**RESEARCH ARTICLE**

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# Maize biofortification using SSR markers for Zn, Fe and provitamin A

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**ABSTRACT**

Biofortification was carried out for Zn, Fe and pro-vitamin A in the maize lines HPK-2 and CM-40 using a marker-assisted breeding approach. These parental lines were screened for polymorphism using 227 SSR primers of maize. The mapping population of 172 inbred lines was genotyped using 160 polymorphic SSR primers. The inbreds were phenotyped for the Zn and Fe content in the grain kernel. The Zn content ranged from 5-20 ppm, while the Fe content ranged from 35-60 ppm. A single QTL for Fe on chromosome I and three QTLs for Zn on chromosomes I (2 QTLs) and IX (1 QTL) were identified. Further, a crossing scheme was created by combining lines with higher Zn and Fe content (BAJIM 6-01, BAJIM 6-15, BAJIM 6-08, BAJIM 6-10, BAJIM 6-06) with donor lines having higher β-carotene content (CIMMYT-13, CIMMYT-4). The foreground selection for *crt*RB1 allele was done up to $BC_2F_4$ and $F_6$ generation to obtain β-carotene fortified lines. The $BC_2F_4$ generation of Bajim-06-10 x CIMMYT-13 showed β-carotene content of 12.54 ppm.

**Key words:** Biofortification, Micronutrients, Maize, Fe, Zn, SSR, β-carotene

## Introduction

Micronutrient malnutrition, often known as hidden hunger, is one of humanity's most persistent difficulties (Copenhagen Consensus 2008). It impacts almost two billion individuals, primarily in developing-world and resource-poor households, increasing susceptibility to illness and interfering with physical and psychological intellectual development (Kennedy et al., 2003; WHO, 2005; Bouis and Welch, 2010; Guleria et al., 2013). According to the World Health Organization (WHO, 2002; Glahn et al., 2019), zinc (Zn), iron (Fe), and vitamin A are the three most often deficient micronutrients in diets (WHO, 2002). Zinc (Zn) is an essential cofactor required for the proper functioning of approximately 300 enzymes, making it crucial for the survival and optimal health of living organisms (Coleman, 1998). This element is involved in a variety of activities, including DNA transcription, protein, nucleic acid, carbohydrate, and lipid metabolism (Broadley et al., 2007; Ishimaru et al., 2011; Palmer and Guerinot, 2009), as well as gene transcription regulation and coordination (Rhodes and Klug, 1993; Vallee and Falchuk, 1993). Zn deficiency causes diarrhea, lower respiratory infections, and malaria (Maret and Sandstead, 2006). Iron (Fe), another necessary micronutrient is required for a number of metabolic processes, including respiration and other fundamental redox reactions (Briat and Lobréaux, 1997). Fe deficiency-induced anemia has been found to afflict around one-third of the world's population, disproportionately affecting children and women (Boccio and Iyengar, 2003; FAO, 2006; Stein, 2010). Micronutrient deficiency emerges due to monotonous diets that lack variety and over-dependence on a single staple food (Graham et al., 2001). Biofortification of staple crops is a sustainable and cost-effective way to reduce malnutrition (Meenakshi et al., 2010), with a particular focus on rural communities in distant and low-rainfall areas with fewer variety diets and availability of commercially fortified foods or supplements (Saltzman et al., 2013). Maize is known to be a rich source of both provitamin A and non-provitamin A carotenoids. β-carotene undergoes hydroxylation to produce β-cryptoxanthin, which can further be converted to zeaxanthin and ABA, resulting in the formation of non-provitamin A carotenoids. In normal maize, this conversion of β-carotene to non-provitamin A carotenoids can lead to a micronutrient deficiency, particularly vitamin A deficiency, VAD (Chandrasekharan et al. 2022). The night blindness, visual impairment and death have been reported due to deficiency in provitamin A (Stein, 2010) /vitamin A deficiency (VAD) in at least 190 million preschool children and 19 million pregnant women, primarily in Africa and South Asia. It also causes low resistance to infectious ailments and contributes to over 70% of all childhood fatalities worldwide. Such deficiencies are common in developing-country rural communities, whose primary foods lack micronutrients (Muthusamy et al., 2014). Maize, being a staple in the diets of many people, has been selected as a target crop for the

