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Characterization of carotenoid profiles and presence of functional markers in sub-tropical maize (*Zea mays* L.) inbred lines

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Abstract: Biofortification provides a sustainable, pragmatic strategy to address the lack of vitamin A and the associated health complications. The objectives of the study encompassed the quantification of the carotenoid content of 147 maize inbred lines, the identification of variable regions within the *ZEP1* gene, the correlation of these observed variances in the presence of this gene with carotenoid content, and the identification of lines harbouring the favourable alleles of the *crtRB1* gene. The observed correlations among the carotenoids synthesised by distinct branches of the biosynthetic process were both significant and positive. Utilising gel-based genotyping, 24 lines with contrasting carotenoid profiles were selected, evaluated and sequenced. Analysis of the variation in the sequence classified these lines based on their similarities to give 8 allele groups. The findings highlight that inbred lines both group 1 and group 8 exhibited significant associations with the carotenoid content of the lines. Specifically, *ZEP1_7852*, a discernible variation belonging to group 8, was found to be significantly associated with zeaxanthin content and total carotenoid content. Furthermore, 25 lines were found to have provitamin A content above 15 µg/g, harbouring the favourable alleles of the *crtRB1* gene using KASP SNP zm0016. These lines can serve as parents for source populations and hybrids, leading to the further enhancement of provitamin A in maize.

Keywords: carotenoid; KASP; provitamin A; sub-tropical maize lines; *ZEP1*

Maize is a vital staple food crop globally. All components of the maize plant have significant commercial value, including kernels, leaves, stems, tassels, and ears, which are utilized in producing both food-related and not-food-related product (Manjeru et al. 2019). Maize is a primary supply of sustenance for people, a source of nourishment for animals, and a fundamental ingredient for industrial applications (Morris 2001). In Africa, maize supplies a significant amount of calories required in a day, accounting for 20–30% of the total food consumption (Kaul et al. 2019). Its grains are abundant in vitamins C and E, carbohydrates, and important minerals, and include 9% protein (Shiferaw et al. 2011; Krivanek et al. 2007).

The excessive reliance of Sub-Saharan Africans on foods prepared from white maize, which lacks sufficient quantities of essential vitamins such as vitamin A, can have detrimental effects on the quality

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of their lives throughout their lifespan (Nuss & Tanumihardjo 2010; Bailey et al. 2015; Gebremeskel et al. 2018). Vitamin A deficiency (VAD) leads to multiple health consequences, comprising permanent vision loss, stunted growth, weakened immune function, higher early childhood mortality, more vulnerability to infection, rough and itchy skin, and impaired formation of gums and bones (Amimo et al. 2022; Zhu et al. 2022).

Notwithstanding the implementation of several supplementations and food fortification initiatives with vitamin A, harms both children and pregnant women, causing nearly 800 000 fatalities annually in low-income countries of Southeast Asia and the Americas (Rajwar et al. 2020). Every child is expected to have the option of eating a diverse nutritious diet that includes a wide range of vegetables and fruits. Nevertheless, the diversity of diets is sometimes limited by factors such as the yearly availability of crops, price, and the minimal consumption of carotenoids found in green leafy plants (West et al. 2002). Direct vitamin supplementation has not been widely used because of poor infrastructure in developing nations. The strategy of biofortification, in which staple crops are specifically bred for higher nutrient concentration, focusing on diets mostly utilized by third-world countries (Pfeiffer & McClafferty 2007; Andersson et al. 2017), or the creation of essential micronutrient-dense crop varieties through plant breeding, is considered feasible approach to eradicate mortality and illness caused by malnutrition (Ortiz-Monasterio et al. 2007). Maize is a suitable candidate for improving nutrition, including micronutrient deficiencies due to its high genetic diversity, suitability for genetic studies, and the distinct phenotypic expression of carotenoid variants (Chandrasekharan et al. 2022). The edible maize endosperm is considered a perfect target for biofortification because it naturally accumulates carotenoid compounds, including provitamin A carotenoids and non-provitamin carotenoids (Menkir et al. 2008; Burt et al. 2011).

HarvestPlus, a global program that aims to improve food nutrition by developing and promoting biofortified crops coined biofortification with provitamin A (PVA) goal of 15 µg/g in maize (Bouis & Welch 2010). Gebremeskel et al. (2018), Maazou et al. (2021) and Pixley et al. (2013) asserted maize breeding lines having provitamin A levels of 51.7, 22.30, and 30 µg/g, respectively. Concerted effort through breeding research has resulted in the release of over 40 cultivated maize varieties with high provitamin in Sub-Saharan Africa (SSA) (Andersson et al. 2017; Listman et al. 2019; Nkhata et al. 2020; Prasanna et al. 2020). However, the PVA concentration found in most of the released varieties varies from 6 to 10 µg/g (Andersson et al. 2017) which does not meet the desired 15 µg/g threshold. This benchmark concentration of 15 µg/g (HarvestPlus 2004) was established to supply an estimated 50% of the recommended dietary intake equivalent to 275 µg of retinol for children and approximately 500 µg for women (Simpungwe et al. 2017). For instance, at a concentration of 15 µg/g dry weight (DW) PVA, which equates to roughly 5 µg of retinol using a conversion ratio of 3 : 1, a child could fulfil 50% of their daily retinol requirement (275 µg) by ingesting about 825 µg DW PVA, derived from an average intake of 55 g of PVA maize (Obeng-Bio et al. 2019). Consequently, there is a need to further improve the concentrations of PVA in the kernels of hybrids with desirable agronomic performance for cultivation in Sub-Saharan Africa.

Breeding that integrates molecular markers is capital-intensive. To curtail genotyping expenses and expediting enhancement in carotenoid content, the International Maize and Wheat Improvement Center (CIMMYT) designed kompetitive allele-specific PCR (KASP) single nucleotide polymorphisms (SNPs) markers correlated with carotenoid cleavage dioxygenase 1 (*crtRB1)* gene on the chromosome 10 to identify genotypes with favourable PVA alleles (Gowda et al. 2017). Maazou et al. (2021) and Obeng-Bio et al. (2019) highlighted that the KASP SNP, zm0015 and zm0016 markers associated with *crtRB1* are suitable for rapid selection of PVA enriched maize. KASP markers are a type of genotyping assay used to detect SNPs and insertions/deletions (INDELs) in DNA samples (Intertek Group Plc., Sweden, unpublished). In comparison to gel-based genotyping methods, KASP markers proved to be cost-effective in handling large sample sizes owing to their scalability and multiplexing features. This multiplexing capability not only saves time and reduces costs but also ensures a swift turnaround time for the analysis of genetic variations.

