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Review Article

MARKER ASSISTED BREEDING FOR HIGH β-CAROTENE IN MAIZE

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ABSTRACT
Maize is the economically valuable, commercial crop of global importance, and possesses numerous genotypes worldwide with various specific utilities. Morphological, biochemical and molecular characterization of genotypes and biofortification with nutrients (β-carotene) are the important research areas of maize genotypes including storability of seeds based on kernel colour. Breeding for increasing β-carotene levels in maize (*Zea mays* L.) kernel aims to address the dietary vitamin A deficiency. Due to 3'TE polymorphism, the *crtRB1*gene (that encodes *β-carotene hydroxylase 1*) exists in the three allelic forms, *viz.*, 3'TE allele 1 (termed favorable allele, for it favors higher β-carotene accumulation in kernels), 3'TE allele 2 and 3'TE allele 3 (both termed unfavorable alleles, for they do not favors β-carotene accumulation).

KEY WORDS: Maize, *β-carotene,* biofortification.

INTRODUCTION

One of the target food crops for provitamin a biofortification is maize (*Zea mays* L.). Maize (*Zea mays* L.), the world's most widely grown cereal, is ranked third among major cereal crops and valued as economic crop of global importance widely used in poultry industry and cereal food industry by providing raw materials for starch, gluten, corn oil, corn syrup, sugar, corn meal and corn flour. Hence studies pertaining to these areas in various crops were reviewed here under.

Carotenoids

Carotenoids are responsible for red, orange, and yellow colours present in flowers and fruits and the colour of the specific carotenoid which is determined by the number and location of the double bonds present within their structure (Watson, 1962). Carotenoids are a class of compounds produced by most of the photosynthetic organisms that have been shown to be beneficial to both plants and animals. More than 600 different naturally occurring carotenoids have been identified and are lipophilic (Britton, 1995). It is a C40 is opropenoid compounds that participate in a number of physiological processes in plants and other organisms (Ronen *et al*., 1999; Shewmaker *et al*., 1999). There are two distinct classes of carotenoids - carotenes, which contain only carbon and hydrogen, and xanthophylls, which contain oxygen groups. These compounds are essential in photosynthesis where they function as energy carriers and photo-oxidation protectors (Berg *et al*., 2000).

Carotenoids biosynthesis

In plants, the carotenoid biosynthesis pathway begins with the conversion of geranylgeranyl pyrophosphate (GGPP) into lycopene by a series of enzymes including phytoene synthase (PSY), phytoene dehydrogenase (PDS), ξ-carotene dehydrogenase (ZDS) and carotenoid isomerase (CRTISO) (Buckner *et al*., 1996; Hable *et al*., 1998; Park *et al*., 2002; Matthews *et al*., 2003). Phytoene synthase (PSY) has been shown to be rate-limiting for carotenoid content in several plant tissues, including maize and rice endosperm (Gallagher *et al*., 2004 and Paine *et al*., 2005). Formation of carotenoid pathway precursors competes with the production of chlorophyll (Rodriguez-Concepcion, 2010).

