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PROVITAMIN A BIOFORTIFICATION IN MAIZE THROUGH GENETIC ENGINEERING AND MARKER-ASSISTED SELECTION

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ABSTRACT

Maize is an important cereal in the global economy, which feeds one-third of the world's population and is the third largest food crop after wheat and rice. Nutritional quality of most maize varieties is very low due to the lack of lysine and tryptophan and extremely low provitamin A carotenoids including α-carotene, β-carotene, and β-cryptoxanthin. Therefore, one of the solutions to improve nutritional value in maize is to improve provitamin A carotenoids contents. Many efforts have been made to produce maize plants with enhanced provitamin A carotenoids, especially, β -carotene. This article aims to review the research towards provitamin A biofortification through genetic engineering and marker-assisted selection. The published results and our recent achievements open the window for the improvement of provitamin A carotenoids in maize as well as the issues that need the further investigation.

Keywords: *Zea mays* L, β -carotene, biofortification, genetic engineering, maize, marker-assisted-selection, provitamin A.

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INTRODUCTION

Maize, *Zea mays* L., is an important cereal in the global economy, which feeds one-third of the world's population and is the third largest food crop after wheat and rice. Nutritional quality of most maize varieties is very low due to the lack of lysine and tryptophan and extremely low provitamin A carotenoids including α-carotene, β-carotene, and β-cryptoxanthin (Kurilich and Juvik, 1999).

Humans and animals cannot synthesize vitamin A, so it is necessary to provide provitamin A carotenoids in the diet. Vitamin A deficiency (VAD) is a global health problem that causes 140 to 250 million people at risk for many health problems, which can lead to blindness and increased disease and mortality rates in preschool children (WHO, 2010).

Biofortification is a process of increasing the density of vitamins and minerals in a crop through plant breeding, transgenic techniques, or agronomic practices (Bouis and Saltzman, 2017). Many efforts have been made to produce maize plants with increased provitamin A carotenoids, especially, β–carotene (Wurtzel et al., 2012). Although many strategies including supplementation, dietary diversification, and commercial fortification of foods have been deployed to overcome VAD, biofortification through genetic engineering and marker-assisted selection promise to be an effective and sustainable approach. The important basis for these approaches is identification of the key genes regulating carotenoid biosynthesis pathways and a number of functional molecular markers for genes/alleles related to increase in the levels of carotenoids in plants. Some such genes have been identified (Harjes et al., 2008; Yan et al., 2010), applied for gene transfer (Aluru et al., 2008; Zhu et al.,2008; Farre et al., 2013; Zanga et al., 2016) and selecting maize lines having high content of provitamin A carotenoids (Muthusamy et al., 2014; Liu et al., 2015; Zunjare et al., 2018). In this article, I will review the recent advances in these two approaches toward biofortification of provitamin A carotenoids, especially, β-carotene in maize.

The importance of provitamin a carotenoids

Carotenoids are naturally found in plants. Carotenoids containing β-ionone rings are known as provitamin A carotenoids including β-carotene, β-cryptoxanthin and α-carotene. Provitamin A carotenoids are precursors of vitamin A - an essential micronutrient for humans. Vitamin A is very important for eye health, protects against age-related macular degeneration (US National Institute of Health, 2016), regulates and improves immune system and protects against infections (Ross, 1998; Semba, 1994; Semba, 2009, Huang et al., 2018). Vitamin A is also involved in regulation of gene expression (Combs, 2008), and thus plays an important role in development of the embryo, tissue growth, and cell renewal. Vitamin A deficiency (VAD) is a global health problem that causes 140 to 250 million people at risk for many health problems, which can lead to blindness and increased disease and mortality rates in preschool children (WHO, 2010). In addition, VAD also reduces the immune system in children leading to an increased risk of death from infectious diseases.

Humans cannot synthesize vitamin A *de novo*, and therefore need to obtain it from dietary sources either as preformed vitamin A (retinol) from animal-based foods (liver, fish, egg and dairy), or as precursors of vitamin A from colored vegetables and fruits (carrots, papaya, butternut squash, red bell pepper, grapefruit) in the form of provitamin A carotenoids.