Owens et al. (2014) undertook a genome-wide association study (GWAS) using 281 lines with bright yellow to deep orange. They found zeaxanthin epoxidase 1 (*ZEP1)* as a key gene that influences the carotenoid content in seeds with a potentially significant impact on increasing PVA carotenoid levels in maize through a selection of alleles that increase overall metabolic flow towards carotenoid production or hinder carotenoid breakdown in the pathway. Future breed-

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ing programs can leverage on *ZEP1* gene to improve total carotenoid levels by selecting genotypes that harbour non-functional *ZEP1* alleles. This strategy preserves zeaxanthin, a carotenoid that contributes to the total carotenoid content. This research was thus conducted to (*i*) determine the carotenoid content of 147 inbred lines, (*ii*) identify regions within the *ZEP1* gene with variation and their association with variations in carotenoid content, and (*iii*) evaluate the efficacy of *crtRB1* KASP SNP zm0015 and zm0016 markers in lines selected for varying carotenoid levels. The study posits that the inclusion of favourable alleles of the *ZEP1* gene in maize, alongside *crtRB1*, will significantly increase the PVA carotenoid concentration in maize kernels, potentially exceeding 15 µg/g by a staggering margin. Furthermore, there are variations within the *ZEP1* gene associated with carotenoid accumulation, and *crtRB1* KASP SNP markers (zm0015 and zm0016) are effective in predicting inbred lines harbouring the favourable alleles for the *crtRB1* gene. This study is the first of its kind to explore the role of the *crtRB1* and *ZEP1* genes in carotenoid accumulation in adapted sub-tropical maize inbred lines. These discoveries present new opportunities for focused breeding techniques aiming at further improving the PVA concentration in maize.

MATERIAL AND METHODS

Plant material. A sum of 147 lines derived from 28 backcrosses was utilized in this study (Table S1 in Electronic Supplementary Material (ESM)). Twelve exotic lines of tropical and temperate genetic backgroud with high PVA and non-PVA carotenoids were incorporated as donor parents (Liu et al. 2003; Islam et al. 2004) and crossed with adapted lines in earlier research (Menkir et al. 2008). The resulting first filial generation (F_1) progeny were crossed either with the same or other elite inbred lines to produce 28 backcrosses. Each backcross was planted in a row of 5 m length with 22 plants and self-pollinated to generate bulk seeds at harvest. These bulk seeds were subsequently sown in plots consisting of 10 rows, with intra and inter-space, and plot row length of 0.25, 0.75 and 5 m, respectively, yielding over 160 plants that underwent self-pollination. From the S1 to S3 inbreeding generations, ear-to-row sowing was conducted at Ikenne (3°42'E, 6°54'N, altitude 30 m) and Saminaka (8°39'E, 10°34'N, altitude 760 m) in Nigeria where individual plants showing synchronous pollen shed and silking, low ear placement, good standability, and resistance to diseases, including *Puccinia polysora* rust, *Bipolaris maydis* blight, and *Curvularia lunata* leaf spot were self-pollinated. The breeding approach leveraged to generate the inbred lines encompassed bright yellow to orange kernel colouration due to significant levels of carotenoid content, semi-flint to flint kernel texture alongside carotenoid amount quantified via high performance liquid chromatography (HPLC). At the S4 stage and beyond, similar selections were made, with self-pollination performed within each line to generate bulk seeds for continued inbreeding. From these efforts, 147 S5 to S7 lines incorporating exotic germplasm were selected for the current study.

Field evaluation and DNA extraction. In the current study, 147 sub-tropical maize inbred lines were planted in IITA's maize breeding station (7°29'11.99''N, 3°54'2.88''E, and altitude 190 m), Ibadan, and at Ikenne (3°42'E, 6°54'N, altitude 30 m) in 2023 maize growing seasons. The experimental design was 21 × 7 alpha lattice design with two replications. The intra and inter-space and plot row lengths were 0.25, 0.75 and 5 m, respectively. Maize production management practices were utilized. More than 4 illustrative plants from each row were self-pollinated to create seeds for carotenoid analyses. After the physiological maturity stage, the ears were collected, dried, and threshed. One hundred and twenty-four sampled kernels were taken and used for carotenoid analyses. For PCR-designed genotyping of the ZEP1 DNA marker, the leaves of 4 to 7 random plants per line were assembled after 21 days of planting. The leaves were lyophilized at −80 °C and subsequently used for DNA extraction. The extraction was performed utilizing the protocol outlined by Azmach et al. (2013). NanoDrop ND-800 Spectrophotometer (Thermo Scientific, Waltham, MA, US) was utilized to determine the DNA purity and quality.

Carotenoid extraction. Carotenoid extraction was carried out following the protocol outlined by Howe and Tanumihardjo (2006). At the University of Wisconsin, USA, maize kernel carotenoids were isolated and measured utilizing HPLC equipment from Waters Corporation (Milford, MA, USA). Per sample, 0.7 g of milled maize was used. The extraction process was carried out with two replications following the same extraction process reported by Azmach et al. (2013).

The measured carotenoids included lutein, zeaxanthin, α-carotene, β-carotene (cis and trans isomers), and β-cryptoxanthin. Total carotenoid content estimated as addition of lutein, zeaxanthin, α-carotene, β-cryptoxanthin, and β-carotene concentrations.

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Provitamin A content was determined by totalling β-carotene levels with half the concentrations of α-carotene and β-cryptoxanthin (US Institute of Medicine 2001). All concentrations were reported as μg/g on a DW basis.

Primer design for zeaxanthin epoxidase (*ZEP 1*) gene. The QIAGEN CLC Genomic Workbench, a primer design software was used to generate the primers targeting two SNP positions namely 44448432(C/T) and 44448438(T/G). The SNP position coordinates are from chromosome 2 and version 5 of the B73 reference genome. The primer length ranges from {18–19 bp} with GC content lower than 60%. The primer melting temperature varied from {60 °C–68 °C}. The designed primers were blasted using NCBI-BLAST (National Center for Biotechnology Information (NCBI)-Best Local Alignment Search Tool) to test the specificity. CATCGATTGGCTTGAGCA was used as the forward primer, while GTACGCCCCATATCCCTTC was used as the reverse primer.

Amplification and visualization. The PCR reaction mix for the *ZEP1* gene was prepared using 5 μL of 10× NH_4 PCR buffer, 2 μL per primer, 2 μL of 50 mM $MgCl_2$, 0.2 μL of BIOTAQ™ polymerase, 4 μL of DNA and 3 μL of dimethyl sulfoxide (DMSO), and ultra-pure water constituting 50 μL final volume. Of the total volume, 45 μL was purified and used for sequencing, the remaining 5 μL volume was used for gel-electrophoresis. Gel electrophoresis was conducted to visualize the amplified DNA fragments of the samples. A 2% agarose gel was made using sodium borate buffer and ethidium bromide for DNA staining. After electrophoresis, the gel was removed, and the DNA bands were pictured utilizing a gel documentation system (Enduro GDS, Labnet International Inc.).