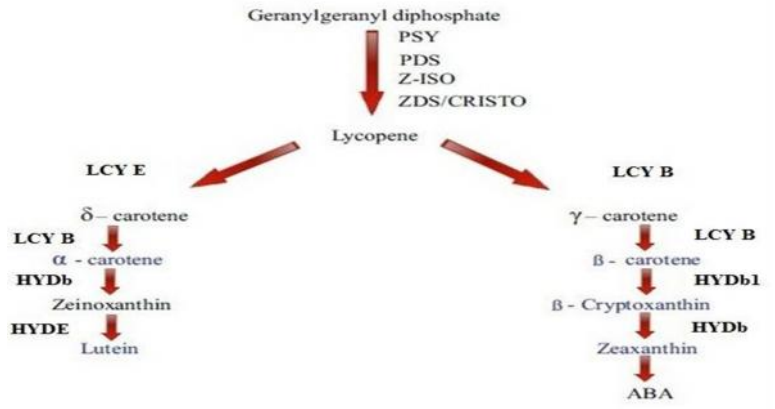

FIGURE 1: Simplified carotenoid biosynthetic pathway in plants

LCYB was cloned by transposon tagging and mapped on chromosome 5 (5.04 bin) (Singh *et al*., 2003). Lycopene represents a branch point (Zhu *et al*., 2003) and the pathway diverges towards two alternate routes to produce (i) ß-carotene by lycopene ß-cyclase (LCYB) (Hirschberg 2001), from which ß-cryptoxanthin and zeaxanthin are further synthesized by ß-ring hydroxylase (crtRB) (Dellapenna and Pogson, 2006), and (ii) α-carotene by lycopene e-cyclase (LCYE), or in combination with LCYB, is further converted into lutein by crtRB and e-ring hydroxylase (Tian *et al*., 2004). As compared to other cereal grains, maize has the greatest phenotypic diversity in β-carotene, β-cryptoxanthin and α-carotene content which are the provitamin A units (Harjes *et al*., 2008).

Enzymatic reactions are represented by arrows. The first arrow depicts multiple enzymatic steps. The compounds are ABA, abscisic acid. The enzymes are PSY, Phytoene Synthase, PDS, Phytoene Desaturase, Z-ISO, 15-cis zetacarotene isomerase; ZDS, Zeta Carotene Desaturase; CRISTO, Carotene isomerase; HYD, Carotene Hydroxylase Enzymes, which include e- and ß- ring hydroxylases (Harjes *et al*., 2008)

Metabolic engineering approaches for Manipulation of carotenoid pathway

Attempts to modify the carotenoid content of seeds have focused on seed-specific manipulation of various steps in the carotenoid pathway. Many different approaches have also been taken to increase provitamin a content in crop plants, such as tomato, potato, and rice, by manipulating various genes of the carotenoid pathway (Romer *et al.*, 2002; Diretto *et al.*, 2006 and Sandmann *et al.*, 2006). In rice, Shewmaker *et al.* (1999) reported that rice endosperm lacks provitamin A due to over expression of the daffodil PSY led to the production of phytoene, but when coupled with expression of the bacterial crtI gene (which mediates the four desaturation reactions) and or the daffodil lycopene β-cyclase gene (LCYB) there was accumulation of lutein, β -carotene, and zeaxanthin (Ye *et al.*, 2000). However, according to Paine *et al. (*2005), 'Golden Rice 2' is the only monocot that has been shown to accumulate substantial amounts of pro-vitamin A to meet the daily requirements to overcome VAD. Aluru *et al*. (2008) also revealed that modification of the PSY step by plant genes led to a significant enhancement in the provitamin A content in transgenic rice.

In canola, over expression of the bacterial crtBα (for PSY) led to 50-fold increase in total carotenoids (Shewmaker *et al.*, 1999). These increases occurred mainly in α- and β-carotene. Using an endogenous PSY gene, enhanced seed-specific accumulation of α- and β-carotene was also achieved in Arabidopsis, but unlike canola, there was less flux into α-carotene versus other carotenoids, primarily lutein and violaxanthin. In maize, according to Aluru *et al.* (2008) modification of crtI as well as PSY seems to be promising next steps to boost kernel provitamin content. They also expressed that whether the attempts to manipulate the carotenoid pathway in maize would be successful because of the differences between rice and maize.

Maize biofortification

Pfeiffer and McClafferty (2007) expressed that "biofortification" is the process of development of micronutrient-enriched staple plant foods through plant breeding which holds significant promise for sustainable food-based solutions to hidden hunger. The maize breeders have now focused on the said approach of "biofortification" that targets the most vulnerable populations (Bouis, 2003) as seed endosperm of maize and other grasses (Poaceae) represents 70 per cent of worldwide food production. Qaim *et al.* (2007) also explained that after the initial development and distribution of the biofortified seeds, there is a multiplying effect resulting from dissemination of the seeds via existing seed distribution systems. The biofortified seeds can be easily reproduced by poor farmers, and thus the seeds are a sustainable means to target remote rural communities not served by conventional seed markets. Maize breeders have succeeded in developing biofortified maize lines containing 15µg β-carotene /g dry kernel weight (Yan *et al.*, 2010). Biofortification is more sustainable and cost efficient compared with the traditional nutrition interventions such as supplementation, food fortification, and dietary diversification (Bouis and Welch, 2010).