HarvestPlus biofortification initiative (Nestel et al., 2006). Maize, one of the principal crops, is consumed by over a billion people in Sub-Saharan Africa, Latin America, and many nations of the world (Prasanna et al., 2011; Guleria et al., 2013). The presence of genetic heterogeneity in this species is essential for the creation of an efficient breeding program to boost mineral concentration in maize kernels (Menkir, 2008). The genetic diversity of kernel micronutrients in adapted genetic materials is a fundamental necessity for biofortification breeding programs and must studied in advance. Developing micronutrient-enriched staple plant foods, either through traditional plant breeding methods or molecular biology approaches, is a popular tool to reach the most vulnerable populations (Bouis, 2011). Several studies have shown that phytate has a deleterious influence on Zn and Fe absorption, resulting in nutritional deficits in animals and humans (Lonnerdal, 2002). For decades, the marker-assisted selection strategy has been used successfully for nutrient biofortification. MAS is a very effective breeding strategy that allows for accurate target gene selection, hence reducing the breeding cycle (Gupta et al., 2013). It is the most efficient method of introducing target genes to an important agronomic cultivar. The marker-assisted backcross breeding (MABB) for nutritional quality development in maize has been effectively used for over a decade to create advanced genotypes with increased endosperm quality and reduced anti-nutritional components (Gupta et al., 2009; Pandey et al., 2015).

Micronutrient content in maize has also been studied using QTL mapping by various researchers. However, the results were inconclusive, which may be attributed to the disparities in mapping populations, genotypes, and habitats employed in the investigations. The main focus of the current study is to detect additional loci for Zn and Fe content using suitable QTL analysis. The aim is to identify consistent QTLs and β-carotene lines that can be used for future fine mapping, marker-assisted selection (MAS), and map-based cloning.

## Materials and Methods

### *DNA isolation and PCR amplification*

Leaf samples from 172 inbred lines were collected from HAREC Bajaura, HP India, for DNA isolation using cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). DNA was isolated in replicates from all 174 (172 RILS and 2 parents) lines. The extracted DNA's quality and content was determined on a 0.8 percent ethidium bromide stained agarose gel, 100 bp DNA ladder (Fermentas, Lithuania) was used as reference. Maize SSR primer pairs (227) were selected from the MaizeGDB (http://www.maizegdb.org/) based on their uniform coverage of all maize chromosomes. Following that, two parents, HPK-2 and CM-40, were screened for 227 maize SSR primers on agarose gel. Polymorphic markers obtained were used to screen the mapping population.

PCR reactions of 10 µl volume with 25 ng DNA template, 15 ng of each forward and reverse primer, 200 µM of dNTP mix, 10mM Tris-HCL (pH 8.3), 1.5 mM $MgCl_2$, and 0.5 U of Taq DNA polymerase (High Media Pvt. Ltd., Bombay, India) were set up with initial denaturation of 1 minute at 94°C and final extension of 7 minutes at 72°C. Thirty five cycles of denaturation (1 minute at 94°C), annealing (1 minute) and extension of 1 minute at 72°C amplified the desired products. The annealing temperature (50-55°C) was set up based on corresponding SSR primer pairs used. All PCR reactions had carried out in a 96-well thermal cycler (Applied Biosystems, USA). Amplification products were separated on a 3% agarose gel. The amplicons were visualized under UV light in the Geldoc system (Syngene) with 100 bp DNA ladder (Fermentas, Lithuania). Distinct amplified allele bands were scored and used for linkage mapping and QTL anlysis.

### *Biochemical Estimation of Kernel Fe and Zn Content*

For each genotype, three selfed ears with wrapped husks were hand-harvested and dried in a clean shade to bring the grain moisture concentration down to 14% post-harvest. Precautions were taken to avoid any contact of grain with hands, soil, or metal objects. Representative grain samples were drawn in triplicate by the quartering method, and the individual samples were ground to a fine powder using an iron-free Cyclotech Sample Mill. The flour sample (0.5 g) was digested with a di-acid mixture in a ratio of 10:4 ($HNO_3$: $HClO_4$) using a standard protocol (Zarcinas et al., 1987). The kernel Fe and Zn concentrations in the samples were analysed using an Atomic Absorption Spectrophotometer (AAS Perkin Elmer) at ICAR-Vivekananda Parvatiya Krishi Anusandhan Sansthan (VPKAS), Almora, Uttarakhand, India.