Sequencing and SNP identification. The PCR products of *ZEP1* for the 24 selected lines with contrasting carotenoid levels were sequenced at IITA Bioscience Center. Bidirectional sequencing was performed, employing the forward and reverse primer pairs. SNPs and InDels analysis was performed by sequencing alignment with CodonCode Aligner, as shown in Figure S1 in ESM. After the sequences were aligned, all the variations present in the sequences were scored and their location on the chromosome noted. The variations were then grouped based on their similarities to reduce redundancy. The scoring with letters AA, BB and CC in groups was chosen to show allelic patterns within the group; they represent the actual alleles that follow the same pattern across the lines. For *ZEP 1*_7852, AA and GG are the actual alleles observed.

KASP genotyping. The extracted DNA of the 147 lines served as the template for the KASP genotyping. The DNA concentration was adjusted to an effective amount of 50 ng/uL. Each KASP reaction was performed in a volume of 10 uL, which consisted of 5 uL template DNA, and 5 uL of the primed genotyping mix (2× KASP master mix and primer mix). Two *crtRB1* SNP assays purchased from LGC Genomics, UK, were used for genotyping. Amplification was conducted utilizing the Roche Light Cycler 480 II (LC480 II) System (Roche-Life Science, USA). Amplification was performed utilizing the protocol outlined by Maazou et al. (2021). Allele calls for all SNPs were done using KlusterCaller software (LGC Group) as homozygous for FAM or HEX allele or heterozygous for both alleles.

Data analysis. PROC MIXED method in SAS (Ver. 9.4, SAS Institute 2012) was used to calculate ANOVA for carotenoids. Descriptive statistics were computed with the pastecs package in R Statistical Software (R Core Team 2013). R Statistical Software (R Core Team 2013) psych package was employed to compute the correlation among the mean values of carotenoid concentrations of the 147 lines. Additionally, R Statistical Software (R Core Team 2013) psych package was employed to compute the correlation between the two KASP markers and carotenoids. $P < 0.05$ served as the threshold mark for statistical significance.

RESULTS

Phenotypic analysis. The 147 lines exhibited marked differences in provitamin A and other carotenoids (Table 1). The zeaxanthin concentration ranged between 1.25 to 32.44 μg/g followed by β-carotene, 1.71 to 32.35 μg/g, and α-carotene varying from 0.22 and 5.07 μg/g. The concentration of total carotenoid level reached a maximum of 65.62 μg/g due to high contents of zeaxanthin, β-carotene and lutein. PVA concentration and mean value recorded were 2.24 to 34.20 and 12.11 μg/g, respectively (Table 1).

Correlation among carotenoids. The phenotypic correlations between distinct carotenoids followed their established relationships in the carotenoid biosynthesis pathway (Farré et al. 2010). The correlation among the distinct carotenoids exhibited significant positive or negative relationship. Lutein located on α-branch was not significantly corre-

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Table 1. Minimum, maximum and mean carotenoid concentration of the 147 maize inbred lines analysed utilizing high performance liquid chromatography (HPLC)

Variable	Minimum	Maximum	Mean	SD
	(μg/g)			
Lutein	1.65	28.04	7.01	3.74
Zeaxanthin	1.25	32.44	9.45	8.08
β-cryptoxanthin	0.84	10.48	3.92	1.43
α-carotene	0.22	5.07	1.35	0.47
β-carotene	1.71	32.35	9.47	4.55
Provitamin A	2.24	34.20	12.11	4.71
Total carotenoid	12.99	65.62	31.06	9.81

SD – standard deviation

lated with β-cryptoxanthin and β-carotene, located on β branch of the carotenoid biosynthesis pathway (Table 2). These findings indicate that the inbred lines possessed allelic configurations that controlled distinct stages of individual carotenoid accumulation within a specific branch of the biosynthetic pathway, rather than influencing both branches simultaneously. The correlation between lutein and zeaxanthin was very small (r = –0.05) indicating the possibility of simultaneous improvement of these beneficial carotenoids. Enhancing the concentrations of both lutein and zeaxanthin carotenoids simultaneously may be achievable through the selection of favourable alleles that govern distinct stages of the carotenoid biosynthesis pathway (Muthusamy et al. 2015). This finding aligns with the presence of multiple genes harbouring diverse allelic variants that interact both within and across loci to modulate metabolic flow in the two branches of the pathway (Wurtzel et al. 2012). This genetic complexity presents breeders with the potential to generate significant variation in carotenoid composition and concentration in maize kernels for selective breeding (Menkir et al. 2014). The correlation between β-cryptoxanthin and zeaxanthin (r = 0.43, P < 0.001) was significant and positive, suggesting that substantial conversion occurred from β-cryptoxanthin to zeaxanthin on β-branch. B-cryptoxanthin recorded a positive correlation with both α-carotene and provitamin A but a negative correlation with β-carotene (Table 2). β-carotene possesses the highest provitamin A potency, making it the most desirable endosperm carotenoid for nutritional enhancement. β-carotene had a strong and positive correlation with provitamin A (r = 0.98, P < 0.001), indicating likelihood of enhancing provitamin A to elevated amounts through redirecting the metabolic flow from the α to the β branch of the carotenoid biosynthetic pathway, or through increasing the supply of precursors at the beginning of the pathway while simultaneously restricting its hydroxylation into downstream xanthophyll derivatives that lack provitamin A functionality. HPLC analysis for quantifying β-carotene is costly and labour-intensive. Therefore, marker-assisted selection targeting beneficial alleles of genes that drive β-carotene accumulation in the metabolic pathway offers a more efficient strategy for breeding provitamin A-enriched maize varieties (Yan et al. 2010; Babu et al. 2013) to combat vitamin A deficiency (VAD) globally.