Carotenoid variation for high β-carotene in maize breeding

The first step in breeding maize for enhanced carotenoid contents involves an assessment of the extent of genotypic variation existing in adapted germplasm, to achieve the desired improvement. Yellow maize kernels contain several carotenoid isoforms, including two carotenes (α-carotene and β-carotene) and three xanthophylls (β-cryptoxanthin, zeaxanthin, and lutein) (Weber, 1987). Yellow kernel maize is thought to be associated with high levels of β-carotene, a source of vitamin A (Buckner *et al.*, 1990). Furthermore, carotenoids are known to have heritable traits in temperate maize (Wong *et al.*, 1998). Egessel *et al*. (2003) reported a range of 0.5 to 3.4 µg/g (mean; 1.5 µg/g) β-carotene concentration among a set of maize hybrids. Higher carotenoid content in maize is associated with darker orange color and carotenoid content could be improved by selecting for orange color. Amongst the three major cereals, only maize kernel contains coloured carotenoid compounds that can be converted into vitamin A in the human and animal body (Wurtzel, 2004).

Li *et al.*, (2008) showed that, β-carotene-derived xanthophylls accounted for the bulk of total carotenoids in wild-type embryo tissues, with zeaxanthin being predominant (74.5%), followed by antheraxanthin (11.8%) and violaxanthin (7.8%). The α-carotene-derived xanthophylls lutein comprised 5.9 per cent of total embryo carotenoids. Among the diverse panel of inbred lines analysed, β-carotene level was found up to 13.6 µg/g whereas most of the yellow maize grown and consumed throughout the world has only 0.5 to 1.5 µg/g β-carotene (Harjes *et al.*, 2008). However, limited reports have been published on the range of variation of carotenoids in diverse tropical-adapted yellow maize inbred lines (Abebe *et al.*, 2008). Survey of such inbred lines, facilitates the selection of suitable breeding materials for successful development of maize cultivars that combine high carotenoid levels with desirable agronomic performance (Abebe *et al.*, 2008). In marked contrast, in endosperm tissue, the α-carotene-derived xanthophylls, lutein and zeinoxanthin accounted for 66 per cent of total carotenoids (55 and 10.8%, respectively), with β-carotene and various β-carotene derived xanthophylls comprising 34% of the total (Bai *et al.*, 2009) and the differential accumulation of α- and β-carotene-derived

carotenoids suggested that cyclization of lycopene is differentially regulated in embryo and endosperm tissues.

A range of 0.7 to 4.7 µg/g across four trials with a mean of 1.9 µg/g of kernel β-carotene was reported by Menkir *et al*. (2008) while evaluating a group of tropical yellow maize inbreds. Chander *et al*. (2008) reported that kernel β-carotene varied from 0.01 to 1.72 µg/g in a set of inbreds from the Chinese maize breeding programme. Mishra and Singh (2010) reported that the maize inbreds were characterized for grain colour was found to be poor determinant of either total kernel carotenoids or relative provitamin A carotenoids and revealed that selection based on kernel colour may not always be a true reflection for high or low carotenoids in maize. Chandler (2013) opined that colour scores in a mapping population of maize have been used to detect QTL for colour variation in maize that is associated with known carotenoid genes.

Vignesh *et al*. (2012) characterized 105 maize inbreds of diverse pedigree from India- and CIMMYT- origin for kernel β-carotene concentration and revealed the significant variations with a range of 0.02 to 16.50 µg/g. Dhyneshwaran , (2012) reported that among the 11 selected inbreds of 210 accessions showed the highest total carotenoid content in CIM Entry68 (29.100µg/g) and the lowest total carotenoid content in the genotype DMWHOY4318 (10.173µg/g) and the β-carotene concentration varies from 0.29µg/g (DMWHOY4318) to 7.07µg/g (DMWNY4091).