In the human body, provitamin A carotenoids can be converted into physiologically active vitamin A (retinol) catalyzing by β-carotenoid-15,15'-dioxygenase. β-carotene can generate two molecules of vitamin A, while α-carotene and β-cryptoxanthin can produce only one molecule of vitamin A as they only have a single non-hydroxylated β-ring (Davis et al., 2008). The conversion efficiency of dietary β-carotene to retinol was reported to be in the

range of 3.6-28:1 by weight, but this varies according to the body weight of the test subjects and other factors (Tang, 2010). In 2001, the United States Institute of Medicine revised the standard ratio for bioconversion of β-carotene to retinol to 12:1 by weight (United States Institute of Medicine, 2001). Since 2001, the US Institute of Medicine uses retinol activity equivalents (RAE) for the Dietary Reference Intakes (1 μg RAE = 2 μg all-*trans*-β-carotene from supplements = 12 μg of all-*trans*-β-carotene from food = 24 μg α-carotene or β-cryptoxanthin from food). World Health Organization recommends estimated average requirements of 250 and 500 RE (Retinol Equivalents) per day for children and adults respectively, for their normal growth and development (Bouis, Welch, 2010). RE was developed in 1967 by the United Nations Food and Agriculture Organization and World Health Organization (1 μg RE = 1 μg retinol = 6 μg β-carotene = 12 μg α-carotene or β-cryptoxanthin).

Carotenoids in maize

Maize is the third most important food crop after wheat and rice, consumed by more than one billion people in sub-Saharan Africa, Latin America and parts of Asia and provitamin A content from 3,167 to 9,792 μg/g would be preferred. In Vietnam, maize is the second largest food crop after rice. The concentration of provitamin A carotenoids, including β-carotene in maize kernels is very low. According to Harjes et al. (2008), most yellow maize grown and consumed throughout the world has only 0.5 to 1.5 μg/g β-carotene. Kurilich and Juvik (1999) used HPLC to analyze carotenoids in five sweet maize lines and found that β-carotene contents ranged from 0.14 to 7.97 μg/g dry weight (DW). The results from carotenoid concentration analysis of 36 yellow maize genotypes showed 13-cis-carotene contents ranging from 0.48 to 8.32 μg/g, trans-β-carotene 0.05 to 16, 79 μg/g and 9-cis-carotene from 0.3 to 4.06 μg/g (Muzhingi et al., 2008). Vignes *et al.* (2012) analyzed β-carotene contents of 105 hybrid maize varieties in India - and CIMMYT (International Maize and Wheat Improvement Center) found a significant variation from 0.02 to 16.50 μg/g (Vignes et al., 2012). Thirusendura Selvi et al. (2014) analyzed the β-carotene contents of 24 lines of maize and showed a range from 0.23 to 7.92 μg/g. Muthusamy et al. (2015) reported the significant difference in carotenoid contents in 105 native (India) and exotic tropical (CIMMYT) maize lines, namely; lutein (0.2–11.3 μg/g), zeaxanthin (0.2–20.0 μg/g) and β-carotene (0.0–15.0 μg/g). At the same time, the authors found that environmental factors have little effect on the variation in carotenoid content and that genetic factors are important for the high carotenoid content. In the genetic study of carotenoids for improvement of provitamin A in adapted tropical maize varieties, Halilu et al. (2016) determined the content of β-carotene and provitamin A in 31 lines of adapted tropical yellow maize that have medium to high contents; for β-carotene, the content ranged from 1,867 to 8,277 μg/g and provitamin A content from 3,167 to 9,792 μg/g.

Thus, for normal maize varieties, the carotenoid contents are not high. Therefore, the improvement of carotenoid content is highly desired. And therefore maize is considered an important candidate for improving metabolites in order to increase levels of zeaxanthin, lutein, and provitamin A in food crops (Messias et al., 2014). Moreover, maize is closely related to some other food crops in the Poaceae family, so that it may also be possible to use the methods for improving provitamin A in maize for other cereal species (Wurtzel et al., 2012).

Genetic improvement of staple crops for improved nutritional quality (enhanced level of micronutrients and provitamin A) is termed biofortification and is a promising approach for reducing vitamin A and other micronutrient deficiencies in humans. The biofortification of maize with higher levels of provitamin A carotenoids can play a significant role in reducing vitamin A deficiency in regions where maize is a major staple crop (Wurtzel et

al. 2012; Burt et al. 2011; Meyers et al. 2014). The HarvestPlus, a biofortification program initiated by CIMMYT, has set the target level of 15 μg/g β-carotene to reduce vitamin A deficiency, considering the loss of 50% after post-harvest and cooking, and bioconversion rate of 12:1 of provitamin A into retinol. Thus, daily maize consumption of 200 and 400 g could meet daily requirement for children and women, respectively (Ortiz-Monasterio et al., 2007).

