearly separated the high and low β-carotene genotypes. In general, increases in β-carotene and provitamin A content were associated with decreases in lutein and zeaxanthin in many inbred lines included in our study. Consequently, further research is needed to develop high throughput markers with other genes to complement the KASP assay for accurate screening and identification of inbred lines with high levels of provitamin A and other beneficial carotenoids.

## 5. Conclusions

The favorable alleles of the gel-based and KASP SNPs markers associated with individual and total carotenoids were similar, indicating the effectiveness of the two assays in identifying inbreds with high levels of PVA carotenoids. Inbred lines containing favorable alleles of the gel-based *crtRB1*-5′TE and *crtRB*-3′TE markers and KASP SNP markers had the highest levels of PVA carotenoids. However, there are some inbred lines carrying favorable alleles of the gel-based markers but no favorable alleles of the KASP markers that showed high or low PVA content. The SNP1 identified in the present study, once validated in a larger sample size, could be used to design a KASP SNP marker to select maize inbred lines with high β-carotene content. Further work is also needed to develop additional high throughput markers that complement the KASP marker for accurate identification of inbred lines with high levels of both PVA and non-PVA carotenoids.

**Supplementary Materials:** The following are available online at www.mdpi.com/2073-4395/11/10/2022/s1, Figure S1: Plot for 64 Provitamin A maize inbred lines genotyped using 3 *crtRB1*-KASP markers, Table S1: Maize inbred lines used in the present study, Table S2: Total number of favorable alleles and summary of descriptive statistics of carotenoids in 64 maize inbred lines, Table S3: Carotenoids concentration and genotypes of 64 tropical maize inbred lines, Table S4. Primers used to sequence the 3′-UTR of *crtRB1* and 5′-UTR of *LCYE*.

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