International Journal of Advanced Biotechnology and Research (IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol 6, Issue1, 2015, pp86-95
http://www.bipublication.com

Review Article

Enhancing Provitamin A of Maize using functional gene markers

Deepti B. Sagare*, S. Sokka Reddy,
Prashant Shetti and M. Surender

Department of Molecular Biology and Biotechnology,
Professor Jayashankar Telangana State Agricultural University
(Formerly part of Acharya N. G. Ranga Agricultural University,
Rajendranagar, Hyderabad-500030, Telangana, India
* Corresponding Author: Email: deepti.sagare@gmail.com

[Received-11/01/2015, Published- 30/01/2015]

ABSTRACT:

Vitamin A deficiency is a global health problem and can be effectively alleviated by improved nutrition. Development of cereal crops with increased provitamin A carotenoids can provide a sustainable solution to eliminating vitamin A deficiency worldwide. Maize is the only major cereal crop that can naturally accumulate appreciable levels of provitamin A in the kernels. Maize germplasm resources exhibit wide genetic variation and allelic diversity for carotenoid components and carotenoid biosynthesis genes. The favourable alleles of key genes in the carotenoid biosynthetic pathway are associated with higher accumulation of β-carotene in the maize kernel. Functional markers were developed and demonstrated for use in selecting favorable alleles of the key genes, *LcyE* (*lycopene epsilon cyclase*), *CrtRB1* (*β-carotene hydroxylase*) and *PSY1* (*phytoene synthase1*) loci. Selection for these alleles will greatly benefit in identification of genotypes with higher β-carotene concentration, thus reducing the need for large scale phenotypic assays. Here, we discuss maize carotenoid biosynthesis pathway, genetic variability for the key genes and functional markers for the enhancement of provitamin A concentration in edible maize endosperm. We also discuss challenges for optimizing provitamin A carotenoid biofortification of maize. This knowledge will be helpful to understand the involvement of the functional markers of key genes of carotenoid pathway for enhancing provitamin A level of the maize kernels through biofortification.

Keywords: maize, provitamin A, β-carotene, LcyE & CrtRB1, PSY1, functional markers, biofortification.

INTRODUCTION:

Provitamin A refers to the carotenoids that can be converted into physiologically activated vitamin A (retinol) in the human body and includes α-carotene, β-carotene, and β-cryptoxanthin. Moreover, β-carotene can generate two molecules of vitamin A, while α-carotene and β-cryptoxanthin, which only have a single non-hydroxylated β-ring, can only produce one vitamin A [13]. Vitamin A is an essential nutrient needed by the humans for normal functioning of visual system, growth and development and maintenance of epithelial cell integrity, immune system and reproduction. World Health Organisation (WHO) recommends estimated average requirements of 250 and 500 RE (Retinol Equivalents) per day for childrens and adults respectively, for their normal growth and development [6]. Humans are unable to

synthesize their own vitamin A requirements and dietary needs for vitamin A are normally provided as preformed retinol or provitamin A carotenoids from plant based foods. WHO defines Vitamin A deficiency (VAD) as "if the tissue concentration of vitamin A is low enough to have adverse health consequences even if there is no evidence of clinical xerophthalmia" [45]. VAD alone affects over 250 million people worldwide and accounts for about 70% of the childhood deaths across the world [46]. WHO estimates that 250,000 to 500,000 children become blind every year due to VAD. It contributes to predisposition to several infectious diseases such as anemia, diarrhea, measles, malaria and respiratory infections. VAD also constributes to maternal death, malnourished pregnancy and poor lactation, making the young children, pregnant women and lactating mothers most vulnerable [5].India is the home of 120 million pre-school children of which 36 million are estimated to be vitamin A deficient. Among them 1.17 million pre-school children are affected by night blindness and it is estimated that nearly 20,000 children go blind every year in India due to severe VAD [44]. India also faces a severe burden of maternal VAD with 50% of the total six million reported case of maternal night blindness living in the country [44]. A provisional estimate suggests that VAD could be a significant problem in pre-adolescent (1 -15 years) children as well [36]. Thus any effort directed to minimize VAD will have a positive impact on the health of humans. VAD is continuous and serious health problem in many countries, a multipronged strategy were used worldwide to alleviate and combat the VAD related health problems. Dietary diversity, food biofortification, supplement tactics and crop biofortification have been suggested to solve VAD problem. The first three solutions are expensive and inaccessible for the poverty in developing countries, reducing their efficiency and application (FAMOBIB FAO/Food Nutrition Division). Also, few studies using high doses of synthetic β-carotene supplements have shown an increased risk of susceptibility to some diseases, not a decrease. This suggests food sources containing natural carotenoids may be more beneficial than vitamin supplements [39]. Biofortification is the breeding of staple food crops to increase micronutrient density [6, 29]. Because of the widespread consumption of staple crops, crop biofortification may be an effective and sustainable way of addressing micronutrient malnutrition including VAD [16].