### *QTL analysis*

To determine the QTLs for Fe and Zn, data from 160 primers and phenotyping data for Zn and Fe were analyzed. Multipoint analysis and the computer application QTL IciMapping 4.2 (http://www.isbreeding.net) were used to generate SSR marker linkage maps. Linkage groups were inferred at a log of the odds (LOD) threshold of 3.0, and map distances were determined using the Kosambi mapping function (Kosambi, 1943). The inclusive composite interval mapping procedure was used to identify QTL and estimate their effects (Li et al., 2007; Li et al., 2008; Wang, 2009). The integrated software QTL IciMapping 4.2 was used for QTL mapping analysis. Forward regression analysis parameters were set to a window size of 10 cM and a walk speed of 1 cM. The significance threshold for QTL detection

was determined by performing 1,000 random permutations of the phenotypic data at a 5% level.

***Development of β-carotene inbred lines***

To develop inbred lines a crossing scheme was devised using higher Zn and Fe RILs (BAJIM 6-01, BAJIM 6-15, BAJIM 6-08, BAJIM 6-10, BAJIM 6-06) available from the HAREC Bajaura, CSKHPKV, Palampur and lines having higher β-carotene kernel content (CIMMYT-13 and CIMMYT-4) obtained from IARI, New Delhi. Various crosses were attempted. Foreground selection in the $F_2$ population for allelic variation of the *crt*RB1 (β-carotene hydroxylase 1) gene was identified using HYD 65F: ACACCACATGGACAAGTTCG, HYD 62R: ACACTCTGGCCCATGAACAC, and HYD 66R: ACAGCAATACAGGGGACCAG primers (Yan et al., 2010; Muthusamy et al., 2014). $F_2$ generation plants with higher β-carotene content were selected up to $F_4$/$F_5$ generation. A biochemical analysis was carried out to determine the status of converted lines for β-carotene. Atomic Absorption Spectroscopy (AAS Perkin Elmer) was used to determine the β-carotene content at ICAR-Vivekananda Parvatiya Krishi Anusandhan Sansthan (VPKAS), Almora, Uttarakhand, India.

## Results

DNA was isolated from 172 RILS and 2 parental lines. Out of the 227 maize SSR primers screened on two parents (HPK-2 and CM-40), 160 SSR primers were found to be polymorphic.

***Genotyping of a mapping population (HP2 X CM140R) to discover Zn and Fe QTLs***

Polymorphic primers (160) were used for genotyping of the mapping population (as shown in Figures 1 and 2). IciMap QTL software was used to create linkage maps.