Nucleotide variation. Amongst the selected lines, 16 had medium-to-high β-carotene concentration, while the remaining 8 lines had low levels (Table 3) and their gel electrophoresis bands, as shown in Figure 1. The inbred lines passport information, specifically the values for individual carotenoids measured in this study such as zeaxanthin, lutein, alpha-carotene, beta-carotene, provitamin A, and total carotenoids, were arranged in increasing order. For each carotenoid, four inbred lines were selected: two lines with the lowest values and the other two lines with the highest values resulting in 24 inbred lines. Since the study primarily focuses on β-carotene,

Table 2. Pearson's correlation coefficients among mean values of carotenoid concentrations of the 147 maize inbred lines

	Lutein	Zeaxanthin	β-cryptoxanthin	α-carotene	β-carotene
Zeaxanthin	–0.05				
β-cryptoxanthin	–0.051	0.43***			
α-carotene	0.14	0.17*	0.56***		
β-carotene	–0.013	–0.21*	–0.041	0.27***	
Provitamin A	–0.019	–0.13	0.14	0.40**	0.98***

*, **, ***Significant at P < 0.05, 0.01, and 0.001 respectively

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Table 3. The 24 selected maize inbred lines with contrasting carotenoid amount quantified utilizing high performance liquid chromatography (HPLC) used for sequencing

Category	Entry	Line	Zeaxanthin	β-cryptoxanthin	α-carotene	β-carotene	Provitamin A	Total carotenoids
			(μg/g)					
L	27	TZMI2015-3	16.83	3.08	0.98	3.60	5.66	26.14
L	37	TZMI2019	1.25	3.84	1.42	1.71	18.17	25.30
L	40	TZMI2023-5-1	29.48	5.96	2.43	2.57	13.21	60.91
L	41	TZMI2023-5-2	32.44	4.81	2.07	5.42	11.47	57.19
L	53	TZMI2027-2	2.25	1.82	0.59	4.57	5.72	14.01
L	57	TZMI2028-2-2	2.02	1.97	0.67	4.05	5.45	12.99
L	61	TZMI2028-5-1	2.09	1.67	0.48	4.09	5.24	12.99
L	75	TZMI2056	14.08	3.47	1.03	2.88	5.05	29.10
M-H	76	TZMI2058	28.82	4.68	1.66	10.12	13.20	58.87
M-H	83	TZMI2078	32.25	10.48	2.55	7.92	14.40	65.62
M-H	99	TZMI2043-2	2.59	2.41	0.75	7.97	6.99	14.54
M-H	110	TZMI2113	25.77	6.87	2.34	11.50	16.12	52.04
M-H	118	TZMI2032-1-2	3.13	3.65	5.07	11.06	15.55	30.58
M-H	131	TZMI2050	21.37	8.24	1.81	9.67	14.69	43.72
M-H	138	TZMI2075	31.14	8.72	2.27	7.94	13.42	55.07
M-H	151	TZMI2104	1.67	3.00	1.25	10.49	12.64	23.94
M-H	161	TZMI2110	4.56	3.05	0.57	7.83	9.56	19.24
M-H	171	TZMI2067-1-1	11.10	1.29	1.30	22.05	25.30	38.56
M-H	172	TZMI2067-1-2	9.62	1.73	0.87	27.52	28.79	42.84
M-H	174	TZMI2114	10.03	2.21	1.31	32.35	34.20	51.37
M-H	175	TZMI2119	7.91	2.10	1.69	30.44	32.35	46.74
M-H	176	TZMI2120	10.47	2.00	1.55	24.98	26.74	44.80
M-H	182	KU1409	11.72	0.84	0.22	15.49	2.24	21.09
M-H	183	TZI2354	13.93	3.39	0.69	9.00	4.63	23.55
Mean			13.60	3.80	1.48	11.46	14.2	36.3
SD			11.03	2.54	1.02	9.16	9.20	17.01
CV			0.81	0.66	0.68	0.79	0.64	0.47

SD – standard deviation; *CV* – coefficient of variation; L – maize inbred lines with low carotenoid amount; M-H – maize inbred lines with medium to high carotenoid amount

lines with β-carotene values below 6 μg/g were classified as low, while those with values above 7 μg/g were categorized as medium-high (Table 3). Sequencing was carried out for the discovery of genetic modification, including SNPs, insertions, deletions, and structural variants in the lines and the precise order of the nucleotides (A, T, C, and G). Alignment of the 24 lines sequence results using Codon Code aligner software (Figure S1 in ESM) found 8 allele groups (Table 4). Amongst the 8 allele groups, two showed significant relationships with the carotenoid content of the 24 selected lines (Table 5). Group 1 showed a significant association with zeaxanthin content, with samples in the group carrying allele "BB" having significantly reduced zeaxanthin content while those carrying allele "AA" had significantly high zeaxanthin content (Table 5). Although allele group 8 also showed a significant relationship with zeaxanthin content, samples having allele "BB" had a significantly greater carotenoid content compared to samples with allele "AA" (P = 0.002) (Table 5). The variant of *ZEP1* (*ZEP1*_7852) present in allele group 8 was significantly correlated with both zeaxanthin (P = 0.00) and total carotenoid content

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Table 4. A categorization of the observed loci into groups based on similarities in the allelic pattern across the 24 selected maize inbred lines of the loci

Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
*ZEP18*018	*ZEP18*032	*ZEP1*798	*ZEP18*281	*ZEP18*282-8284	*ZEP18*318	*ZEP18*324	*ZEP17*852
*ZEP17*852	*ZEP17*803	*ZEP17*785	*ZEP18*254-8259				*ZEP17*914
*ZEP17*914	*ZEP17*805	*ZEP17*851					*ZEP17*819
*ZEP17*819	*ZEP1*780	*ZEP17*769					*ZEP18*005
*ZEP18*005	*ZEP17*825	*ZEP17*851					*ZEP18*016
*ZEP18*016	*ZEP17*846	*ZEP18*020					*ZEP18*081
*ZEP18*081	*ZEP17*856	*ZEP18*028					*ZEP18*138-8145
*ZEP18*092	*ZEP17*859	*ZEP18*302					
*ZEP17*733-34	*ZEP17*849	*ZEP1*794-7944					
*ZEP17*735-43	*ZEP17*827	*ZEP18*034-8051					
*ZEP17*865-7882	*ZEP17*837						
*ZEP1*787-793	*ZEP17*843						
*ZEP18*138-8145	*ZEP17*849						
	*ZEP17*887						
	*ZEP17*891						
	*ZEP17*897						
	*ZEP17*898						
	*ZEP17*993						
	*ZEP18*009						
	*ZEP18*011						
	*ZEP18*089						
	*ZEP18*100						
	*ZEP18*106						
	*ZEP18*108						
	*ZEP18*1202						
	*ZEP18*184						
	*ZEP18*190						
	*ZEP18*245						
	*ZEP18*305						
	*ZEP17*727						
	*ZEP17*750						
	*ZEP17*796-7800						