MAIZE AS A MODEL CROP FOR NUTRITIONAL ENHANCEMENT

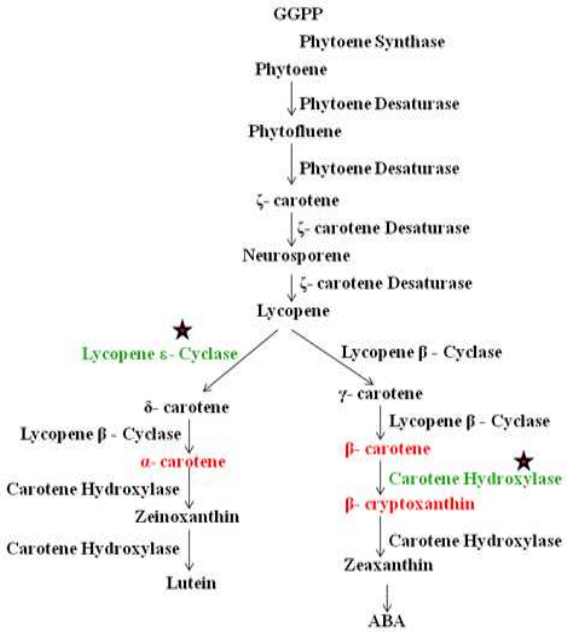
In the 1930s, the discovery of increased nutrition in yellow maize grain [25] led to selection of pigmented grain as a desirable quality trait for both human food and animal feed [4, 43]. At present, the developed world uses more maize than the developing world, but forecasts indicate that by the year 2050, the demand for the maize in the developing countries will double [34]. Maize is the only major cereal crop that can naturally accumulate appreciable levels of provitamin A in the kernels [48]. Maize germplasm resources exhibit wide genetic variation and allelic diversity for carotenoid components and carotenoid biosynthesis genes [9, 18, 49] and thus, are an obvious target for biofortification project. Maize has been targeted for biofortification for other nutrients for decades and the efforts were largely successful. 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 [18]. Provitamin A biofortification of maize has become a feasible approach to address the challenge of VAD in developing countries [18, 49].

PROVITAMIN A SYNTHESIS IN MAIZE

In maize carotenoid biosynthesis occurs during seed development [21] and the accumulation of carotenoids imparts a yellow-orange color to the endosperm, an easily scored phenotype. In maize seed endosperm, the primary carotenoids that accumulate in diverse cultivars are either lutein or zeaxanthin or a combination of both. Provitamin A compounds are biosynthetic pathway intermediates and therefore usually not

the predominant carotenoids in endosperm, the target of provitamin A biofortification. Vitamin A is a C_{20} enzymatic cleavage product made in humans from plant carotenoids containing an unmodified β-ring [42]. α-carotene and β-cryptoxanthin have provitamin A potential, due to their single unmodified β-ring, but β-carotene is the most efficient source, as two retinol molecules may be derived from each β-carotene molecule. Carotenoids are derived from products of Glycolysis or Isoprenoid biosynthesis. Plastid localized methylerythritol 4-phosphate (MEP) supplies isoprenoid precursors for carotenoids. Glyceraldehyde-3-phosphate and pyruvate are combined to form deoxy-D-xylulose 5-phosphate (DXP), a reaction catalyzed by *DXP synthase* (DXS), and a number of steps are then required to form geranylgeranyl diphosphate (GGPP), the precursor to carotenoid biosynthesis as well as to other biosynthetic pathways [33]. The first carotenoid, phytoene, is produced by the condensation of two GGPP molecules, a reaction that is catalyzed by *phytoene synthase*1 (*PSY1*). Two desaturases (*PDS*, *phytoene desaturase*; *ZDS*, *ζ-carotene desaturase*) and two isomerases (*Z-ISO*, *ζ-carotene isomerase; CRTISO, carotenoid isomerase*) introduce a series of double bonds and alter the isomer state of each biosynthetic intermediate to produce all trans lycopene. At this point the main biosynthetic pathway branches, depends on cyclization activity. Asymmetric cyclization of lycopene by both *ε-lycopene cyclase* and *β-lycopene cyclase* (*LcyE* and *LcyB*, respectively) produces α-carotene with one ε and one β-ionone ring [12]. Symmetric cyclization by *LcyB* yields β-carotene, with two unmodified β-ionone rings. Hydroxylation of the carotene β-ionone ring by one of two classes of structural distinct carotene hydroxylase enzymes eliminates provitamin A potential. The hydroxylated carotenes include the non-provitamin A xanthophylls, lutein, and zeaxanthin, which may be further modified to other xanthophylls, some of which are cleaved to form ABA [28]. Therefore pathway branching and hydroxylation are the key determinants in controlling provitamin A levels in maize kernels (Figure 1). The details of the carotenoid pathway are depicted in figure 1, based on the information provided by [1].