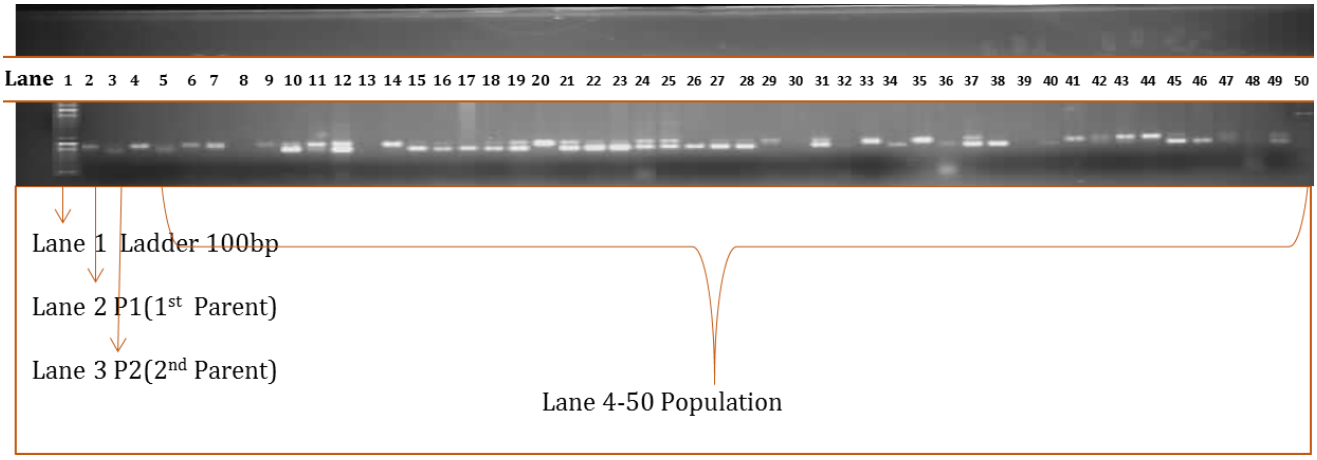


**Figure 1.** *Genotyping of Maize population with BNLG 1083*

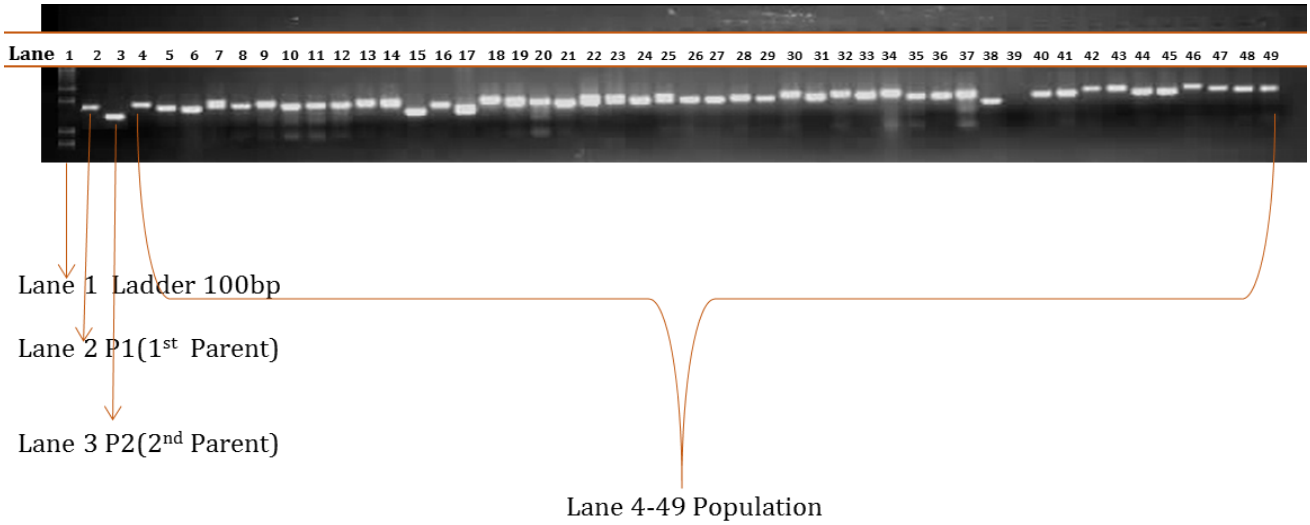


**Figure 2.** *Genotyping of Maize population with UMC1944*

***The mapping population's phenotyping***

The mapping population was phenotyped for Zn and Fe concentration in the kernel using the acid digestion procedure, as stated in the material and methods section. Fe concentrations ranging from 35 to 60 were identified by AAS, as were Zn values ranging from 5 to 20 ppm.

### *Identification of QTLs*

Three major QTLs for Zn were discovered on chromosomes I and IX in this study. First QTL for Zn was located on chromosome I at 152 cM distance, LOD 8.9209, with flanking markers BNLG1055 and BNLG1784 on the left and right positions, respectively, showed PVE (%) of 19.1693 and an additive effect of 1.9286. The second QTL of Chromosome I was located at 179 cM between 166.5 and 187.5 cM, with a LOD of 2.5816 and flanking markers BNLG1784 and UMC2319 on the left and right positions, respectively, with a PVE (%) of 9.7839 and an additive effect of 1.9148. The other major Zn QTLs were found on

**Table 1.** *Quantitative trait loci detected for Zn and Fe*

| Trait Name | Chromosome | Position | Left Marker | Right Marker | LOD | PVE (%) | Add | Left CI | Right CI |
|---|---|---|---|---|---|---|---|---|---|
| Zinc | 1 | 152 | BNLG1055 | BNLG1784 | 8.9209 | 19.1693 | 1.9268 | 149.5 | 155.5 |
| Zinc | 1 | 179 | BNLG1784 | UMC2319 | 2.5816 | 9.7839 | 1.9148 | 166.5 | 187.5 |
| Zinc | 9 | 0.5 | UMC1818 | UMC2189 | 4.0621 | 10.3484 | 0.9444 | 0 | 3.5 |
| Iron | 1 | 180 | BNLG1784 | UMC2319 | 5.7711 | 0.7517 | 12.246 | 178.5 | 180.5 |

