Maize : potential crop for provitamin A biofortification

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Abstract

Cereals are the only source of nutrition for one-third of the world's population especially in developing and underdeveloped nations of Sub-Saharan Africa and South-east Asia. Among the cereals, only the yellow maize exhibits tremendous natural variation for provitamin A carotenoids to exploit through plant breeding and being a carotenogenic plant, it is considered as one of the model cereal crops, showing promise for provitamin A biofortification. This paper deals with the maize carotenid biosynthetic pathway, genetic variability for kernel carotenoids, native genetic system for kernel carotenoids, marker assisted backcross breeding for enhancing provitamin A, recent advances in provitamin A biofortification and quantification of provitamin A carotenoids.

KeyWords *Maize, provitamin A, biofortification, marker assisted backcross breeding*

Introduction

Maize posseses tremendous natural variation for provitamin A carotenoids to exploit through plant breeding and is one of the model cereal crops, showing promise for provitamin A biofortification (Zhang et al., 2012). Maize has been targeted for biofortification of other micronutrients for decades and the efforts were largely successful (Vasal, 2001; Babu et al., 2005; Gupta et al., 2009; Atlin et al., 2012 and Gupta et al., 2013).

The significant variation in carotenoid content and composition of maize suggests that maize diversity may hold clues as to the target genes that could be manipulated by breeding or transgenics for improvement of cereal crop provitamin A content (Harjes et al., 2008). Efforts are underway under CIMMYT-Harvestplus maize programme to biofortify maize with provitamin A carotenoids by exploiting the natural variation for kernel carotenoids (Pixley et al., 2011). Maize has been successfully biofortified with β-carotene using natural variations for kernel carotenoids (Muthusamy et al., 2014 and Liu et al., 2015). β-carotene rich maize is efficacious when consumed as a staple food as compared to vitamin A supplementation and commercial fortification; and the risks of hyper vitaminosis A from provitamin A rich foods are almost non-existent (Gannon et al., 2014).

Kernel carotenoids in maize and their significance

As compared to rice and wheat, only the yellow kernel maize has significant levels of provitamin A carotenoids in the kernel (Buckner et al., 1990) and therefore maize is called as, a carotenogenic plant (Rodriguez-Amaya, 2001). There are two distinct classes of carotenoids in maize, (i) Carotenes, which contain only carbon and hydrogen (ii) xanthophylls, which contain oxygen group (Van den Berg et al., 2000).

The yellow maize kernel contains several carotenoid isoforms including, two carotenes (α-carotene and β-carotene) and three xanthophylls (β-cryptoxanthin, zeaxanthin and lutein). All yellow genotypes contain carotenoids, although the fraction of carotenoids with provitamin A activity (α-carotene, β-carotene and β-cryptoxanthin which can be converted to vitamin A) is typically small as compare to zeaxanthin and lutein (Muthusamy et al., 2015b). Besides their potential role as dietary source of vitamin A, carotenoids play diverse and fundamental roles viz., (i) as accessory pigment for photosynthesis; (ii) as protection against photo-oxidation; (iii) as structural determinants in plastid pigment protein complexes and (iv) in attracting pollinating insects etc.

Carotenoid biosynthetic pathway

Though biochemical regulation of carotenoid biosynthesis in maize endosperm has yet not been fully characterized, key genes encoding major structural enzymes have been isolated, characterized and cloned (Buckner et al., 1990; Buckner et al., 1996; Li et al., 1996; Sun et al., 1996, Tian and DellaPenna, 2001; Singh et al., 2003 and Li et al., 2007). These genetic studies provide not only a better understanding of genetic control of carotenoids in maize grain, but the opportunity to use marker assisted selection (MAS) to enhance the expression of the trait through breeding (Bouis and Welch, 2010). In higher plants carotenoid biosynthesis occurs in plastids by enzymes that are encoded by nuclear gene and exported into the organelle post-transcriptionally (Cunningham and Grant, 2002; Fraser and Bramley,

2004 and Howitt and Pogson, 2006). The details of the carotenoid biosynthetic pathway are depicted in Figure 1 based on the information provided by Aluru et al. (2008).