Carotenoid biosynthesis and accummulation

Carotenoid biosynthesis pathway (Fig. 1) initiates with the synthesis of geranylgeranyl diphosphate (GGPP), a C20 intermediate and this reaction is catalyzed by GGPP synthase (*GGPPS*) enzyme. Two molecules of GGPP are condensed into 15-cis-phytoene mediated by phytoene synthase (*PSY*) enzyme. Later on, a series of desaturation reactions are carried out by phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*) and two isomerases (*Z-ISO*, ζ-carotene isomerase; *CRTISO*, carotenoid isomerase) result in a series of double bonds and alter the isomer state of each biosynthetic intermediate to produce all-trans-lycopene (Chen et al., 2010, Wurtzel et al., 2012). At this step, depending on cyclization activity, two branches of pathways occur as lycopene acts as substrate for two competing enzymes, lycopene β-cyclase (*LYCB*) and lycopene β-cyclase (*LYCE*). Asymmetric cyclization of lycopene by both ε- and β-lycopene cyclase, respectively, produces α-carotene with one ε- and one β-ionone ring. Symmetric cyclization by *LCYB* results in β-carotene, with two unmodified β-ionone rings (Wurtzel et al., 2012). In the presence of β-carotene hydroxylase (*BCH*) enzyme, α-carotene is converted into lutein, whereas β-carotene is converted into zeaxanthin. Prior to zeaxanthin formation, an intermediate β-cryptoxanthin is also produced but it is quickly converted into abscisic acid through the xanthophyll cycle (Seo, Koshiba, 2002). Lutein is the end product of α-carotene branch.

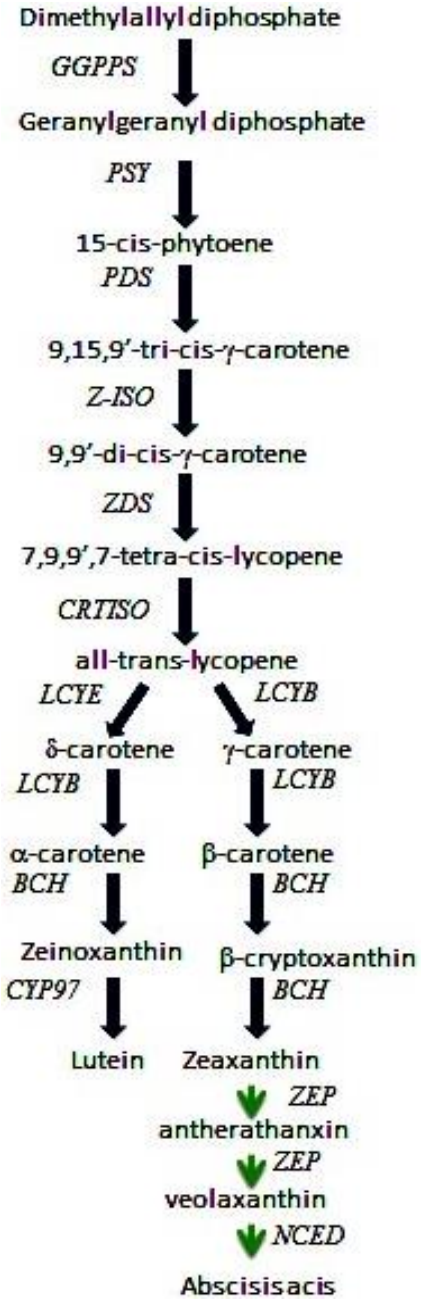

Figure 1. Simplified carotenoid biosynthesis pathways: GGPP: Geranylgeranyl diphosphate; PSY: Phytoene synthase; PDS: Phytoene desaturase; ZDS: ζ-carotene desaturase; CRTISO: carotene isomerase; LCYB: Lycopene beta cyclase; LCYE: Lycopene epsilon cyclase; BCH: β-carotene hydroxylase; ZEP: Zeaxanthin epoxidase; NCED: 9-cis-epoxycarotenoid dioxygenase