***Legend:*** PVE (%): Phenotypic variation explained by QTL at the current scanning position.
Add: Estimated additive effect of QTL at the current scanning position.
Left CI and Right CI: Confidence interval calculated by one-LOD drop from the estimated QTL position

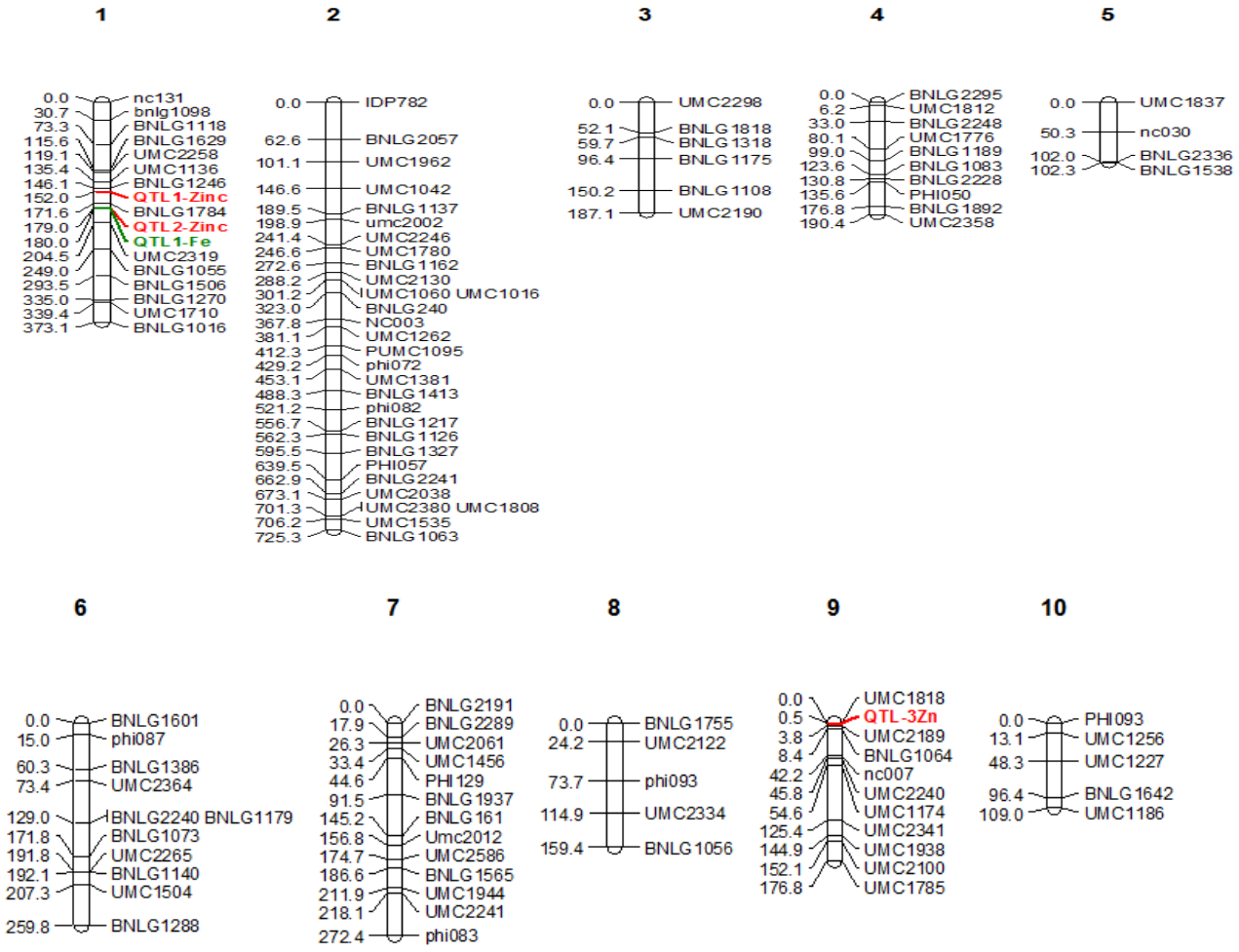


**Figure 3.** *Distribution of QTL for kernel Zn and Fe concentration on linkage map.*
*Line segment indicates the marker interval of QTL, the node on line segment indicates the position of the QTL on linkage map. Colour distinguishes the Fe and Zn.*

chromosome IX at a starting position of 0.5 cM, LOD 4.0621 flanking markers BNLG1784 and UMC2319 on the left and right positions, respectively, with a PVE (%) of 0.7517 and an additive effect of 12.426 (Table 1, Figure 3).