Figure 1. *Carotenoid biosynthetic pathway in maize.*

Among the genes involved in the carotenoid biosynthesis pathway, the *yellow1 (Y1)* gene, also referred to as *psy1 (phytoene synthase)* plays a pivotal role by condensing two geranyl-geranyl pyrophosphate molecules into one molecule of phytoene (Buckner et al., 1990 and 1996). Plants that contain *phytoene synthase1* gene *(PSY1/Y1)* produce carotenoid in both endosperm and leaves. The kernel carotenoid in maize is determined by allelic constitution of *Y1* which largely determines the variation of kernel colour from white to intense orange (Buckner et al., 1996). The *Y1Y1* and *Y1y1* alleles produces yellow kernel as a result of the accumulation of carotenoids, while *y1y1* allele produces white kernels that contains no carotenoids (Linden et al., 1993). Overexpression of the *psy1* gene in white kernels leads to significant carotenoid accumulation, confirming the essential role of *psy1* for carotenoid biosynthesis in maize (Zhu et al., 2008). The *Y1* gene was mapped to chromosome 6 (bin 6.01) and was cloned by Robertsons' mutator transposon tagging (Buckner et al., 1996). The strong influence of dosage effect of *Y1* on quantitative variation for carotenoids has been well documented (Palaisa et al., 2004; Wong et al., 2004; Chander et al., 2008a; Fu et al., 2010 and 2013).

The first branch point of this pathway occurs at cyclization of lycopene where action of lycopene beta cyclase (lcyB or βLCY) at both ends of linear lycopene produces a molecule with two β rings (Pogson et al., 1996). Alternatively, the coactions of lycopene beta cyclase (lcyB) and lycopene epsilon cyclase (lcyE or εLCY) generate a β, ε-carotene that is a precursor to lutein. Relative activities of lcyB and lcyE are hypothesized to regulate the proportion of carotenes directed to each branch of this pathway (Pogson et al., 1996 and Cunningham and Gantt, 2001). Studies on targeted mutagenesis of the *pink scutellum1/viviparous7 (ps1/vp7)* locus in maize showed, *ps1* to encode lycopene β-cyclase which maps to chromosome 5 (bin 5.04) is necessary for the accumulation of both abscisic acid and the carotenoid zeaxanthin in immature maize embryos (Singh et al., 2003). Downregulation of *lcyE* reduces the ratio of the α-carotene branch to the β-carotene branch (Harjes et al., 2008). The *lcyE* gene has been mapped to chromosome 8 (bin 8.05) near the SSR marker *bnlg1599* (Harjes et al., 2008).

Another key gene in the pathway is β-carotene hydroxylase 1 (*crtRB1*; also known as, *HYD3*) that causes the hydroxylation of α-carotene and β-carotene into the non-provitamin A carotenoids lutein and zeaxanthin respectively. Hydroxylation of carotenes depletes the provitamin A carotenoides thereby increasing non-provitamin A xanthophylls (Matthews and Wurtzel, 2007).

To identify target genes for blocking carotene hydroxylation, maize genes encoding carotene hydroxylases were investigated. Two structurally distinct classes of enzymes were found to be encoded by a total of eight genes in maize (Vallabhaneni and Wurtzel, 2009). Using the maize diversity core collection produced by metabolite sorting, it was possible to pinpoint the one carotene hydroxylase encoded by the *Hydroxylase3 (HYD3)* locus, whose transcript levels negatively correlated with high β-carotene levels and positively correlated with zeaxanthin levels. *HYD3* was mapped to chromosome 10 (Bin 10.06) and *crtRB1* alleles were found to be associated with reduced transcript expression of the gene which correlates with higher β-carotene concentrations in the kernel composition (Yan et al., 2010).

The β-carotene and β-cryptoxanthin are two predominant provitamin A carotenoids in maize produced by β, β branch of the biosynthetic pathway, whereas the third common provitamin A carotenoid, α-carotene is produced by β, ε pathway (Figure 1). Therefore, pathway branching and hydroxylation are the key determinants in controlling provitamin A levels (Yan et al., 2010). Concerns have been raised earlier that reducing the amount of carotenoids may lead to compromised abiotic stress tolerance in crop plants (Tan et al., 1997). The transcript profiling efforts for these two loci by Harjes et al. (2008) and Yan et al. (2010) revealed that the differences in expression levels were very high in endosperm, not very different in embryos and not at all different in leaves, which suggest tissue-specific regulation of *lcyE* and *crtRB1*. Thus selecting for mutant allele of *lcyE* and/or *crtRB1*, whose expression is limited to endosperm is unlikely to cause any undesirable effects in the carotenoid metabolism of leaves or other vegetative tissues (Babu et al., 2013).