Fig. 1 : Carotenoid biosynthetic pathway



CANDIDATE GENES INVOLVED IN ACCUMULATION OF PROVITAMIN A

The genes encoding key enzymes in the carotenoid biosynthesis pathway namely; *phytoene synthase 1* (*PSY1*), *phytoene desaturase* (*PDS*), *ζ-carotene desaturase* (*ZDS*), *lycopene □-cyclase* (*LcyE*), *lycopene β-cyclase* (*LcyB*), *β-carotene hydroxylase 1* (*CrtRB1*) have been elucidated and cloned in plants. The genes encoding key enzymes in the carotenoid biosynthesis pathway and their location in the genome are represented in table 1.

Table 1 : Maize genes encoding key enzymes in the carotenoid biosynthesis pathway and their location.

Gene encoding enzyme	Location		Reference
	Chromosome	Bin	
phytoene synthase 1(*PSY1*)	6	6.01	Buckner et al. (1996)
phytoene desaturase (*PDS*)	1	1.02	Hable et al. (1998)
ζ-carotene desaturase (*ZDS*)	7	7.02	Matthews et al. (2003)
lycopene β-cyclase (*LcyB*)	5	5.04	Singh et al. (2003)
lycopene □-cyclase (*LcyE*)	8	8.05	Harjes et al. (2008)
β-carotene hydroxylase 1 (*CrtRB1*)	10	10.06	Yan et al. (2010)

***phytoene synthase* (*PSY1*)**

As early as 1940, the yellow 1 (*Y1*) locus was found to have a gene dosage effect on seed carotenoid content [32]. Cloning and subsequent sequence analyses identified the *Yellow 1* (*Y1*) gene as encoding *PSY1* (*phytoene synthase1*) which play a vital role by condensing two Geranylgeranyl pyrophosphate (GGPP) molecules into one molecule of phytoene [7, 8]. 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 [7]. 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 [23]. Overexpression of the PSY1 gene in white kernels leads to significant carotenoid accumulation, confirming the essential role of PSY1 for carotenoid biosynthesis in maize [50]. The *Y1* gene was mapped to chromosome 6 (bin 6.01), and was cloned by Robertsons' mutator transposon tagging [8]. The strong influence of dosage effect of *Y1* on quantitative variation for carotenoids has been well documented [10, 47]. There are two polymorphic sites within PSY1, 378-bp InDel upstream of the transcription start site and a SNP in the fifth exon resulting in a Thr to Asn substitution [15], which accounts 7 and 8 % of the total carotenoid variation respectively, and these may be functional sites associated with total carotenoid levels in maize grain. Analysis of the evolution of PSY1 strongly suggests that there was positive selection for these polymorphic sites after the divergence of yellow maize from white maize.