It is clear that the pathways include several physiological events from initiation with geranylgeranyl diphosphate (GGPP) to the last step at abscisic acid or lutein. By understanding the critical steps in the pathway, we can modulate the pathway for enhancement of the desired products. In order to do that, the key genes regulating the pathway should be identified and modulated to increase the biosynthesis of provitamin A carotenoids. As in figure 1, the key genes are the genes coding for phytoene synthase (*PSY*), phytoene desaturase (*PDS*), ζ -carotene desaturase (*ZDS*), lycopene β-cyclase (*LCYB*), lycopene ε-cyclase (*LCYE*) and β-carotene hydroxylase 1 (*crtRB1*). However, all of these genes are not equally effective in the production of provitamin A carotenoids. So far *PSY1* gene is known to play a critical role in formation of phytoene by condensation of two molecules of geranylgeranyl pyrophosphate (GGPP). Earlier, *PSY1* was known as *Y1* gene that determines the variability in the kernel color ranging from white to orange (Buckner et al., 1990, Buckner et al., 1996). The role of *PSY1* was validated by transgenic overexpression of this gene in white kernels which produced transgenic plants with yellow grains (Zhu et al., 2008). *PSY1* gene has two polymorphic sites; a 378-bp InDel is located upstream of the transcription start site and a SNP on fifth exon, and both of these polymorphs account for 7% and 8% of the total carotenoid variance, respectively. *PDS* (phytoene desaturase) and ζ-carotene desaturase (*ZDS*) genes regulate the desaturation of phytoene to produce lycopene - the first colored pigment to be produced in the pathways (Li et al., 1996). Two enzymes lycopene β-cyclase (*LCYB*) and lycopene ε-cyclase (*LCYE*) react with lycopene to generate β-carotene and α-carotene. Harjes et al. (2008) described that the progression for β-carotene branch was increased over α-carotene by down-regulating the *LCYE* gene. β-carotene hydroxylase 1 *(BCH/crtRB1)* is involved in hydroxylation of α-carotene and β-carotene producing non-provitamin A carotenoids lutein and zeaxanthin, respectively. *crtRB1* is also known as HYD3 (Hydroxylase 3), however, hydroxylation of carotenes increases the proportion of xanthophylls having no provitamin A activity (Matthews, Wurtzel, 2007). *HYD3* encodes one of the carotene hydroxylases. Transcripts of HYD3 were positively correlated with zeaxanthin and negatively associated with β-carotene levels. *HYD3* accounted for 36% of the phenotypic variance for β-carotene concentration (Vallabhaneni, Wurtzel, 2009).

Carotenoids in plants are synthesized in the membranes of plastids and stocked in chromoplasts of flowers, fruits and roots (Howitt, Pogson, 2006). Chromoplast has a special mechanism for storing large amounts of carotenoids by creating structures called carotenoid-lipoprotein structures inside the chromoplast. These structures are also known as carotenoid isolation structures. The isolation structures act as reservoirs to store/isolate carotenoids and prevent the end product of carotenoid synthesis from affecting the synthesis of carotenoids in chromoplast membranes (Al Babili et al., 1999). In chromoplasts, carotenoids accumulate often in esterified form. The esterification enhances carotenoid stability. While, in chloroplasts, carotenoids associate with chlorophylls and pigment binding proteins giving the proper function of the photosynthetic apparatus. The binding of carotenoids with different classes of pigment binding proteins in chromoplasts (Vishnevetsky et al., 1999) and chloroplasts (Neilson, Durnford, 2010) contributes to the stability of carotenoids.

Once the potential genes such as *PSY1*, *PDS*, *ZDS*, *LCYB*, *LCYE* and *crtRB1* involved in the biosynthesis of provitamin A carotenoids are recognized, we can employ them for provitamin A biofortification in maize either by genetic engineering or marker-assisted selection. For the latter, the molecular markers associated with these genes need to be identified to precisely exploit them for provitamin A biofortification. The role of other genes in the synthesis pathway and accumulation of provitamin A carotenoids

can be explored for faster and more effective provitamin A biofortification in maize.

Strategies for improvement of carotenoid content

Based on the knowledge of biosynthesis and accumulation, improving the carotenoid levels in plants can be done with the interference of carotenoid biosynthesis cycle by enhancing the expression of genes that encode key enzymes involved in synthesis, or inhibition of certain genes that encode substances that degrade carotenoids, and the use of genes regulating carotenoid accumulation.

Enhancing the expression of one or more genes that encode key enzymes involved in synthesis of carotenoids is the first strategy. This had been done in rice (Ye et al., 2000), potato (Diretto et al., 2007), cassava (Wesch et al., 2010), wheat (Wang et al., 2014), and bananas (Paul et al., 2017). In maize, Zhu et al (2008) transformed different combinations of 5 carotenogenic genes controlled by different endosperm-specific promoters into a white maize variety and generated transgenic plants expressing different enzyme combinations and showing different carotenoid compositions. However, overexpression of synthetic enzymes may lead to the depletion of synthetic precursors, thus limiting the accumulation of the desired product.