Bioavailability and target level of provitamin A carotenoids in maize

Bioavailability is defined as the amount of the nutrient that is potentially available for absorption from a meal and once absorbed, thus utilizable for metabolic processes in the body (Welch and Graham, 2004). The bioconversion ratio given by the Institute of Medicine for dietary sources is 12:1 for β-carotene from maize to vitamin A (retinol) in humans. The important question that is to be answered is, the target levels for provitamin A carotenoids in the inbreds/hybrids to meet Recommended Dietary Allowances (RDA). In general, to compute the target level that can be achieved through breeding, it is necessary to understand: (1) per capita consumption of the staple food; (2) retention of nutrients during post-harvest processing and cooking; (3) bioavailability and (4) intake from other foods. It is also important to consider the level of other nutrients in diet that may act as an enhancer, such as, fat/lipids, iron/zinc for provitamin A carotenoids (Welch and Graham, 2004).

Based on the available information, 200 and 400g of daily maize consumption is required to provide

Table 1. Available genetic variation for various carotenoid components (µg/g) in maize lines

Sl No.	Lutein	Zeaxanthin	β-cryptoxanthin	β-carotene	α-carotene	PVAC*	TC**	Germplasm	Reference
1	4.70 - 17.50	8.90 - 30.70	1.10 - 4.90	0.50 - 3.40	-	-	17.90 - 51.40	US	Egesel et al., 2003
2	1.33 - 32.31	0.38 - 34.88	0.00 - 6.13	0.00 - 5.81	0.00 - 2.31	0.24 - 2.80	-	CIMMYT	Ortiz-Monasterio et al.,2007
3	0.01 - 20.00	1.29 - 20.70	0.29 - 9.88	0.37 - 8.79	0.03 - 0.86	-	9.90 - 39.96	US	Hulshof et al., 2007
4	0.10 - 18.20	0.60 - 24.50	0.40 - 5.50	0.70 - 4.70	0.00 - 1.90	1.10 - 7.80	-	Africa	Menkir et al., 2008
5	0.04 - 17.50	0.02 - 6.72	0.01 - 3.66	0.01 - 1.72	0.00 - 0.85	-	0.09 - 22.49	China	Chander et al., 2008b
6	0.70 - 31.33	0.47 - 43.97	0.09 - 10.84	0.06 - 13.63	0.01 - 2.03	-	5.61 - 47.20	US	Harjes et al., 2008
7	1.03 - 21.00	0.01 - 35.00	-	-	-	-	1.09 - 61.10	Italy	Berardo et al., 2009
8	-	-	-	-	-	1.73 - 2.30	20.50 - 26.40	Brazil	Rios et al., 2009
9	-	-	-	-	-	-	0.03 - 25.8	India	Mishra and Singh, 2010
10	-	-	-	-	-	-	0.94 - 38.25	India	Das and Singh, 2012
11	-	-	-	-	-	-	12.2 - 30.10	India	Tiwari et al., 2012
12	1.44 - 23.27	3.23 - 97.77	0.80 - 2.66	0.40 - 18.80	-	1.22 - 19.47	-	India, CIMMYT	Vignesh et al., 2012a
13	0.45 -13.51	0.04 - 25.90	0.08 - 8.55	0.03 - 16.38	0.00 - 1.68	0.06 - 17.25	4.43 - 42.71	Africa, CIMMYT	Azmach et al., 2013
14	-	-	-	-	-	-	6.50 - 67.3	India	Sivaranjani et al., 2013
15	-	-	-	0.00 - 4.81	-	-	3.30 - 27.4	India	Rashmi and Singh, 2014
16	-	-	-	0.23 - 7.92	-	-	-	India	Selvi et al., 2014
17	-	-	-	-	-	-	0.10 - 11.40	India	Vikal et al., 2014b
18	4.07 - 21.66	1.26 - 19.91	0.23 - 5.33	0.17 - 2.33	0.00 - 0.41	-	6.53 - 39.78	Italy	Alfieri et al., 2014
19	-	-	-	1.10 - 18.80	-	-	-	India, CIMMYT	Chaudhary et al., 2015
20	1.30 - 11.30	1.70 - 20.00	0.10 - 3.30	0.00 - 1.80	-	-	-	India	Muthusamy et al., 2015a
21	-	-	-	-	-	1.52 - 9.97	-	China	Liu et al., 2015
22	0.36 - 15.75	0.25 - 22.76	0.06 - 4.37	0.07 - 17.41	-	-	-	India, CIMMYT	Muthusamy et al., 2015b
23	1.00 - 19.40	0.40 - 30.80	0.10 - 7.90	0.00 - 16.60	-	0.01 - 17.40	5.50 - 48.60	Africa, CIMMYT	Menkir et al., 2015