***phytoene desaturase* (*PDS*) and *ζ-carotene desaturase* (*ZDS*)**

Lycopene, the first colored component, generated from phytoene via desaturation by *phytoene desaturase* and *ζ-carotene desaturase* [20]. *Phytoene desaturase* is the second enzyme in the carotenoid biosynthetic pathway and is responsible for a two step desaturation, taking phytoene to zeta (ζ)-carotene. *Phytoene desaturase* has been associated with the mutant *viviparous 5* (*vp5*), a white endosperm mutant deficient in both carotenoids and ABA. The *vp5* gene has been cloned and mapped to chrosome 1 (bin 1.02) in maize [20, 17]. *ζ-carotene desaturase* is the third enzyme in the carotenoid biosynthetic pathway and is responsible for a two step saturation from ζ-carotene to lycopene. Recently, it was discovered that the maize y9 locus encodes *ζ-carotene isomerase* (*Z-ISO*), a previously unknown pathway enzyme that is necessary for carotenogenesis in all plants [11]. Without *Z-ISO* function, provitamin A carotenoids cannot be produced in the endosperm, the target tissue for biofortification. A cDNA encoding *ZDS* has been cloned and mapped on chromosome 7 (bin 7.02) in maize [24, 26].

***lycopene beta cyclase* (*LcyB*) and *lycopene epsilon cyclase* (*LcyE*)**

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. Alternatively, the coactions of *lycopene beta cyclase* (*LcyB*) and *lycopene epsilon cyclase* (*LcyE* or □*LCY*) generates 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 [31]. 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 immature maize embryos [35]. [18] reported that downregulation of *LcyE* reduces the ratio of the α-carotene branch to the β-carotene branch. The *LcyE* gene has been mapped to chromosome 8 (bin 8.05) near the SSR marker *bngl1599.* The selection of favourable allele for high β-carotene content was invested using allele mining strategy and four natural *LcyE* polymorphisms explained 58 % of the phenotypic variation of provitamin A with a threefold increase compounds between the lines

with the lowest and highest carotenoid contents were reported by [18].

***β-carotene hydroxylase 1* (*CrtRB1/HYD3*)**

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 [27]. 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 [38]. 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 close to a known QTL for β-carotene composition. PCR genotyping of 51 maize lines showed that the HYD3 locus could explain 36% variation and fourfold difference in β-carotene levels [38]. Allele mining of *CrtRB1* gene by [49] confirmed that *CrtRB1* underlines a principle quantitative trait locus associated with β-carotene concentration and its further conversion to other non-provitamin A carotenoids in maize kernels. This gene was mapped on 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.

FUNCTIONAL GENE MARKERS FOR ENHANCING PROVITAMIN A

In maize, three genes have been proposed to play crucial roles in the final accumulation of provitamin A carotenoids in the grain. *Phytoene synthase1* (*Y1* or *PSY1*) catalyses the first committed step in the pathway leading to formation of phytoene from geranylgeranyl diphosphate and is primarily responsible for the shift from white to yellow maize [21]. Two genes, *lycopene epsilon cyclase* (*LcyE*) and *β-carotene hydroxylase 1* (*CrtRB1*) have been shown to regulate the accumulation of provitamin A compounds [18, 49]. [18] identified 3 polymorphic sites in *LcyE* gene (*LcyE*-5'TE, *LcyE*-SNP216 and *LcyE*-3'InDel) associated with reduction in lutein content and concomitant increase in total provitamin A concentration. [49] identified 3 polymorphic sites in C*rtRB1* (*CrtRB1*-3'TE, *CrtRB1*-InDel4 and *CrtRB1*-5'TE) accounting for 40% of the observed variation in β-carotene concentration in maize endosperm and found *CrtRB1*-3'TE polymorphisms are more consistent than that of *CrtRB1*-5'TE. [40] identified 10 SNPs and 6 InDels in the 3'- untranslated region (UTR) of allele1 of *CrtRB1*-3'TE gene which could be associated with the variation in kernel β-carotene accumulation by regulating the translation and stability of the mRNA. These SNPs and InDels associated with higher level of β-carotene will be used as a gene based markers in selection of genotype and to develop biofortified maize to alleviate vitamin A. [15] identified two polymorphisms in the gene encoding *phytoene synthase* (*PSY1*-InDel1 and *PSY1*-SNP7), explaining 7 to 8% of the variation in total carotenoids. Molecular markers based on functional polymorphisms within *Psy1, LcyE* and *CrtRB1,* hold great potential for accelerated and resource efficient development of provitamin A enriched lines. With the development of PCR-based markers for these genes and an understanding of the different frequencies of favorable alleles for each gene among different germplasm, breeders can now use targeted strategies to improve the trait in any germplasm pool [15]. Nomenclature of functional DNA markers and their allelic series in represented in table 2. The *PSY1, LcyE* and *CrtRB1* genes affect provitamin A levels and composition differently. The high level of natural variation for provitamin A components largely relates to the upregulation of *PSY1* [14], but downregulation of *LcyE* and *CrtRB1*[49], which coincide with the biological functions of these genes. Combining favorable alleles for *LcyE* and *CrtRB1* will increase the content of β-