**Table 2.** *Crosses used for the development of inbreds for higher Zn, Fe and β- carotene*

| S. No. | Generation | Number of plants |
|---|---|---|
| 1 | BAJIM06-15x CIMMYT 13($F_5$) | 6 |
| 2 | BAJIM06-6 x CIMMYT 13 ($F_4$) | 4 |
| 3 | BAJIM06-8 x CIMMYT 4($F_4$) | 9 |
| 4 | BAJIM06-8 x CIMMYT4($F_4$) | 11 |
| 5 | BAJIM06-1 x CIMMYT4($F_4$) | 10 |
| 6 | BAJIM06-06 x CIMMYT13($F_4$) | 7 |
| 7 | BAJIM06-08 x CIMMYT4 | 3 |

among 0-3.5 cM with UMC1818 on the right and UMC2189 on the left with PVE (%) 10 and additive effect of 0.9444. For Fe, one QTL was detected on chromosome I at 180 cM, LOD 5.7711 between 178.5 and 180.5 cM, with

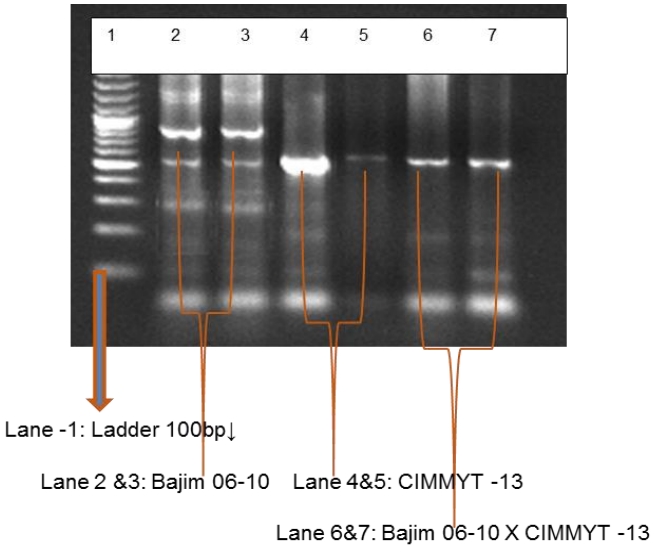


**Figure 4.** *β-carotene lines*

### *Development of inbreds with high β- carotene*

Thirty plants from the $F_2$ generation were selected in the $F_2$ generation for higher β-carotene content in the $F_4/F_5$ generation (Table 2, Figure 4, 5). The presence of the β-carotene allele and the number of populations in progress are shown in Figure 6. The

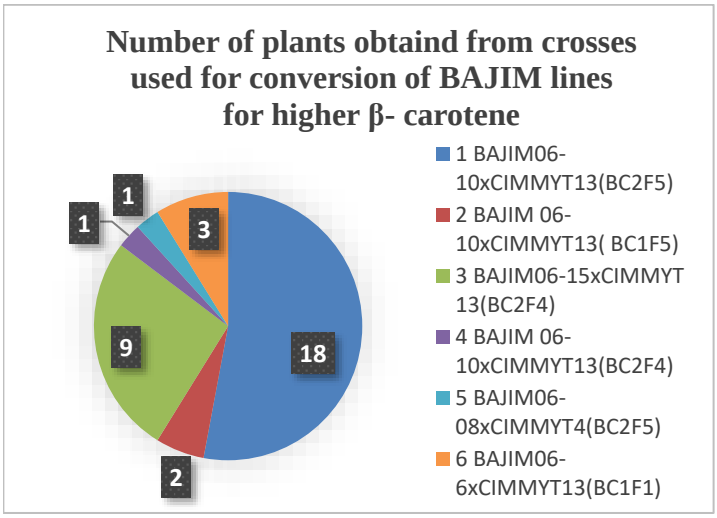


**Figure 6.** *Plants obtained through crosses of BAJIM lines for higher β- carotene*

content of β-carotene in biochemical analyses ranged from 4 to 12.54 ppm. The highest β-carotene content (12.54 ppm) was recorded in Bajim-06-10 x CIMMYT-13 ($BC_2F_4$) (Figure 7).