Expression of enzymes that inhibit certain genes encoding for substances that degrade carotenoids is the second strategy for the improvement of carotenoids content. Romer et al (2002) have generated transgenic potato with elevated zeaxanthin content 4 to 130 fold by antisense inactivation and co-suppression of carotenoid epoxidation. Using a similar approach, *ZEP* (Diretto et al., 2006) and *LCYE* (Pons et al., 2014) genes were silenced to improve zeaxanthin and β-carotene, respectively. Alternative strategy is the combination of enhancing and inhibiting gene expression as shown by overexpression *crtB* gene combined with silencing of carotenoid hydroxylase gene (*CHY*) in wheat that resulted the increase of β-carotene up to 31 fold (Zheng et al., 2015). In maize, transgenic plants with high value carotenoid astaxanthin in the kernel endosperm were produced by combining the overexpression of *PSY* gene for enhanced carotenoid production and silencing of *LCYE* gene for directing more precursors into the β-branch (Farre et al., 2016).

The third strategy is the use of gene regulating carotenoid accumulation. This strategy was employed first by Lu's and Lopez's work, showing that the mutated *Brassica oleracea Or* gene (*BoORMUT*) showed its effect by triggering chromoplast differentiation and enhancing storage sink strength for carotenoid biosynthesis and accumulation (Lu et al., 2006; Lopez et al., 2008). The overexpression of wild-type *Or* gene also enhanced the carotenoid accumulation in sweet potato (Kim et al., 2013) and in the calli of rice (Bai et al., 2014). The carotenoid accumulation driven by the *Or* gene associated with the differentiation of non-pigmented plastids into chromoplasts and the formation of the metabolic sinks (Lu et al., 2006, Zhou et al., 2008). The *Or* gene codes for plastid protein containing a DnaJ Cys-rich domain that is associated with high carotenoid accumulation. In addition to the role in carotenoid accumulation, a recent study showed that the Or protein of *Arabidopsis thaliana* interacts directly with PSY protein and functions as a major regulator of active PSY protein abundance in mediating carotenoid biosynthesis (Zhou et al., 2015). The overexpression of the *Or* gene from *Ipomea batata*s (*IbOr*) in transgenic plants of sweetpotato, potato and alfalfa (Park et al., 2015; Cho et al., 2016; Li et al., 2012; Guo et al., 2015; Wang et al., 2015) has led to increased carotenoid accumulation without affecting carotenoid synthesis. A recent report on the role of *Arabidopsis thaliana Or* gene (*AtOr*, Berman et al., 2017) and sweet potato *Or* gene (*IbOr*, Tran et al., 2017) in maize suggested the potential use of *Or* gene to improve the carotenoid accumulation in staple crops including maize. In our recent work, the *IbOr* gene isolated form local sweet potato cultivar Hoang Long (*Ipomea batata*s cv.

Hoang Long) and placed under the control of maize seed-specific promoter globulin 1 (Glo1), has been successfully transferred into inbred maize lines and total carotenoid and β-carotene content of generated transgenic plants were significantly higher than those of wild-type and in the best line, the total carotenoid and β-carotene content were increased up to 10.36 and 15.11 fold, respectively (Tran et al., 2017). To our best knowledge, our research is the first to successfully overexpress the *IbOr* gene in maize.

Marker-assisted selection (MAS), particularly marker-assisted back crossing and introgression (MABC, MASI) are the fourth strategy for the improvement of carotenoid contents. This strategy is realized on the natural variation of carotenoid content and DNA marker associated with the genes/QTLs or alleles that are crucial for carotenoid synthesis and accumulation.

There is a wide range of levels of provitamin A carotenoids in temperate, tropical and subtropical germplasm (Ortiz-Monasterio et al. 2007; Menkir et al. 2008). Tropical maize contains less β-carotene than temperate maize, so the initial breeding sources of high provitamin A carotenoids can be selected from temperate maize (Pixley et al. 2013; Menkir et al. 2017). However, evaluation of a wider range of tropical germplasm in future may identify potential donors for enhanced *β*-carotene content. Babu et al. (2013) reported the range to be 15–20 μg/g provitamin A contents in improved inbred lines, whereas Suwarno et al. (2015) reported 2.34–22.25 μg/g provitamin A contents in maize kernels. Breeding lines of maize that can accumulate up to 26 μg/g β-carotene (and 30 μg/g of provitamin A carotenoids) in the endosperm have been reported (Pixley et al. 2013). The maize lines having high provitamin A carotenoids content can be used as the provitamin A donors for the improvement of provitamin A carotenoids in maize.