carotene at the expense of other carotenoid components, whereas *PSY1* can increase β-carotene content by increasing the amount of substrate flowing into the carotenoid biosynthesis pathway. The favourable allele of *PSY1* are almost fixed in the tropical background and favorable alleles of *LcyE* and *CrtRB1*, however, are very rare in tropical germplasm and may be targeted for high provitamin A maize breeding the tropical region [15]. *CrtRB*-3'TE had large, significant effect on enhancing β-carotene and total provitamin A content, irrespective of genetic constitution for *LcyE*-5'TE and Marker Assisted Selection (MAS) for favorable 'allele 1' of *CrtRB1* can lead to rapid doubling, or more, of total provitamin A concentration. In contrast, MAS for favorable 'allele 4' of *LcyE* generally results in 20-30 % increase in total provitamin A concentration. Thus, MAS for the *CrtRB1* locus alone appears to be a reliable strategy for rapidly achieving genetic gains for β-carotene and total provitamin A carotenoids in tropical maize breeding programs [3]. Tropical maize inbred lines harbouring the favourable alleles of the *CrtRB1*-5′TE and C*rtRB1*-3′TE functional markers produce higher levels of provitamin A which can be used as donor parents to speed up the development of provitamin A biofortified tropical maize [2]. The β-carotene concentration of Indian maize genotypes is low eventhough they posses favourable allele of *CrtRB1*-3'TE [41]. However, the CIMMYT-HarvestPlus bred maize inbreds with high kernel β-carotene and *CrtRB1*-3'TE favourable allele [37] offer tremendous possibility to breed for high β-carotene maize through MAS. Using these β-carotene enriched genotype as donors, marker assisted backcross breeding (MAB) programme has been already initiated at several research institutes in India. The *CrtRB1*-3'TE favourable allele from high β-carotene genotypes is being introgressed into tropical inbreds to reach the HarvestPlus's target level of 15 µg/g of kernel β -carotene in the tropical maize hybrids. Functional markers located within the target genes offer efficient means of tracking the favorable alleles in backcross or pedigree breeding programs. However, genetic background in which these favorable alleles reside, population size, nature of gene action (additive or epistatic), trait heritability and marker trait relationships influence the effectiveness of such MAS in routine breeding programs. Though the potential applications of MAS are attractive for selecting natural variants of large effect carotenoid genes, it is rarely a straight forward exercise in a practical breeding program. Applying these markers CIMMYT breeders have already conducted preliminary evaluations of tropical maize lines from crosses introducing favourable alleles for *LcyE* and *CrtRB1,* the key genes in β-carotene biosynthesis pathway [30]. Large scale validation of the effect of molecular marker polymorphism in these two genes was carried out at CIMMYT, and the results suggests that by doing MAS with markers reported for these genes, it would be possible to breed tropical maize varieties with high concentration of β-carotene for alleviating the widespread VAD in Humans [3].

Table 2 : Nomenclature of functional DNA markers and their allelic series

Gene	Polymorphic site	Nature of polymorphism	Allelic series	Favourable allele	Reference
PSY1	*PSY1*-SNP 7	A-C SNP	A, C	A	Fu et al. (2013)
	PSY1-InDel1	378 bp InDel	0, 378	378	
LcyE	*LcyE*-5'TE	250 bp InDel	1,2,3,4	1,4	Harjes et al. (2008)
	LcyE-SNP 216	G-T SNP	G,T	G	
	LcyE-3'InDel	8 bp InDel	8, 0	8	
CrtRB 1	*CrtRB1*-5'TE	397/206bp InDel	1,2,3	2	Yan et al. (2010)
	CrtRB1-InDel4	12 bp InDel	12, 0	12	
	CrtRB1-3'TE	325/1250 bp InDel	1,2,3	1	