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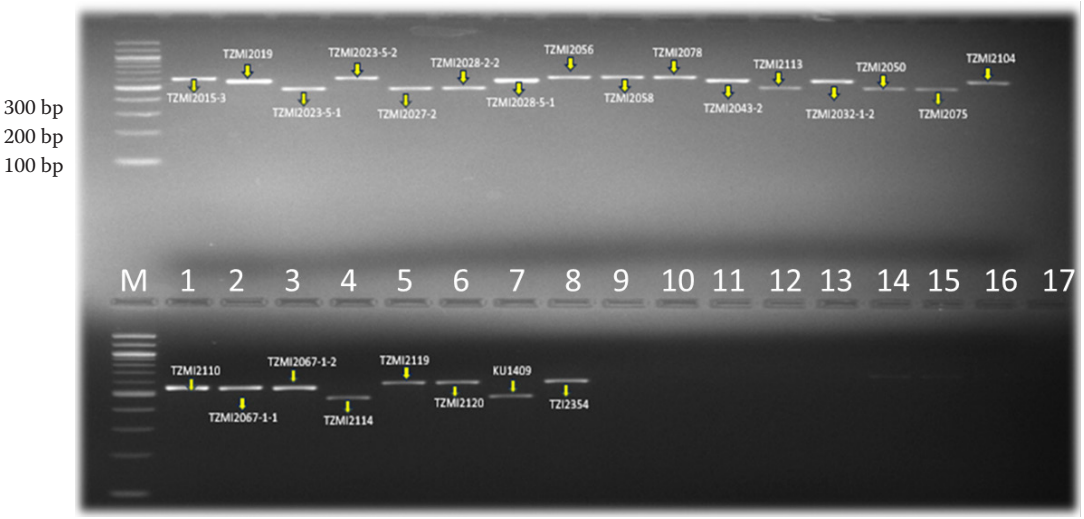

Figure 1. Agarose gel image depicting the molecular profiling of maize inbred lines using ZepSNP (438, 432) functional markers

M – 100 bp DNA ladder; lanes 1–17 correspond to the DNA amplification products of selected 24 lines

(P = 0.006). The inbred lines with allele "AA" had higher zeaxanthin and total carotenoid content followed by lines having allele "GG" (Table 5).

Screening inbred lines with favourable alleles of *crtRB1*. Genotyping of 147 lines with KASP SNP markers zm0015 and zm0016 discovered the effectiveness of KASP SNP zm0016 in successfully differentiating the favourable homozygous and heterozygous alleles for the crtRB1 gene (Figure 2). Amongst the 147 lines, 132 exhibited heterozygote allele (G: A [green]), with only one inbred line showing no amplification. The remaining 14 inbred lines carried the favourable allele (G: G [red]) (Table 6). The 5 non-template controls (NTC) were used to verify the amplification and effectiveness of the KASP SNP zm0016. As shown in Figure 2, these controls formed a distinct cluster, consistent with the findings of, (Maazou et al. 2021). It is significant to highlight that most of the lines with unfavourable *crtRB1* alleles for KASP SNP zm0015 (Figure 3) displayed increased contents of PVA carotenoids. The aforementioned findings suggest that genes other than *crtRB1*, like *LCYE* and *LUT1* might have contributed to the accumulation of PVA carotenoids in this group of lines (Owens et al. 2014). Inbreds TZMI2015-2, TZMI2022-2, TZMI2025-2, TZMI2056, TZMI2033-1, TZMI2053, TZMI2106-1, and TZMI2110 carried homozygous favourable alleles of the two KASP SNPs markers (Figure 3). The two markers (zm0016 and zm0015) were not significantly associated with either high or low carotenoid content of the inbred lines (data not shown). Analysis of pedigrees of the inbred lines resulted in the grouping of the lines based on their source population. As the groups exhibited marked discrepancies in their carotenoid content, this could contribute to the absence of a significant association between carotenoid content and the two KASP SNP markers (zm0015 and zm0016). The inbred lines belonging to group 1 (TZMI1017/SW 1 (S) C14-7-1-1-B)-57-1-1-2-B) displayed the highest lutein content among all the groups. In contrast, the lines in group 26 (TZMI1029/SW 5 (S) C6-18-2-1-B)-18-4-1-1-B)

Table 5. The 3 loci groups of the 24 selected maize inbred lines with significant association with carotenoid content

Group 1			Group 8			*ZEP1*_7852		
Alleles	mean	SD	alleles	mean	SD	allele	mean	SD
AA	19.591	10.001	AA	4.501	3.774	AA	19.233	10.088
BB	4.501	3.774	BB	18.157	10.654	GG	4.226	3.626
CC	16.723	11.769						

SD – standard deviation

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Table 6. Lines housing the favourable and heterozygote alleles of kompetitive allele-specific PCR (KASP) snpZM0016 marker of the 147 maize inbred lines (in µg/g)

Carotenoids	Minimum	Maximum	Mean	No. of lines
Inbred lines with favourable alleles of crtRB1-KASP zm0016 marker				
Lutein	2.88	16.96	6.99	
Zeaxanthin	2.09	25.67	10.75	
β-cryptoxanthin	1.29	7.82	3.73	14
α-carotene	0.48	1.98	1.26	
β-carotene	2.88	22.05	8.7	
Provitamin A	5.05	25.3	11.35	
Inbred lines with heterozygote alleles of crtRB1-KASP zm0016 marker				
Lutein	1.65	28.04	6.97	
Zeaxanthin	1.25	32.44	9.34	
β-cryptoxanthin	0.84	10.48	3.92	132
α-carotene	0.22	5.07	1.35	
β-carotene	1.71	32.35	9.53	
Provitamin A	2.24	34.2	12.18	

had the lowest lutein content (Table S5 in ESM). Lines belonging to the 28 pedigree groups did not exhibit a discernible relationship with α-carotene and β-cryptoxanthin (data not shown). In contrast, lines belonging to groups 1, 26, 31, 43, and 34 (TZMI1029/SW 5 (S) C6-18-2-1-B)-21-2-2-1-B) exhibited significant differences in zeaxanthin. Amongst these, group 26 displayed the highest zeaxanthin content among the inbred lines (Table S6 in ESM). Lines belonging to group 43 (TZMI1046/SW 5 (S) C6-18-3-1-B)-35-1-1-1-B) displayed significant difference in β-carotene and provitamin A content. Similarly, lines in group 38 (TZMI1029/SW 5 (S) C6-18-2-1-B) -32-4-1-1-B) exhibited striking differences in its β-carotene and provitamin A amount (Tables S2, S4 in ESM). Furthermore, the total carotenoid con-