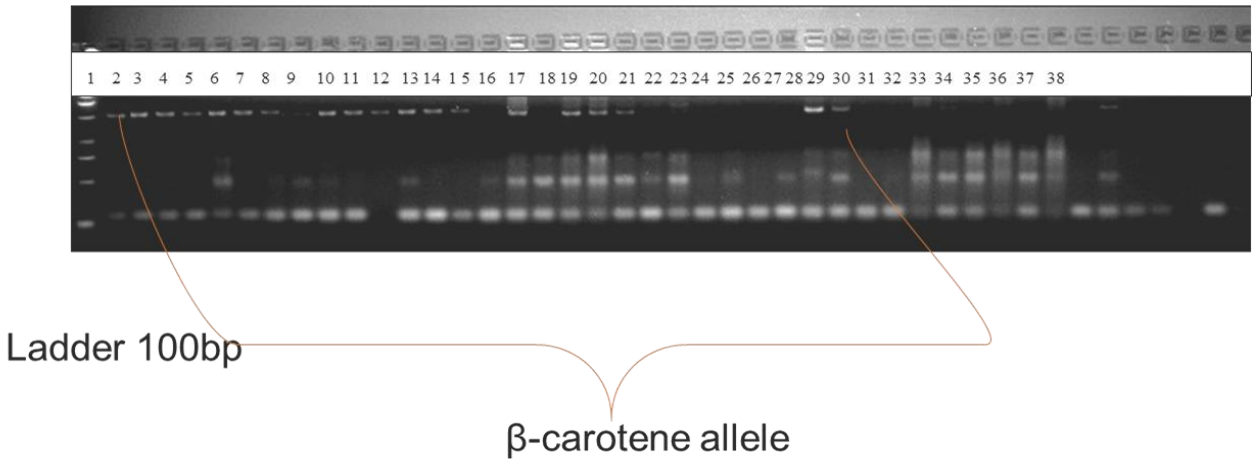


**Figure 5.** *Foreground selection for β-carotene allele*

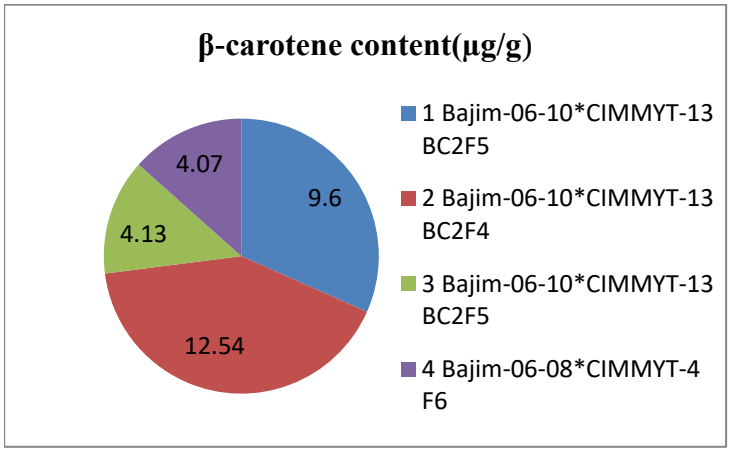


**Figure 7.** *Biochemical analysis of the converted lines for β-carotene*

## Discussion

### *QTL mapping for Zn and Fe*

QTL mapping for improving the mineral concentration in maize kernel biofortification has gained significant attention, especially in regard to increasing levels of zinc, iron, and provitamin A. Previous maize research has primarily focused on evaluating germplasm for the genetic potential to increase Zn and Fe grain density (Simic et al., 2009). However, the current trend focuses on QTL analysis to study the genetic basis and molecular-physiological mechanisms conferring high levels of grain micronutrients in maize (Lung'aho et al., 2011; Qin et al., 2012). QTL mapping of mineral content in maize grain is advantageous and a practical approach to proceed through biofortification at the genetic and molecular levels. The RIL mapping population used in the current study discovered three QTLs for zinc (Zn) and one for iron (Fe) in maize on chromosomes I and IX, respectively. Two QTLs for Zn at 152, 179 cM distance, with LODs 8.9209, 2.5816, showing PVE (%) of 19.1693, 9.7839 on chromosome I, respectively. The third QTL for Zn was located at chromosome IX at a starting position of 0.5 cM, LOD 4.0621 with PVE (%) 10. For Fe one, QTL was found on chromosome I at 180 cM, LOD 5.7711 between 178.5 and 180.5 cM, and flanking markers BNLG1784 and UMC2319 on the left and right, respectively, with PVE (%) of 0.7517 and an additive effect of 12.426. Similar findings were recorded by Qin et al. (2012) showing Zn QTL at a distance of 116 to 180 cM on chromosomes I and IX in maize. Stangoulis et al. (2007) and Tiwari et al. (2009) also observed the Zn QTL on chromosome IX. It is complicated and tedious to compare QTL results from diverse studies, because the QTL mapping could be prejudiced by factors such as genetic effects (genotypes, populations, generations), environments, mapping methods, and even markers and population sizes (Li et al., 2011).

Several factors result in positive or negative correlations among different traits in a population, viz. linkage, pleiotropy and environmental effects (Aastveit and Aastveit, 1993; Qin et al., 2012).

Micronutrients in different crops significantly show positive correlations between Zn and Fe concentrations, as reported by Blair et al. (2009). The positive correlation between zinc and iron possibly reveals the most common regulated mechanisms for Zn and Fe in plants and will probably simultaneously improve the concentration of both elements. However, Tiwari et al. (2009) observed no significant correlation between Zn and Fe concentration. The weak relationship between Zn and Fe concentration may