PVAC * : Provitamin A carotenoids; TC ** : Total carotenoids

250µg and 500µg retinol daily requirement for children and women respectively, considering only 50% retention after post-harvest processing and a 12:1 bioconversion rate of β-carotene to retinol (Ortiz-Monasterio et al., 2007). Thus, 15µg/g of provitamin A in kernel has been set as a target level in maize biofortification by HarvestPlus (Bouis et al., 2011). However, the study conducted by Howe and Tanumihardjo (2006) to investigate the bioefficacy of provitamin A carotenoids from maize concluded that bioconversion of β-carotene to retinol was 2.8:1 and it is comparable to β-carotene supplementation. The discovery and confirmation of lower bioconversion ratio for β-carotene from maize is due to its association with oil in the grains which leads to better absorption rate. Thus, breeding for provitamin A in high oil inbred would lead to significant effect on maize biofortification (Vignesh et al., 2012a).

Genetic variability for kernel carotenoids in maize

Maize grain carotenoid concentrations are among the highest produced in cereals (Howitt and Pogson, 2006) and exhibit considerable diversity in the composition of grain carotenoid profiles with respect to the predominant carotenoids (lutein and zeaxanthin), provitamin A carotenoids (α-carotene, β-carotene and β-cryptoxanthin) and other non-provitamin A carotenoids (zeinoxanthin) (Harjes et al., 2008 and Pixley et al., 2011). Plant breeding has been the primary focus of programs to enhance staple food crops with sufficient levels of iron, zinc and provitamin A carotenoids to meet the demand of populations at risk (White and Broadley, 2009). The first step in breeding maize for enhanced carotenoid contents involves an assessment of variability existing in adapted germplasm. When there is sufficient genetic variation, breeders can use various breeding schemes in order to exploit the additive gene effects, transgressive segregation and heterosis to improve the trait.

Various research efforts worldwide have reported the existence of wide genetic variation for carotenoids (Egesel et al., 2003; Hulshof et al., 2007; Ortiz-Monasterio et al., 2007; Chander et al., 2008b; Harjes et al., 2008; Menkir et al., 2008; Berardo et al., 2009; Rios et al., 2009; Mishra and Singh, 2010; Das and Singh, 2012; Tiwari et al., 2012; Sivaranjani et al., 2013; Azmach et al., 2013; Rashmi and Singh, 2014; Vikal et al., 2014; Selvi et al., 2014; Alfieri et al., 2014; Choudhary et al., 2015; Muthusamy et al., 2015a and 2015b; Liu et al., 2015 and Menkir et al., 2015). The extent of variability of lutein, zeaxanthin, β-cryptoxanthin, β-carotene, α-carotene and total carotenoids along with their source of germplasm are presented in Table 1.

The carotenoids in maize are reported to have high heritability (Egesel et al., 2003; Menkir et al., 2008 and Muthusamy et al., 2015b). Genetic studies also show that accumulation of carotenoids in maize grain is quantitatively inherited (Islam et al., 2004; Wong et al., 2004 and Kandianis et al., 2013). Preponderance of additive genetic variance for carotenoids in maize further offers possibility of higher response to selection in developing carotenoid rich maize genotypes (Senete et al., 2011; Suwarno et al., 2014 and Muthusamy et al., 2015b). Many reports have also suggested that the influence G × E interaction is very less and the carotenoids are stable across locations (Menkir et al., 2008 and Muthusamy et al., 2015b). Thus, breeding maize for increased levels of provitamin A carotenoids would be an economical and efficient way to address VAD, especially in the developing world (Yan et al., 2010 and Zhang et al., 2012).

Native genetic system for provitamin A enrichment in maize

The eight candidate genes *y1*, *zds1*, *lcyE*, *crtRB3*, *lut1*, *crtRB1*, *zep1*,and *ccd1* are all in chromosome regions associated with QTL for carotenoids (Wong et al., 2004; Chander et al., 2008a; Zhou et al., 2012; Chandler et al., 2013 and Kandianis et al., 2013). Six of eight genes were also associated with QTL for intensity of orange color, *crtRB3* and *lut1* being the exceptions (Chandler et al., 2013). A darker orange color is associated with higher total carotenoids, particularly lutein and zeaxanthin in maize (Pfeiffer and McClafferty, 2007 and Burt et al., 2011).