Results of the studies on the role of the *crtRB1* and *LCYE* genes in the accumulation of β-carotene in maize (Harjes et al., 2008; Yan et al., 2010; Babu et al., 2013; Zhang et al., 2012; Azmach et al., 2013) showed different roles of different alleles of these two genes on β-carotene content in maize kernels. Harjes et al. (2008) suggested that the reduced functionality of *LCYE* gene shifts more lycopene into the β-branch of the pathway, thereby enhancing the flux towards provitamin A carotenoids. The authors reported the presence of four alleles in the 5'TE region of the *LCYE* gene, including allele 1 (150 + 280 bp), allele 2 (250 bp), allele 3 (250 bp + 380 bp) and allele 4 (650 bp). Of these, allele 1 and allele 4 are two regulating alleles that increase the provitamin A carotenoids through the β-branch, and these alleles are known as favorable alleles. While allele 2 and allele 3 are unfavorable alleles as they negatively affect the provitamin A levels in maize kernels. There were two alleles in the 3'TE region of the *LCYE* gene: allele 1 (399 + 502 bp) and allele 2 with 8 bp deletion (144 + 502 bp), and allele 2 is the favorable allele (Harjes et al., 2008).

crtRB1 gene is involved in conversion of β-carotene into β-cryptoxanthin which has only a half of provitamin A activity compared to β-carotene. Yan et al. (2010) have discovered the natural genetic variation of *crtRB1* that reduced functionality of *crtRB1* gene leading to increased accumulation of β-carotene in the maize endosperm. Through linkage mapping, three regions of the *crtRB1* gene have been identified that are significantly associated with the changes in β-carotene levels: The 5'TE end (in non-coding region), InDel4 (in the coding region) and the 3'TE end (extends from the 6th exon and the untranslated 3'-region). *crtRB1 3'TE* polymorphism produces three alleles: allele 1 (543 bp without insertion), allele 2 (296 bp + 875 bp, with insertion of 325 bp) and allele 3 (296 bp + 1221 bp + 1880 bp, with insertion of 1,250 bp) associated with β-carotene accumulation (Yan et al., 2010). Allele 1 is known as an allele favorable for increasing β-carotene by reducing the transcriptional expression of the *crtRB1* gene, whereas allele 2 and allele 3 are unfavorable alleles. *crtRB1*

5'TE polymorphism also produces three alleles: allele 1 with 397-bp insertion, allele 2 with 206-bp insertion, and allele 3 without insertion. Among these, alleles 2 (600+206 bp) is the favorable.

DNA markers associated with favorable alleles of *crtRB1* and *LCYE* genes were identified, validated and used in marker-assisted selection and introgression in temperate and tropical maize (Babu et al., 2013). Azmach et al (2013) had analyzed the association of functional gene markers for provitamin A levels across diverse tropical yellow maize inbred lines and revealed tropical maize inbred lines harboring the favorable alleles of the crtRB1-5′TE and crtRB1-3′TE functional markers produce higher levels of provitamin A. Such maize lines can be used as donor parents to cross with elite germplasm of maize that had high yield potential and good agronomic traits, such as disease resistance and drought tolerance. However, the challenge for marker-assisted selection of high provitamin A carotenoids is the low frequency of favorable alleles in traditional maize population. Muthusamy et al., (2015b) has screened a diverse set of 385 maize inbred lines of indigenous and exotic origin and detected the presence of two alleles (amplicon size: 250 and 650 bp) of *LCYE* and three alleles (amplicon size: 296, 543 and 875 bp) of *crtRB1* in the inbred panel. Favorable alleles of both genes were rare among the traditional maize germplasm; 3.38% of the inbred possessed the favorable allele (650 bp) of *LCYE*, and 3.9% inbred had the favorable allele (543 bp) of *crtRB1*. Five inbred (1.3%) with favorable alleles of both genes were found. Inbred with favorable alleles of *crtRB1* and *LCYE* serves as rich genetic resources for effective utilization in the maize biofortifcation program.

Improvement of provitamin a carotenoid content in maize through genetic engineering

Although the improvement of provitamin A carotenoids content in crops can be done by conventional breeding, this method relies on the existing gene pool so it must take long time to develop a new variety. In maize, the gene pool for high provitamin A content is very limited. Therefore, enhancing provitamin A carotenoids in maize will be more effective by using genetic engineering including overexpression of genes that encode key enzymes involved in synthesis of carotenoids, or inhibition of certain genes that encode substances that degrade carotenoids, and the use of the genes regulating carotenoid accumulation through transgenic approaches.