LOOKING FORWARD TO ENHANCE PROVITAMIN A IN MAIZE

Although β-carotene accumulation via *CrtRB1* and *LcyE* is currently the primary selection target for provitamin A biofortification, any potential improvement is limited unless the total flux of GGPP into the carotenoid biosynthesis pathway can be increased [3]. An additional promising approach that remains to be tested is the endosperm specific upregulation of isoprenoid precursor synthesis. Transcript abundance of several isoprenoid genes was found to positively correlate with endosperm carotenoid content [38] and thus those genes are potential biofortification targets. Genetic control of maize grain carotenoid profiles is coordinated through several loci distributed throughout three secondary metabolic pathways (MEP, Isoprenoid and Carotenoid), most of which exhibit additive, and more importantly, pleiotropic effects [19]. Transgenic manipulations may offer the most expedient approach to control these additional targets given the absence of known regulatory factors to control multiple steps. Transgenic maize plants have been engineered to accumulate a wide variety of carotenoid intermediates and unusual keto-carotenoids and seeds ranged from white and yellow to dark-red, despite the white-endosperm genetic back-ground [1, 50]. This achievement demonstrates remarkable plasticity in carotenoid accumulation and indicates that the targets identified by metabolite sorting and transcript profiling can be successfully manipulated. Combining systems biology tools in an effective way will make for significant advances in metabolic engineering and biofortification of provitamin A carotenoids in cereal food crops. Improving the nutritional composition of such staples could have a positive impact on the health of millions of people worldwide.

ACKNOWLEDGMENTS:

DBS is thankful to Department of Science and Technology, Government of India, New Delhi for providing INSPIRE Fellowship for pursuing PhD.

REFERENCES :

1. Aluru M, Xu Y, Guo R., Wang ZG, Li SS, White W, Wang K, Rodermel S (2008) Generation of transgenic maize with enhanced provitamin A content. J Exp Bot 59: 3551-3562.
2. Azamach G, Gedil M, Menkir A, Spillane C (2013) Marker-trait association analysis of functional gene markers for provitamin A levels across diverse tropical yellow maize inbred lines. BMC Plant Biol 13: 227.
3. Babu R, Rojas NP, Gao S, Yan J, Pixley K (2013) Validation of the effects of molecular marker polymorphisms in LcyE and CrtRB1 on provitamin A concentrations for 26 tropical maize populations. Theor Appl Genet 126: 389-399.
4. Bauernfeind JC, Adams CR, Marusich WL (1981) Carotenoids as colorants and vitamin A precursors. Academic Press, New York.
5. Black RZ, Allen LH, Bhutta ZA, Caulfield LE, de Onis M, Ezzati M, Mathers C, Rivera J (2008) Maternal and Child under Nutrition study group: Global and regional exposures and health consequences. The Lancet 371: 243-260.
6. Bouis HE, Welch RM (2010) Biofortification - a sustainable agricultural strategy for reducing micronutrient malnutrition in the global South. Crop Sci 50: S20-S32.
7. Buckner B, Kelson TL, Robertson DS (1990) Cloning of the y1 locus of maize, a gene involved in the biosynthesis of carotenoids. Plant Cell 2: 867-876.
8. Buckner B, Miguel PS, Janickbuckner D, Bennetzen JL (1996) The Y1 gene of maize codes for phytoene synthase. Genetics 143: 479–488.
9. Chander S, Guo YQ, Yang XH, Zhang J, Lu XQ, Yan JB., Song TM, Rocheford TR, Li JS (2008a) Using molecular markers to identify two major loci controlling carotenoid contents in maize grain. Theor Appl Genet 16: 223-233.
10. Chander S, Meng YJ, Zhang YR, Yan JB, Li JS (2008b) Comparison of nutritional traits variability in selected eighty-seven inbreds