Among the genes involved in the carotenoid biosynthesis pathway, *psy1* located on chromosome 6, plays a pivotal role by condensing two geranyl-geranyl pyrophosphate molecules into one molecule of phytoene (Buckner et al., 1990). The first branching point of the pathway is the cyclization of lycopene: lycopene-ε-cyclase (*lcyE*) gene located on chromosome 8, converts more lycopene to the β, ε branch, which produces α-carotene and lutein (Harjes et al., 2008). Another key gene, β-carotene hydroxylase (*crtRB1*) present on chromosome 10 causes hydroxylation of α and β-carotene into non-provitamin A carotenoids, viz., lutein and zeaxanthin, respectively (Yan et al., 2010).

Using allele mining strategy, four natural *lcyE* polymorphisms, viz., *lcyE* 5′TE (Transposable Element; in 5′-untranslated region - UTR), *lcyE* SNP216 (in exon 1), *lcyE* SNP2238 (in intron 4) and *lcyE* 3′InDel (in 3′-UTR) were identified, of which, the favourable allele of *lcyE* 5′TE causes more increase in provitamin A in the endosperm (Harjes et al., 2008). Yan et al. (2010) through association mapping approach, detected three polymorphisms, viz., 5′TE (in the 5′-UTR), InDel4 (in the coding

Table 2. Polymorphic sites, nature of polymorphism, allelic series and sequences of functional markers of *lcyE* and *crtRB1* genes

Gene	Polymorphic site	Nature of polymorphism	Allelic series	Favourable allele	Functional Marker sequence	Reference
lcyE	*lcyE*-5'TE	938 bp InDel	1,2,3,4	1,4	F : 5'-AAGCATCCGACCAAAATAACAG-3' R : 5'-GAGAGGGAGACGACGAGACAC-3'	Harjes et al. (2008)
	lcyE-SNP 216	G-T SNP	G,T	G	F : 5'-GCGGCAGTGGGCGTGGAT-3' R: 5'-TGAAGTACGGCTGCAGGACAACG-3'	
	lcyE-3'InDel	8 bp InDel	8,0	8	F : 5'-ACCCGTACGTCGTTCATCTC- 3' R : 5'-ACCCTGCGTGGTCTCAAC-3'	
crtRB1	*crtRB1*-5'TE	397/206 bp InDel	1,2,3	2	F: 5'-CTCTGTGTTAGAGCCTCTGTG-3' R: 5'AATCCCTTTCCATGTACGC-3'	Yan et al. (2010)
	crtRB1-InDel4	12 bp InDel	12,0	12	F: 5'ACCGTCACGTGCTTCGTGCC-3' R : 5'CTTCCGCGCCTCCTTCTC-3'	
	crtRB1-3'TE	325/1250 bp InDel	1,2,3	1	F : 5'-ACACCACATGGACAAGTTCG-3' R1: 5'-ACACTCTGGCCCATGAACAC-3' R2: 5'- ACAGCAATACAGGGGACCAG-3'	

region) and 3'TE (spanning the sixth exon and 3'-UTR) in *crtRB1* that are significantly associated with conversion of β-carotene to β-cryptoxanthin and zeaxanthin in maize kernels and thus it has a significant impact on variation for β-carotene concentration in endosperm (Fu et al., 2013).

Yan et al. (2010) found that provitamin A concentration of haplotypes with *crtRB1*-5'TE and *crtRB1*-3'TE favorable alleles were 5.2 fold higher than those of other haplotypes. According to Azmach et al. (2013) two functional markers of *crtRB1* (i.e. 5'TE and 3'TE markers) are in linkage disequilibrium and display consistent and strong effect on provitamin A carotenoid contents of the inbred lines. Babu et al. (2013) further reported that *crtRB1-3'*TE favorable allele alone causes two to ten fold variation in the β-carotene concentration irrespective of the genetic constitution of *lcyE* and similar results were also reported by Muthusamy et al. (2014).