Improvement of provitamin A carotenoid content in maize through transgenic approaches has started with the production of transgenic maize plants by introducing the bacterial *CRTB* and *CRTI* genes under the control of super γ-seen promoters resulting in an increase of total carotenoids of up to 34-fold with a preferential accumulation of β-carotene in the maize endosperm (Aluru et al., 2008). At the same time, Zhu et al. (2008) transformed the five carotenoid genes including *Zmpsy1 (Zea mays phytoene synthase 1)*, *PacrtI* (*Pantoea ananatis phytoene desaturase*), *Gllycb (Gentiana lutea lycopene-cyclase*), *Glbch (G. lutea* -carotene hydr*oxylase)*, and *ParacrtW* (*Paracoccus*-carotene ketolase) under the control of endosperm-specific promoters and generated transgenic maize plants with extraordinary levels of β-carotene (57.35 μg/g DW) and other carotenoids, including complex mixtures of hydroxycarotenoids and ketocarotenoids. Naqvi et al. (2009) simultaneously transferred maize *ZmPSY1* cDNA under the control of wheat LMW glutenin promoter along with *CRTI* gene from *Pantoea ananatis* under the control of barley Dhordein promoter, *dhar* gene from rice (for ascorbate) and *E. coli folE* gene (for folate) to maize. They were able to produce a transgenic line containing 169-fold higher the normal amount of β-carotene, 6-fold the normal amount of ascorbate, and double the normal amount of folate.

Genetic engineering of maize for improvement of provitamin A carotenoid also can be done by boosting β-carotene

production and reduce α-carotene production or by the inhibition of genes that encode substances that degrade provitamin A carotenoids. Ferre et al. (2016) introduced *PSY* and *LCYE* into a white maize genetic background to extend the carotenoid pathway to astaxanthin. Simultaneously, phytoene synthase, the controlling enzyme of carotenogenesis, was overexpressed for enhanced carotenoid production and lycopene ε-cyclase was knocked-down to direct more precursors into the β-branch of the extended ketocarotenoid pathway to form astaxanthin. High-carotenoid maize (Carolight) was developed by introducing the *PSY1* gene from maize and *CRT1* gene from *Pantoea annatis* in the genetic background of M37W, a white endosperm maize variety. Total carotenoid contents were comparable under greenhouse conditions; and field studies showed the mean of approximate 106 ± 7 μg/g which was significantly higher than 86 ± 0.65 μg/g as reported in another study on T1 generation of Carolight line (Farre et al., 2013; Zanga et al., 2016).

The use of the genes regulating carotenoid accumulation was started with the works of Lu et al. (2006) introducing the *Or* gene isolated from an orange cauliflower (*Brassica oleracea* var. botrytis) mutant that confers high level accumulation of β-carotene in tissues that normally lack this pigment into potato (*Solanum tuberosum* L. cv. Desiree) and transgenic tubers have thus been produced with 6-fold increased levels of total carotenoids. The results demonstrated that the *Or* gene can serve as a new molecular tool to manipulate carotenoid content and composition in food crops. In maize, recently, Berman et al. (2017) have overexpressed the *Arabidopsis OR* gene (*AtOR*) under the control of the endosperm-specific wheat LMW glutenin promoter in a white maize variety M37W that normally accumulates only trace amounts of carotenoids. The total endosperm carotenoid content in the best-performing *AtOR* transgenic maize line was 32-fold higher than wild-type controls (25 μg/g DW) and the other principal carotenoids remained the same. Recently, we have successfully produced transgenic maize plants with considerably increased total carotenoids and β-carotene (Tran et al., 2017). The *Or* gene cloned from yellow-fleshed sweet potato cultivar Hoang Long (named *IbOr* gene) was transferred into two inbred maize lines (H145 and H95) through *A. tumefaciens* under the control of maize seed-specific promoter globulin 1 (Glo1). In the best transgenic line (H145-IbOr.10), the total carotenoids and β-carotene content were increased up to 10.36 (27.6 μg/g DW) and 15.11 (19.35 μg/g DW) fold, comparing to wild-type (2.67 and 1.28 μg/g DW), respectively (Tran et al., 2017). These results indicate the potential use of the *Or* gene to improve the carotenoid content in maize and other staple crops.

Improvement of provitamin a carotenoid content in maize through marker-assisted selection

Although genetic engineering is a potential tool for provitamin A carotenoid enrichment in food crops, the biosafety, public perception and other related regulatory issues must be considered prior to generalizing transgenic crops for public consumption. These are the important challenges for the application of genetic engineering in provitamin A biofortification in maize and other crops. Much progress has been made towards elucidating the genes that regulate carotenoid biosynthesis in maize endosperm and several functional markers associated with genes/alleles related to the enrichment of provitamin A content in maize have been identified. This provides a basis for utilization of marker-assisted selection of high provitamin A carotenoids in maize.