Molecular Screening of Maize for enhanced β-carotene

Studies in several different plant species have examined the relationship between candidate genes and quantitative variation (Faris *et al.,* 1999; Prioul *et al.,* 1999 and Thorup *et al*. 2000). Faris *et al*. (1999) suggested that if the candidate gene can be validated, then it can be used as an efficient molecular marker to aid in selecting desirable alleles. Three major QTL were identified for accumulation of carotenoids in maize (Wong *et al.,* 2004). Two of these QTL co localized with y1 and zeta carotene desaturase (zds); the third QTL mapped to a region without a candidate gene.

The candidate gene association approach has been successful in identifying genes controlling various quantitative traits in maize. Comparisons between β-carotene and total carotenoids with grain colour (scaled according to shade of yellow) revealed poor correlations with low R^2 values, which indicated that marker assisted selection (MAS) may prove much more efficient than selection based on colour alone (Harjes *et al*., 2008). Polymerase chain reaction (PCR)-based functional markers have been developed for molecular breeding of pro-vitamin A carotenoids particularly for ß-carotene (Harjes *et al*., 2008).

In maize, three genes identified for the accumulation of proA 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 (Li *et al*., 2009). Two genes, lycopene epsilon cyclase (*LcyE*) and beta-carotene hydroxylase 1 (*crtRB1*) have been shown to regulate the accumulation of proA compounds. LcyE converts lycopene into zetacarotene and eventually to alpha-carotene through the action of other associated genes. Naturally existing mutant alleles of *LcyE* with reduced functionality have been identified that apportion more lycopene into the beta branch of the pathway, enhancing the flux toward proA compounds (Harjes *et al*., 2008).

Yan *et al*. (2010) with their research on maize beta carotene opined that c*rtRB1* is a hydroxylase gene that converts β-carotene (BC) into β-cryptoxanthin (BCX), whose proA activity is theoretically only half that of BC. Natural genetic variation for *crtRB1* has recently been discovered that results in the retention of more BC in the maize endosperm. They confirmed that *crtRB1* (β-carotene hydroxylase) is an important gene associated with the β-carotene concentration in maize kernels. Through association mapping approach, three polymorphisms viz., 5'TE (in the 5'- Untranslated Region), InDel4 (in the coding region) and 3'TE (spanning the sixth exon and 3'-Untranslated Region), were identified for *crtRB1* gene that were significantly associated with variation for the target trait. They also reported that the crtRB1 3'TE favourable allele (allele 1, 543 bp) associated with reduced transcript expression of the gene correlated with higher β-carotene concentrations, with an average increase of 6.50 µg/g β-carotene in maize endosperm above the unfavourable allelic class.

PCR-based co-dominant markers were identified paving the way for rapid improvement of provitamin A status in maize kernels through marker-assisted selection (Zhang *et al*., 2012). Dhyneshwaran (2012) reported that the inbred DMWNY4042 had 8bp deletion in the *LcyE* gene in the 3'Indel region as it had only amplicons of size 144bp and 502bp and the inbred DMWSCY4405 with orange kernels yielded amplicons of size 144bp, 399bp and 502bp which indicate the absence of the 8bp deletion in the 3'Indel region. Babu *et al*. (2012a) validated the effects of *LcyE* and *crtRB1* and concluded that *crtRB1*clearly has a much larger effect than *LcyE* on proA concentration, and MAS for favorable 'allele 1' of *crtRB1* can lead to rapid doubling, or more, of total proA concentration. Vignesh *et al*. (2012) characterized 95 inbreds for crtRB1 3'TE gene using gene-specific markers indicated that only 5 inbreds viz., DM-RIL47, QPM-MAS-4972, QPM-MAS-4974, QPM-MAS-4979 and HPLET08-Entry10 revealed the favourable allele while rest showed unfavourable alleles.