The favorable allele of *crtRB1* 3'TE with reduced transcript expression causes enhanced accumulation of β-carotene. Transcript expression of these two key genes (*crtRB1* and *lcyE*) is tissue specific; where the difference in expression of wild and mutant alleles is very high in endosperm, while it is not much in embryos and similar in leaves (Babu et al., 2013). PCR based co-dominant functional markers have been designed for both *lcyE* and *crtRB1* based polymorphisms (Table 2) which can pave way for rapid improvement of provitamin A in maize through MAS (Babu et al., 2013). Since these favorable alleles are reported to cause higher accumulation of β-carotene to other carotenoids, identification of genotypes with the favorable allele of these two key genes thus can help in identification of provitamin A rich genotypes without intensive HPLC assay (Vignesh et al., 2012a; Dhyaneswaran, 2012; Babu et al., 2013; Muthusamy et al., 2014 and 2015a; Sagare et al., 2015a and 2015b). Vignesh et al. (2012a) reported very low frequency of favorable allele for both *lcyE* (3.38%) and *crtRB1* (3.90%) while screening large set of maize inbreds in India. Similar results of nil to low frequency of the favorable alleles were also reported (Rashmi and Singh, 2014; Selvi et al., 2014 and Vikal et al., 2014).

Inbreds bred under the CIMMYT-HarvestPlus programme possess ~15 µg/g of β-carotene (Vignesh et al., 2012a). Interestingly, the Indian genotypes with the favorable allele of these genes were quite low in β-carotene, in contrast to the CIMMYT-HarvestPlus genotypes. This phenotypic variation could be attributed to the effect of the genetic background as the concentration of β-carotene is regulated by various genes other than *lcyE* and *crtRB1* in the carotenoid biosynthesis pathway. This could also be attributed to the presence of nucleotide variation within the favorable allele thereby leading to phenotypic variation.

Vignesh et al. (2012b) identified SNPs and InDels in the 3'UTR region of the *crtRB1* favorable allele while comparing a set of high and low β-carotene inbreds and concluded that, those SNPs and InDels can be used as target regions in provitamin A enrichment programme.

In genome wide association studies for various carotenoids Suwarno et al. (2015) identified *crtRB1*, *lcyE* and other key genes/genomic regions governing rate-critical steps in the upstream (*DXS1*, *GPSS1* and *GPSS2* : accumulates precursor isoprenoids) as well as downstream pathway (*HYD5*, *CCD1* and *ZEP1* : causes hydroxylation and carotenoid degradation). They also identified SNPs at or near all of these regions which may be useful target regions for carotenoid biofortification breeding efforts in maize.

Brenda et al. (2014) conducted genome wide association studies for maize grain carotenoids and reported two novel genes : *zep1* (zeaxanthin epoxidase) and *lut1* associated with maize grain carotenoids. They

identified SNPs associated with zeaxanthin and total β-xanthophylls in the coding region of *zep1*, which fits well with the activity of the encoded enzyme in converting zeaxanthin to violaxanthin via antheraxanthin. They also identified a SNP in the *lut1*-coding region associated with α-carotene/zeinoxanthin, zeinoxanthin/lutein, and zeinoxanthin. This SNP is consistent with the enzymatic activity of lut1 in forming lutein by hydroxylation of thee ring of zeinoxanthin.

In the *zep1* region, QTL have been identified for levels of β-branch carotenoids, zeaxanthin, β-cryptoxanthin and β-carotene (Kandianis et al., 2013) and for degree of orange color (Chandler et al., 2013), a trait associated with higher levels of zeaxanthin (Pfeiffer and McClafferty, 2007).

Marker assisted backcross breeding for enhancing provitamin A

Marker assisted selection (MAS) is regarded as a key method for increasing provitamin A concentrations in maize (Prasanna et al., 2010). The effectiveness of molecular marker polymorphisms in linking *lcyE* and *crtRB1* to provitamin A concentrations has been verified using 26 tropical maize populations and the functional gene markers for high provitamin A concentration have been used in MAS (Azmach et al., 2013). As Benchimol et al. (2005) pointed out that through a backcross breeding program, source genes related to high provitamin A concentration can be integrated into genotypes with elite agronomic traits of the recurrent parents. However, one of the major limitations is the long period of time required for the backcross procedure. Therefore, molecular markers are important tools for accelerating the recovery of recurrent parent genome as well as assisting in the selection of plants that carry a desired marker linked to high provitamin A concentration (Bouchez et al., 2002). Marker assisted backcrossing is highly suited to monitoring the degree of similarity of the lines to the recurrent parent.