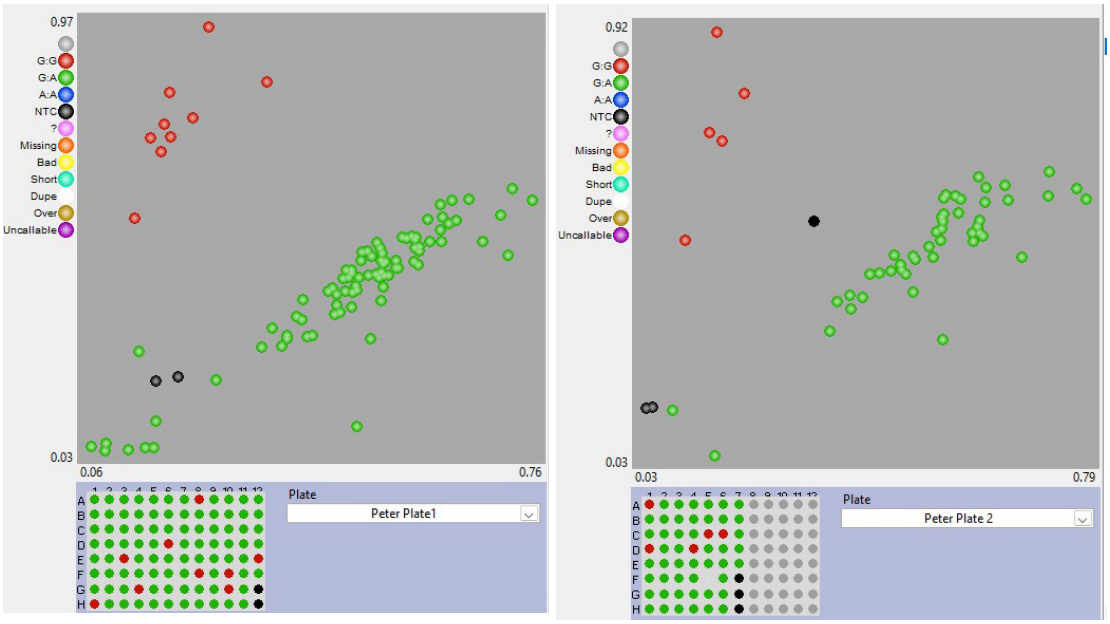

Figure 2. Genotype plot for 147 lines genotyped utilizing crtRB1-KASP marker zm0016
Red – favourable alleles; green – heterozygous; pink – no amplification; black – no template controls

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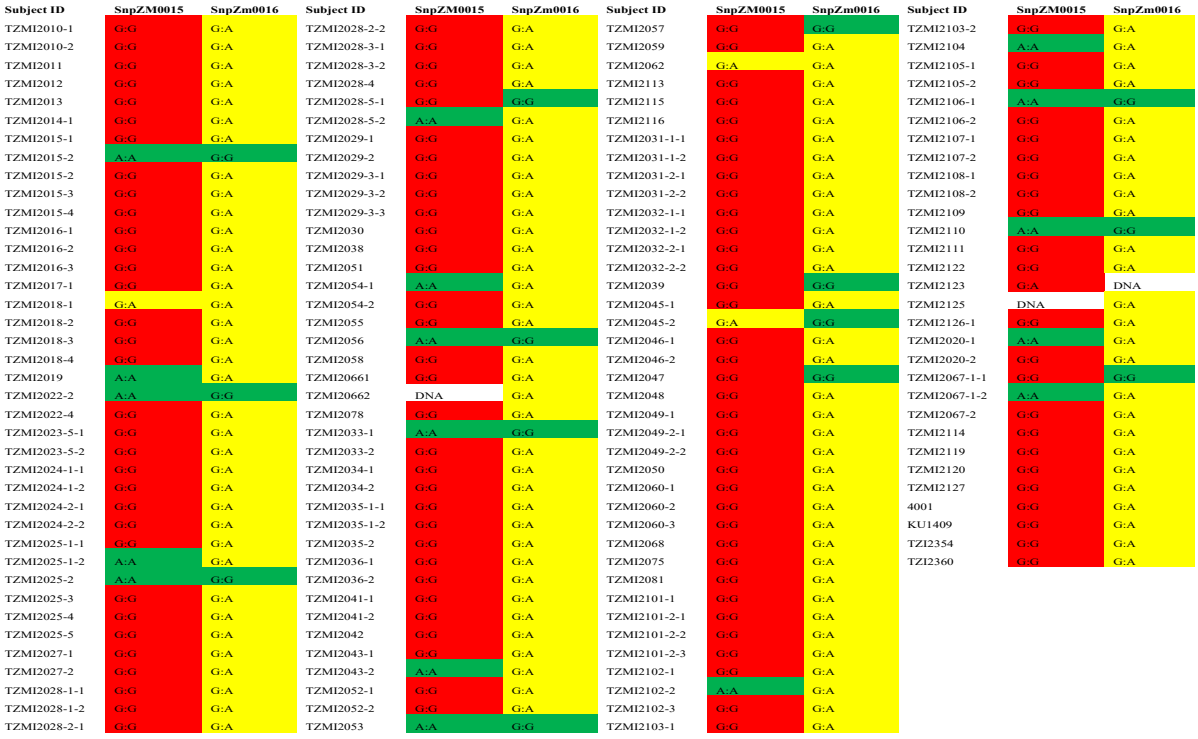