be due to some QTL co-localization (Stangoulis et al., 2007; Tiwari et al., 2009). There is evidence to suggest that under certain environmental stress conditions, zinc can replace iron in some cellular processes (Morrissey and Guerinot, 2009). In addition to the possibility that these QTLs may be related to the same pleiotropic QTL that controls the network of metal uptake, transportation, and sequestration mechanisms (Clemens, 2001), another possible explanation for co-localization is that these QTLs are closely linked to each other (Qin et al., 2012).

### *Development of β-carotene lines*

Vitamin A deficiency (VAD), prevalent worldwide, affects people mainly dependent on β-carotene deficient cereals. Maize kernels though show tremendous variation for carotenoids, yet they are inherently deficient in provitamin A content (Mehta et al., 2021). In the present investigation, the concentration of β-carotene content of reconstituted hybrids was significantly higher than the RILs used in the study. Babu et al. (2013) reported a similar trend while validating the effect of a favorable allele of the *crt*RB1 gene in tropical maize using F2 populations. The increased concentration of β-carotene in the kernel is due to a decrease in the transcript expression of the *crt*RB1 gene, which reduces the hydroxylation of β-carotene to further carotenoids in the pathway (Muthusamy et al., 2014).

Although all of the CIMMYT-HarvestPlus donors used in this study had the same favorable allele of the *crt*RB1 gene, their kernel β-carotene content ranged from 11.3 to 17.8 mg/g, supporting the variation in β-carotene content. Various QTLs for β-carotene accumulation in maize kernels have previously been identified (Chander et al., 2008). Increased accumulation of kernel β-carotene in hybrids could also be attributed to non-additive gene action (Egesel et al., 2003). HarvestPlus, a CGIAR initiative, has set a target of 15 mg/g β-carotene content in maize kernels to achieve the goal of mitigating vitamin A deficiency in humans

(www.harvestplus.org). Traditional maize varieties with yellow kernels have low levels of β-carotene ranging from 0.01 to 4.7 mg/g (Vignesh et al., 2013).

## Conclusion

The use of a marker-assisted breeding strategy in the development of maize with enhanced levels of micronutrients such as Zn, Fe, and β-carotene has enormous potential because it allows for the precise selection of desirable plants without the need for large-scale biochemical estimation procedures in the segregating generations. Additionally, improved hybrids with sufficient Zn and Fe levels, alongwith *crt*RB1 alleles, can be utilized as donors for β-carotene enrichment in biofortification programs, which can directly alleviate micronutrient deficiency worldwide.

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