Maize breeders at IARI (Indian Agricultural Research Institute), New Delhi successfully introgressed *crtRB1* favourable allele from CIMMYT maize lines in to seven elite parental inbreds using MAS (Muthusamy et al., 2014). These inbreds are parents of high yielding commercial maize hybrids in India. The reconstituted hybrids developed from improved parental inbreds also showed enhanced kernel β-carotene as high as 21.7µg/g compared to 2.6µg/g in the original hybrid (Muthusamy et al., 2014). These improved hybrids possessed similar grain yield potential as compared to original hybrids. Improved version of Vivek QPM hybrid-9 developed through MABB possesses high β-carotene coupled with higher lysine and tryptophan, thereby providing multi-nutrients through maize based diet.

In China, Liu et al. (2015) successfully introgressed *crtRB1* favourable alleles (*crtRB1*-5'TE-2 and *crtRB1*-3'TE-1) from maize inbred Hp321-1 into QPM inbred lines, CM161 and CM171. The mean provitamin A concentration was improved from 1.60µg/g to 5.25µg/g in CM161 and from 1.80µg/g to 8.14µg/g in respective BC2F3 offsprings while maintaining similar QPM characteristics of recurrent parent. In Africa maize β-carotene enrichment program is at the peak and they have developed biofortified maize rich in tryptophan and lysine, Fe, Zn and β-carotene. Three maize hybrids from Zambia (GV662A, GV664A, GV665A), and two hybrids (Ife maize hyb-3, Ife maize hyb-4) from Nigeria (Crops Research Institute (CRI) of the Council for Scientific and Industrial Research (CSIR), Honampa) were released that contain 6 to 8 µg/g of provitamin A (www.harvestplus.org). At CIMMYT, MABB program is being carried out to breed tropical maize varieties with 15µg/g β-carotene, the target level as set by HarvestPlus for alleviating the widespread VAD in humans (Babu et al., 2013).

Development of provitamin A rich maize in India using natural mutants

Considering the low levels of β-carotene in the Indian maize germplasm, CIMMYT-HarvestPlus genotypes with favourable allele of *lcyE* and *crtRB1* with high β-carotene have been used as donors in the Indian maize biofortification programme. Maize breeders at IARI successfully introgressed *crtRB1* favourable allele in to seven elite parental inbreds, viz., VQL1, VQL2, V335, V345, HKI1105, HKI323 and HKI161; using MAS (Muthusamy et al., 2014). These inbreds are parents of four high yielding commercial maize hybridsin India, viz., Vivek QPM-9, Vivek Hybrid-27, HM-4 and HM-8. The improved inbreds contained kernel β-carotene ranging from 8.6 to 17.5µg/g; much closer to 15µg/g, the target level set by HarvestPlus for alleviating VAD. The reconstituted hybrids developed from improved parental inbreds also showed enhanced kernel β-carotene as high as 21.7µg/g, compared to 2.6µg/g in the original hybrid (Muthusamy et al., 2014). These improved hybrids possessed similar grain yield potential as compared to original hybrids. This is the first-ever demonstration of conversion of elite maize hybrids into β-carotene-rich version using MABB approach.

MAS derived hybrid Vivek QPM-9 possesses high β-carotene coupled with higher lysine and tryptophan, thereby providing multi nutrients through maize-based diet. This is the first successful example of combination of nutrients, viz., provitamin A and QPM (Muthusamy

et al., 2014). The β-carotene enriched hybrid Vivek QPM-9 recorded a grain yield compared to the original hybrid Vivek QPM-9 and is presently under testing in the All India Coordinated Maize Improvement Project (Gupta et al., 2015a and 2015b). Parental lines of the hybrids HM-4 and HM-8 are also targeted for improvement of lysine and tryptophan in a separate breeding programme (Hossain et al., 2014) and the efforts are on to combine QPM and provitamin A.

MAS is being used to pyramid favourable alleles, viz., *lcyE* and *crtRB1* to further enhance kernel β-carotene in QPM hybrids. Currently, maize breeding programme at IARI, VPKAS, CSK-HPKV, PJTSAU (Formerly part of ANGRAU), GBPUAT and Tamil Nadu Agricultural University (TNAU), Coimbatore are actively involved in generating/selecting diverse inbreds with high β-carotene. A diverse set of inbreds with favourable alleles of lcyE and/or crtRB1 have been characterized for their effective utilization in the breeding programme (Choudhary et al., 2014, Choudhary et al., 2015 and Sagare et al., 2015a and 2015b).

Recent advances in development of provitamin A rich maize

The major challenge in breeding for enhanced provitamin A in maize is the loss of β-carotene during post-harvest/processing stages (De-Moura et al., 2013). Since, carotenoids are highly heat labile, it is essential to develop biofortified maize to sustain the carotenoid level during the post-harvest handling and processing.