from Chinese maize (Zea mays L.) germplasm. J Agr Food Chem 56: 6506-6511.
11. Chen Y, Li FQ, Wurtzel ET (2010) Isolation and characterization of the Z-ISO gene encoding a missing component of carotenoid biosynthesis in plants. Plant Physiol 153: 66-79.
12. Cunningham FX, Gantt E (2001) One ring or two? Determination of ring number in carotenoids by lycopene epsilon-cyclases. Proceedings of the National Academy of Sciences U.S.A 98, 2905.
13. Davis C, Jing H, Howe JA, Rocheford T, Tanumihardjo SA (2008) β-Cryptoxanthin from supplements or carotenoid enhanced maize maintains liver vitamin A in Mongolian gerbils (*Meriones unguiculatus*) better than or equal to β-carotene supplements. Br J Nutr 100: 1-8.
14. Fu ZY, Yan JB, Zheng YP, Warburton ML, Crouch JH, Li JS (2010) Nucleotide diversity and molecular evolution of the PSY1 gene in Zea mays compared to some other grass species. Theor Appl Genet 120: 709-720.
15. Fu Z, Chai Y, Zhou Y, Yang X, Warburton ML, Xu S, Cai Y, Zhang D, Li J, Yan J (2013) Natural variation in the sequence of PSY1 and frequency of favorable polymorphisms among tropical and temperate maize germplasm. Theor Appl Genet 126: 923–935.
16. Graham RD, Welch RM, Bouis HE (2001) Addressing micronutrient malnutrition through enhancing the nutritional quality of staple foods: principles, perspectives and knowledge gaps. Adv Agron 70: 77-142.
17. Hable WE, Oishi KK, Schumaker KS (1998) Viviparous-5 encodes phytoene desaturase, an enzyme essential for abscisic acid (ABA) accumulation and seed development in maize. Mol Gen Genet 257: 167-176.
18. Harjes CE, Rocheford T, Bai L, Brutnell T, Kandianis CB, Sowinski SG, Stapleton AE, Vallabhaneni R., Williams M, Wurtzel ET, Yan JB, Buckler ES (2008)Natural genetic variation in lycopene epsilon cyclase tapped for maize biofortification. Science 319: 330-333.
19. Kandianis CB, Stevens R, Liu W, Palacios N, Montgomery K, Pixley K, White WS, Rocheford T (2013) Genetic architecture controlling variation in grain carotenoid composition and concentrations in two maize populations. Theor Appl Genet 126: 2879-2895.
20. Li ZH, Matthews PD, Burr B, Wurtzel ET (1996) Cloning and characterization of a maize cDNA encoding phytoene desaturase, an enzyme of the carotenoid biosynthetic pathway. Plant Mol Biol 30: 269-279.
21. Li FQ, Vallabhaneni R, Yu J, Rocheford T, Wurtzel ET (2008) The maize phytoene synthase gene family : Overlapping roles for carotenogenesis in endosperm, photomorphogenesis, and thermal stress tolerance. Plant Physiol 147: 1334-1346.
22. Li FQ, Tzfadia O, Wurtzel ET (2009) The phytoene synthase gene family in the Grasses. Plant Signal Behav 4 N: 208-211.
23. Linden H, Lucas MM, Rosarlo de FM, Sandman G (1993) Immunogold localization of *phytoene desaturase* in higher plant chloroplasts. Physiologia Plantarum 88: 229-236.
24. Luo R, Wurtzel ET (1999). A maize cDNA encoding zeta carotene desaturase. Plant Physiol 120 : 1206.
25. Mangelsdorf PC, Fraps GS (1931) A direct quantitative relationship between vitamin A in corn and the number of genes for yellow pigmentation. Science 73: 241-242.
26. Matthews PD, Luo R, Wurtzel ET (2003) Maize phytoene desaturase and zeta carotene desaturase catalyze a poly Z desaturation pathway: implications for genetic engineering of carotenoid content among cerealcrops. J Exp Bot 54: 2215-2230.
27. Matthews PD, Wurtzel ET (2007) Food colorants: chemical and functional properties [Socaciu, C. (ed.)]. CRC, Boca Raton, FL, pp. 347-398.
28. North HM, De Almeida A, Boutin JP, Frey A, To A, Botran L, Sotta B, MarionPoll A

(2007) The Arabidopsis ABA-deficient mutant aba4 demonstrates that the major route for stress induced ABA accumulation is via neoxanthin isomers. Plant J 50: 810-824.