Marker assisted breeding

Tanksley (1989) expressed that in traditional backcross breeding the reconstruction of the recurrent parent genotype requires more than six generations, while this may be reduced to only three generations in Marker-assisted backcrossing (MABC). Becker (1993) was also added value to the above view stating that in repeated crossings the original cross is backcrossed with the recurrent parent until most of the genes stemming from the donor are eliminated. Eathington *et al.* (1997) indicated that markers are used for selecting qualitative as well as quantitative traits. MAS can aid selecting for all target alleles that are difficult to assay phenotypically. Especially in early generations, where breeders usually restrict their selection activities to highly heritable traits, as a visual selection for complex traits like yield is not possible as only few plants per plot being available, Marker assisted selection (MAS) is said to be effective, cost and time saving . In order to minimize this linkage drag, marker assays can be of advantage as per Frisch *et al*. (1999).

Marker assisted selection (MAS) reduce the size of introgressions more rapidly and more efficiently (Hospital, 2001). The effectiveness of MAB depends on the availability of closely linked markers and/or flanking markers for the target locus, the size of the population, the number of backcrosses and the position and number of markers for background selection. Backcrossing is used in plant breeding to transfer (introgress) favorable traits from a donor plant into an elite genotype (recurrent parent). Markers can be used in the context of Marker-assisted backcrossing (MABC) to either control the target gene (foreground selection) or to accelerate the reconstruction of the recurrent parent genotype (background selection). Ragot and Lee (2007) opined that in maize MABC is certainly the form of MAS with the most immediate and obvious benefits. Ribaut and Ragot, (2006) also explained that MABC is especially efficient if a single allele is to be transferred into a different genetic background, for example, in order to improve an existing variety for a specific trait. However, if the performance of a plant is determined by a complex genotype it is unlikely that this ideal genotype will be attained through MABC only. Selection of favourable gene alleles with inexpensive molecular markers will now enable breeders in developing countries to produce maize grain with higher provitamin A levels. Harjes *et al*. (2008); Yan *et al*. (2010) identified *lcyE* and *crtRB1respectively as* inexpensive PCR-based markers to differentiate all alleles of genes for use in MAS.

Through the use of donor lines and user-friendly PCR-based marker systems, introgression of the most favorable *crtRB1* and *lcyE* alleles into tropical germplasm has already achieved provitamin A target concentrations of 15 µg/g in preliminary evaluations of breeding lines (Warburton *et al*., 2010) and the use of these markers in applied breeding programs throughout the world will quickly bring more highly nutritious, well-adapted maize cultivars to farmers. Babu *et al.* (2012a) suggested that introgression of variant alleles with enhanced efficiency at multiple points of the carotenoid pathway in maize, of which controlled regulation of BC hydroxylation through *crtRB1* has emerged as one of the important breeding strategies for enhanced accumulation of proA-related compounds. Vignesh *et al*. (2012) revealed that Marker-assisted breeding has been initiated at Maize Genetics Unit, IARI to introgress the crtRB1 3'TE favourable allele using high β- carotene CIMMYT inbreds as donors, to develop provitamin A-rich maize cultivars suitable for maize growing regions in India.

CONCLUSION

Maize is native to South America and adapted to tropical and temperate environments. The increasing interest for maize is also due to its economic importance which provides industrial raw materials for starch, gluten, corn oil, corn syrup, sugar, corn meal and corn flour. With all its higher nutritive value maize is deficit in pro-vitamin A. In India, maize is consumed as food in many hilly tracts on north eastern states and Indian maize consumption in daily food ration is showing increasing trends. Enriching corn with vitamin A, will serve and assure the human population feeding on maize with enriched nutritive factor the solution for major diseases and eye blindness caused by vitamin A. The candidate gene association approach has been successful in identifying two genes namely lycopene epsilon cyclase (*LcyE*) and beta-carotene hydroxylase 1 (*crtRB1*) which regulate the accumulation of proA compounds in maize. Marker assisted selection (MAS) can aid selection of all target alleles that are difficult to assay phenotypically in less time and with minimum linkage drag. MAS plays an important role in developing many provitamin A-rich maize cultivars which can be cultivated in different maize growing regions in India.

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