Studies at CIMMYT have shown that loss of provitamin A is higher at initial stages of storage and becomes stable after 6-8 weeks. However, degradation is also influenced by genetic background and few inbreds with lesser degradation during the storage have been identified (De-Moura et al., 2013; Suwarno et al., 2015). A native variant of CCD1 (carotenoid cleavage dioxygenase 1) that causes reduced loss of provitamin A during storage has been recently identified (Suwarno et al., 2015). Thus, research efforts need to be directed to develop maize genotypes that retain higher levels of provitamin A for a longer period of time while storage.

Transgenic approach for enrichment of provitamin A

Transgenic approach using over expression of *crtB* (phytoene synthase) and *crtI* (carotene desaturase) genes from *Erwinia herbicola* under the control of γ-zein promoter resulted in accumulation of 10µg/g of β-carotene in Hi-II maize genotype (Aluru et al., 2008). This result represents an important step forward in the development of high provitamin A maize. Subsequently, Zhu et al. (2008) and Naqvi et al. (2009) transformed white maize genotypes (M37W) with combination of five genes (*psy1*, *crtI*, *lycb*, *bch* and *crtW*) and achieved ~60µg/g of β-carotene in transgenic plants having *psy1* from *Zea mays* and *crtI* (Carotene desaturase) from *Pantoea ananatis*. Despite development of transgenic maize lines with very high β-carotene in its endosperm, commercial production of β-carotene rich maize cultivar is yet to become a reality. However, the report of Zhu et al. (2008) and Naqvi et al. (2009) have generated high hopes.

Quantification of provitamin A carotenoids

Provitamin A carotenoids in maize kernels may lead to different colors in the endosperm, varying from light yellow to dark orange (Weber, 1987). However, there is a low correlation between visual grain color and total carotenoids, β-carotene and β-cryptoxanthin in diverse inbreds and screening for high provitamin A concentration based on kernel color is not considered reliable (Harjes et al., 2008 and Mishra and Singh, 2010).

For quantification of carotenoids spectrophotometric and chromatographic methods are used. Although the visible light range (400-1100 nm) is important for predicting carotenoid content in maize grain, calibration curves for estimating carotenoid concentrations using near infra-red reflectance spectroscopy (NIRS) have been successful for estimating the major carotenoids (lutein and zeaxanthin) and total carotenoid, but not for provitamin A carotenoid concentrations (Berardo et al., 2009). There are some reports on estimation of total carotenoids through colorimetric methods (Mishra and Singh, 2010; Tiwari et al., 2012 and Sivaranjani et al., 2013 and 2014)

In chromatographic methods, thin layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC) are the commonly used methods. TLC is not adequate for quantitative analysis because of the danger of degradation and isomerization on highly exposed plate. Carotenoids are particularly prone to oxidation by air when adsorbed on TLC plates. Additionally, it is not easy to quantitatively apply the sample on the plate and quantitatively recover the separated carotenoids from the plate for measurement. GC is also inappropriate because of the thermal liability and low volatility of carotenoids.

HPLC method is widely used for measuring provitamin A concentrations. But, HPLC is expensive, time consuming and has low throughput, limiting its use for routine screening in conventional maize breeding programs (Pfeiffer and McClafferty, 2007). Now days, Ultra performance liquid chromatography (UPLC) method is being used for provitamin A carotenoids estimation. UPLC is a very good alternative to HPLC because its costs for reagents are lower and throughput is three times that of HPLC. Neither HPLC nor UPLC enable ef-

ficient and affordable analysis of the many thousands of samples required each year by a breeding programme (Babu et al., 2013).

Favourable allele possessing rare genetic variation in *crtRB1* gene is associated with higher accumulation of provitamin A carotenoids, especially β-carotene and selection of this allele holds immense promise in reducing large scale phenotypic assays (Muthusamy et al., 2015a). Previous studies have been reported a strong relation between allele1 of *crtRB1*-3'TE and β-carotene concentration in maize kernel (Pixley et al., 2011; Vignesh et al., 2012a; Dhyaneswaran, 2012; Babu et al., 2013; Muthusamy et al., 2014 and 2015a and Sagare et al., 2015a and 2015b). Therefore, screening maize inbreds for favourable allele1 of *crtRB1*-3'TE (PCR based assay) is an alternative for HPLC to identify β-carotene rich maize inbreds.

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