Subject ID	SnpZM0015	SnpZm0016	Subject ID	SnpZM0015	SnpZm0016	Subject ID	SnpZM0015	SnpZm0016	Subject ID	SnpZM0015	SnpZm0016
TZMI2010-1	G:G	G:A	TZMI2028-2-2	G:G	G:A	TZMI2057	G:G	G:G	TZMI2103-2	G:G	G:A
TZMI2010-2	G:G	G:A	TZMI2028-3-1	G:G	G:A	TZMI2059	G:G	G:A	TZMI2104	A:A	G:A
TZMI2011	G:G	G:A	TZMI2028-3-2	G:G	G:A	TZMI2062	G:A	G:A	TZMI2105-1	G:G	G:A
TZMI2012	G:G	G:A	TZMI2028-4	G:G	G:A	TZMI2113	G:G	G:A	TZMI2105-2	G:G	G:A
TZMI2013	G:G	G:A	TZMI2028-5-1	G:G	G:G	TZMI2115	G:G	G:A	TZMI2106-1	A:A	G:G
TZMI2014-1	G:G	G:A	TZMI2028-5-2	A:A	G:A	TZMI2116	G:G	G:A	TZMI2106-2	G:G	G:A
TZMI2015-1	G:G	G:A	TZMI2029-1	G:G	G:A	TZMI2031-1-1	G:G	G:A	TZMI2107-1	G:G	G:A
TZMI2015-2	A:A	G:G	TZMI2029-2	G:G	G:A	TZMI2031-1-2	G:G	G:A	TZMI2107-2	G:G	G:A
TZMI2015-2	G:G	G:A	TZMI2029-3-1	G:G	G:A	TZMI2031-2-1	G:G	G:A	TZMI2108-1	G:G	G:A
TZMI2015-3	G:G	G:A	TZMI2029-3-2	G:G	G:A	TZMI2031-2-2	G:G	G:A	TZMI2108-2	G:G	G:A
TZMI2015-4	G:G	G:A	TZMI2029-3-3	G:G	G:A	TZMI2032-1-1	G:G	G:A	TZMI2109	G:G	G:A
TZMI2016-1	G:G	G:A	TZMI2030	G:G	G:A	TZMI2032-1-2	G:G	G:A	TZMI2110	A:A	G:G
TZMI2016-2	G:G	G:A	TZMI2038	G:G	G:A	TZMI2032-2-1	G:G	G:A	TZMI2111	G:G	G:A
TZMI2016-3	G:G	G:A	TZMI2051	G:G	G:A	TZMI2032-2-2	G:G	G:A	TZMI2122	G:G	G:A
TZMI2017-1	G:G	G:A	TZMI2054-1	A:A	G:A	TZMI2039	G:G	G:G	TZMI2123	G:A	DNA
TZMI2018-1	G:A	G:A	TZMI2054-2	G:G	G:A	TZMI2045-1	G:G	G:A	TZMI2125	DNA	G:A
TZMI2018-2	G:G	G:A	TZMI2055	G:G	G:A	TZMI2045-2	G:A	G:G	TZMI2126-1	G:G	G:A
TZMI2018-3	G:G	G:A	TZMI2056	A:A	G:G	TZMI2046-1	G:G	G:A	TZMI2020-1	A:A	G:A
TZMI2018-4	G:G	G:A	TZMI2058	G:G	G:A	TZMI2046-2	G:G	G:A	TZMI2020-2	G:G	G:A
TZMI2019	A:A	G:A	TZMI20661	G:G	G:A	TZMI2047	G:G	G:G	TZMI2067-1-1	G:G	G:G
TZMI2022-2	A:A	G:G	TZMI20662	DNA	G:A	TZMI2048	G:G	G:A	TZMI2067-1-2	A:A	G:A
TZMI2022-4	G:G	G:A	TZMI2078	G:G	G:A	TZMI2049-1	G:G	G:A	TZMI2067-2	G:G	G:A
TZMI2023-5-1	G:G	G:A	TZMI2033-1	A:A	G:G	TZMI2049-2-1	G:G	G:A	TZMI2114	G:G	G:A
TZMI2023-5-2	G:G	G:A	TZMI2033-2	G:G	G:A	TZMI2049-2-2	G:G	G:A	TZMI2119	G:G	G:A
TZMI2024-1-1	G:G	G:A	TZMI2034-1	G:G	G:A	TZMI2050	G:G	G:A	TZMI2120	G:G	G:A
TZMI2024-1-2	G:G	G:A	TZMI2034-2	G:G	G:A	TZMI2060-1	G:G	G:A	TZMI2127	G:G	G:A
TZMI2024-2-1	G:G	G:A	TZMI2035-1-1	G:G	G:A	TZMI2060-2	G:G	G:A	4001	G:G	G:A
TZMI2024-2-2	G:G	G:A	TZMI2035-1-2	G:G	G:A	TZMI2060-3	G:G	G:A	KU1409	G:G	G:A
TZMI2025-1-1	G:G	G:A	TZMI2035-2	G:G	G:A	TZMI2068	G:G	G:A	TZI2354	G:G	G:A
TZMI2025-1-2	A:A	G:A	TZMI2036-1	G:G	G:A	TZMI2075	G:G	G:A	TZI2360	G:G	G:A
TZMI2025-2	A:A	G:G	TZMI2036-2	G:G	G:A	TZMI2081	G:G	G:A			
TZMI2025-3	G:G	G:A	TZMI2041-1	G:G	G:A	TZMI2101-1	G:G	G:A			
TZMI2025-4	G:G	G:A	TZMI2041-2	G:G	G:A	TZMI2101-2-1	G:G	G:A			
TZMI2025-5	G:G	G:A	TZMI2042	G:G	G:A	TZMI2101-2-2	G:G	G:A			
TZMI2027-1	G:G	G:A	TZMI2043-1	G:G	G:A	TZMI2101-2-3	G:G	G:A			
TZMI2027-2	G:G	G:A	TZMI2043-2	A:A	G:A	TZMI2102-1	G:G	G:A			
TZMI2028-1-1	G:G	G:A	TZMI2052-1	G:G	G:A	TZMI2102-2	A:A	G:A			
TZMI2028-1-2	G:G	G:A	TZMI2052-2	G:G	G:A	TZMI2102-3	G:G	G:A			
TZMI2028-2-1	G:G	G:A	TZMI2053	A:A	G:G	TZMI2103-1	G:G	G:A			

Figure 3. Genotypes of 147 lines housing different alleles of crtRB1-KASP SNP markers
Green, red, and yellow represent favourable, unfavourable, and heterozygous alleles, respectively

tent of lines in groups 1, 43, 28, and 17 (TZMI1029/SW 5 (S) C6-18-2-1-B)-15-4-5-2-B) showed significant differences in total carotenoid content. Specifically, lines in group 1 exhibited the greatest total carotenoid content, while those in group 43 (Table S3 in ESM) showing the second-highest total carotenoid content.

DISCUSSION

Non-PVA carotenoids and β-carotene are the vast carotenoids in maize, with lesser amounts of the remaining PVA carotenoids (USDA National Nutrient Database, ndb.nal.usda.gov), which is consistent with the pattern observed in our study. β-carotene recorded the greatest value relative to the other PVA carotenoids. The results of our study and others (Harjes et al. 2008; Azmach et al. 2013; Obeng-Bio et al. 2019) reported much high β-carotene levels, showing the potential to breed maize hybrids having elevated concentrations of provitamin A.

PVA carotenoids are essential precursors of vitamin A, sine qua non for proper execution of various systems in humans, particularly in guiding against diet-related illnesses (Tanumihardjo 2013). Over 40 maize cultivars with enhanced PVA content have been released in SSA (Listman et al. 2019; Nkhata et al. 2020; Prasanna et al. 2020). These biofortified varieties vary in their PVA concentrations depending on the region. These variations could be attributed to both environmental and genetic factors. The genetic architecture of the planting material plays a crucial role in carotenoid accumulation ability and content (Menkir et al. 2014). Kandianis et al. (2013) showed that the genetic control of maize grain carotenoid composition is regulated through multiple quantitative trait loci (QTL) distributed across the methylerythritol phosphate (MEP), isoprenoid, and carotenoid metabolic pathways. Furthermore, environmental factors encompassing growing locations, seasons, temperature, soil pH and nutrient availability, can significantly modify carotenoid accumulation by affecting physiological and biochemical processes at the cellular and whole-plant levels (Shah et al. 2017). Salinity stress, in particular, has a complex impact on carotenoid content, primarily through its effects on photosynthesis, including stomatal closure and mesophyll limitations that hinder gas exchange (Saini & Keum 2018). In Ghana, the PVA content of cultivated maize ranges from 6–11 µg/g, while in Tanzania, it is slightly higher, ranging from