29. Pfeiffer WH, McClafferty B (2007) HarvestPlus: breeding crops for better nutrition. Crop Sci 47: S88-S105.
30. Pixley K, Palacious N, Babu R, Menkir A (2011) Maize Harvest Plus: Biofortying Maize with Provitamin A carotenoids. In: Book of extended summaries of the 11th Asian Maize Conference, Nanning, China, pp. 250-252.
31. Pogson B, McDonald K, Truong M, Britton G, DellaPenna D (1996) Arabidopsis carotenoid mutants demonstrates lutein is not essential for photosynthesis in higher plants. Plant Cell 8: 1627- 1639.
32. Randolph LF, Hand DB (1940) Relation between carotenoid content and number of genes per cell in diploid and tetraploid corn. J Agric Res 60: 51-64.
33. Rodriguez-Concepcion M (2010) Supply of precursors for carotenoid biosynthesis in plants. Arch. Biochem. Biophys 504: 118-122.
34. Rosegrant MR, Ringler C, Sulser TB, Ewing M, Palazzo A, Zhu T (2009) Agriculture and Food security under Global Change: Prospectus for 2025/2050. IFPRI, Washington, D. C.
35. Singh M, Lewis PE, Hardeman K, Bai L, Rose JKC, Mazourek M, Chomet P, Brutnell TP (2003) Activator mutagenesis of the pink scutellum1/viviparous7 locus of maize. Plant Cell 15: 874-884.
36. Singh V, West KP (2004) Vitamin A deficiency and xerophthalmia among school aged children in South Eastern Asia. Eur. J Clin Nutr 58 : 1342-1349.
37. Vallabhaneni R, Gallagher CE, Licciardello N, Cuttriss AJ, Quinlan RF, Wurtzel ET (2009) Metabolite sorting of a germplasm collection reveals the Hydroxylase3 locus as a new target for maize provitamin A biofortification. Plant Physiol 151: 1635-1645.
38. Vallabhaneni R, Wurtzel ET (2009) Timing and biosynthetic potential for carotenoid accumulation in genetically diverse germplasm of maize. Plant Physiol 150: 562-572.
39. Van den Berg H, Faulks R, Granado HF, Hirschberg J, Olmedilla B, Sandmann G, Southon S, Stahl W (2000) The potential for the improvement of carotenoid levels in foods and the likely systemic effects. J Sci Food Agric 80: 880-912.
40. Vignesh M, Nepolean T, Hossain F, Singh AK, Gupta HS (2012a) Sequence variation in 3'UTR region of *crtRB1* gene and its effect on β-carotene accumulation in maize kernel. J Plant Biochem Biotechnol 22: 401-408.
41. Vignesh M, Hossain F, Nepolean T, Saha S, Agrawal PK, Guleria SK, Prasanna BM, Gupta HS (2012b) Genetic variability for kernel β-carotene and utilization of crtRB1 3'TE gene for biofortification in maize (Zea mays L.). Indian J Genet 72 (2): 189-194.
42. Von Lintig J (2010) Colors with functions: elucidating the biochemical and molecular basis of carotenoid metabolism. Annu Rev Nutr 30: 35-56.
43. Weber EJ (1987) Carotenoids and tocols of corn grain determined by HPLC. J Am Oil Chem Soc 64: 1129-1134.
44. West KP (2002) Extend of Vitamin A deficiency among pre-school children and women of reproductive age. J Nutr 32: 2857-2866.
45. WH0/UNICEF (1996) Indicators of VAD and their use in monitoring intervension programmes. World Health Organisation, Geneva. (WHO/NUT/96.10), PP. 66.
46. WHO (2009) Global prevalence of vitamin A deficiency in populations at risk 1995-2005. In: WHO Global Database on Vitamin A Deficiency, pp. 1-55.
47. Wong JC, Lambert RJ, Wurtzel ET, Rocheford TR (2004) QTL and candidate genes phytoene synthase and zeta-carotene desaturase associated with the accumulation of carotenoids in maize. Theor Appl Genet 108: 349-359.

48. Wurtzel ET (2004) Genomics, genetics, and biochemistry of maize carotenoid biosynthesis. Recent Adv Phytochem 38: 85-110.
49. Yan JB, Kandianis CB, Harjes CE, Bai L, Kim EH, Yang XH, Skinner DJ, Fu ZY, Mitchell S, Li Q, Salas Fernandez MG, Zaharieva M, Babu R, Fu Y, Palacios N, Li JS, DellaPenna D, Brutnell T, Buckler ES, Warburton ML, Rocheford T (2010) Rare genetic variation at *Zea mays* crtRB1 increases β-carotene in maize grain. Nat Genet. 42: 322-327.
50. Zhu CF, Naqvi S, Breitenbach J, Sandmann G, Christou P, Capell T (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. Proc Natl Acad Sci U.S.A 105: 18232-18237.