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8–12 µg/g (Ekpa et al. 2018). Southern Africa has also seen notable advancements, with South African maize cultivars having a PVA content of 8.3 µg/g and those from Malawi reaching 12.3 µg/g (Pillay et al. 2011; Hwang et al. 2016). However, there has been a significant leap in breeding efforts to further increase PVA levels. Pixley et al. (2013) asserted maize breeding lines with PVA concentrations as high as 30 µg/g, while Maazou et al. (2021) documented even higher levels of up to 51.65 µg/g. In the current study, among the 147 assayed inbred lines, 25 had provitamin A content close to or exceeding 15 µg/g dry weight. An inbred line TZMI2114 had provitamin A concentration of 34.20 µg/g dry weight, which can be used as parents of source populations and hybrids to increase PVA carotenoids exceeding the biofortification goal of 15 µg/g. This remarkable PVA concentration is particularly important, given the significant losses in carotenoid content that occur during storage and processing (Bouis & Welch 2010). These losses can be ameliorated by selecting raw materials rich in PVA, which ensures a final product with substantial PVA levels, despite inevitable losses during processing. The preservation of PVA in stored processed foods can also be enhanced by maintaining low temperatures, shielding from light, minimizing oxygen exposure, utilizing modified atmosphere packaging, or employing oxygen-barrier packaging (Taleon et al. 2017). Additionally, treatments with salt and sulfiting further improve retention. Processing at the lowest feasible temperature for the shortest duration is advised; however, high-temperature, short-time processing is a viable alternative (Amaya 1997). PVA carotenoids except β-carotene had lower average values than zeaxanthin and lutein in our study, in concordance with results from earlier studies (Gebremeskel et al. 2018; Obeng-Bio et al. 2019). In our study, β-carotene made a greater contribution to provitamin A content compared to β-cryptoxanthin, consistent with (Senete et al. 2011; Maazou et al. 2021), but differed from the findings of (Egesel et al. 2003, Menkir et al. 2008; Suwarno et al. 2015) that reported higher β-cryptoxanthin compared with β-carotene. Such differences could arise from the lines' genetic makeup alongside the climatic and edaphic characteristics of the experimental sites (Menkir et al. 2021).

In this study, KASP markers zm0015 and zm0016 were leveraged to select inbred lines harbouring the favourable alleles of the *crtRB1* gene. Notably, our analysis revealed that KASP marker zm0015 was insufficiently informative for predicting the presence of *crtRB1* favourable alleles in these lines. This finding contrasts with previous results (Obeng-Bio et al. 2019), who reported zm0015 as a reliable marker for detecting *crtRB1* favourable alleles. Given the limited predictive power of zm0015, further analyses were conducted using marker zm0016, which showed effective discrimination for lines harbouring crtRB1 favourable alleles. The finding corroborates the conclusion of Maazou et al. (2021), identifying zm0016 as a marker for the presence of the *crtRB1* allele. The observed discrepancy in the effectiveness of markers zm0015 and zm0016 may stem from their location at distinct loci on the genome, resulting in their independent behaviour. Furthermore, it is noteworthy that the inbred lines analysed were predominantly heterozygous for the zm0016 marker. This observation suggests that the backcrossing process has selectively retained heterozygosity at the zm0016 locus, rather than promoting a homozygous state in these lines.

The development of markers that can detect single nucleotide substitution using sequencing was among the eventual objectives of this study. Primers were designed for two SNP positions of the *ZEP1* gene namely *ZEP1*SNP (432) and *ZEP1*SNP (438). The marker-trait association study using the presence or absence of the sequence of these markers in 24 selected lines in our study found a significant association of the markers with zeaxanthin and total carotenoids, corroborates with the findings of (Owens et al. 2014). Previous research did not demonstrate the impact of *ZEP1* on grain carotenoid composition in association studies. Vallabhaneni & Wurtzel (2009) examined the *ZEP1* gene related to carotenoid accumulation and found that *ZEP1* exhausts the carotenoid reservoir during the process of conversion into abscisic acid. Their investigations found a negative correlation between *ZEP1* transcript levels and the accumulation of carotenoids.

Biofortified yellow maize holds significant promise in addressing VAD in many malnourished regions worldwide and mitigate the risk of toxicity from excessive supplement doses and preformed vitamin A-fortified foods, which can be potentially lethal (Stevens & Winter-Nelson 2008). However, yellow maize widespread acceptance faces a confluence of hurdles, encompassing cultural factors, income disparities, education, ethnicity, geographical location, and social background (Muzhingi et al. 2008; De Groote et al. 2010). Yellow maize is often considered

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'poor man's' food due to the importation as food aid, leading to its perception as inferior to white maize (Nuss & Tanumihardjo 2010). Moreover, mishandling during importation can cause yellow maize to undergo chemical changes, resulting in undesirable tastes and odours. Additionally, the intense yellow or orange hue of biofortified maize, due to its carotenoid content, may be associated with a strong aroma and flavour (Chapman 2012). Quality control during importation can help reduce the risk of these unpleasant organoleptic properties. Furthermore, encouraging local production of yellow maize is essential to shift the perception that it is a food of lower status. This can be supported through educational campaigns, including the distribution of informational leaflets, brochures, and practical cultivation hands-on facilitated by researchers and others with technical expertise. Several studies highlight that the nutritional benefits of yellow maize could significantly influence consumer acceptance. Nuss and Tanumihardjo (2010) found that the nutritional content of yellow maize is a key factor in household purchasing decisions. Public sensitization campaigns focused on the health benefits of yellow maize can positively reshape consumer attitudes, increasing the likelihood of its adoption and consumption (Pillay et al. 2011).

CONCLUSION

Biofortification is a long-lasting and pragmatic approach with immerse potential to address VAD in impoverished nations heavily reliant on staple foods. Ongoing investment in traditional food enrichment methods may not be financially viable in such contexts. The use of marker-assisted breeding offers a resource-efficient approach to developing provitamin A-rich lines, reducing associated costs. In this present study, 25 inbred lines recorded provitamin A levels exceeding the 15 µg/g dry weight biofortication goal by HarvestPlus. These promising lines hold immense prospect for future breeding programs to boost the provitamin A content of maize to mitigate the adverse effects of vitamin A deficiency.

The *ZEP1* gene regulates the transformation of zeaxanthin into abscisic acid, leading to a reduction in carotenoid levels in seeds. If the *ZEP1* gene is nonfunctional or has a weak allele, it is likely to result in greater carotenoid content that may reduce abscisic acid synthesis. Based on our results, it appears that *ZEP1* should be regarded as equally important as *crtRB1* gene.

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