Recent findings demonstrated that downstream genes, particularly *LCYE* and *crtRB1*, from carotenoid biosynthesis pathway play a major role in accumulation of provitamin A carotenoids in maize endosperm (Harjes et al., 2008; Yan et al., 2010). Harjes et al. (2008) suggested that the reduced functionality of *LCYE* shifts more lycopene into the β-branch of the pathway, thereby

enhancing the flux towards provitamin A carotenoids. Besides *LCYE*, favorable alleles of *crtRB1* were also reported to increase β-carotene content in maize kernel (Yan et al., 2010). Favorable alleles for provitamin A contents were initially identified in temperate maize germplasm which are now being introgressed into tropical maize germplasm. DNA markers associated with favorable alleles were identified, validated and being used in a wide range of maize germplasm (Babu et al., 2013). The potential of the crtRB1-specific markers for enrichment of provitamin A carotenoids in maize grain has been used for marker-assisted backcrossing in quality protein maize (QPM) inbred and hybrids (Muthusamy et al., 2014; Liu et al., 2015). Muthusamy et al. (2014) successfully introgressed the favorable alleles (543 bp) of *crtRB1* gene in elite maize genotypes through marker-assisted backcross breeding. crtRB1-specific markers were used for foreground selection, and 90% of the recurrent parent genome was restored within two backcross generations. β-carotene among the crtRB1-introgressed inbred varied from 8.6 to 17.5 μg/g - a maximum increase up to 12.6-fold over recurrent parent. Marker-assisted stacking of *crtRB1, LCYE* and *o2* genes was undertaken in the genetic background of four maize hybrids (HQPM1, HQPM4, HQPM5, and HQPM7) popularly grown in India. HP704-22 and HP704-23 were used as donors, while four elite QPM parents HKI161, HKI163, HKI193-1 and HKI193-2 were used as recipients. Recovery of recurrent parent genome (RPG) among selected backcross progenies ranged from 89 to 93%. Introgressed progenies possessed high concentration of provitamin A (7.38–13.59 μg/g), compared to 1.65–2.04 μg/g in the recurrent parents (Zunjare et al., 2018). Goswami et al. (2019) reported marker-assisted introgression of rare allele of β-carotene hydroxylase (*crtRB1*) gene into elite quality protein maize inbred for combining high lysine, tryptophan and provitamin A in maize. The results showed that introgressed lines possessed higher mean β-carotene (9.22 μg/g), β-cryptoxanthin (3.05 μg/g) and provitamin A (10.75 μg/g) compared to the recipient line HKI1128Q (2.26 μg/g, 2.26 μg/g and 3.38 μg/g, respectively). Moreover, high concentration of essential amino acids such as lysine (0.303%) and tryptophan (0.080%) in endosperm was also recorded. In the other similar work, Sagare et al. (2019) had carried out the marker-assisted backcrossing using β-carotene donor MGU23379 (6.31 μg/g of β-carotene) to cross with high quality protein recurrent parents, CB6-36 (CBML6) and CB7-28 (CBML7). Foreground selection was carried out with *crtRB1*-3′TE and *umc1066* markers. Tryptophan/lysine content in introgressed progenies was retained as in the recurrent parents, but β-carotene content was significantly high (6.25 and 6.80 μg/g) compared to the original inbred line (0.71 and 1.29 μg/g).

Provitamin A biofortification of maize with the target of improving β-carotene concentration in maize grain beyond 15 μg/g to provide an additional 50% of the estimated average requirement for Vitamin A in maize-eating regions (Menkir et al., 2018) was started by the HarvestPlus-Maize Program at CIMMYT and considerable progress has been achieved to date. With the discovery of useful allelic diversity for *LCYE* and c*rtRB1* and development of molecular markers, source lines with >15 μg/g of provitamin A carotenoids have been identified and are now routinely used as parents for new crosses at CIMMYT. This has led to the selection of lines with 40–250% higher provitamin A carotenoid concentrations than lines without the favorable allele (Babu et al., 2013).

The challenges and solutions in the future

Although many impressible achievements have been made in biofortification of carotenoids and provitamin A carotenoids in several crops including maize through genetic engineering, major challenges for biofortified transgenic crops are bio-safety and the lengthy regulatory process needed before they get released for cultivation and consumption. Due to this, none of the genetically biofortified

crops have been released for cultivation or consumption (De Steur et al., 2017). The development of new technologies such as gene editing to knock-out mutations blocking β-carotene hydroxylation, or lycopene β-cyclization, which are able to increase β-carotene content (Belhaj et al., 2013) or make point mutations able to increase β-carotene content, like the mutations Arg > His in *OR* gene (Tzuri et al., 2015) or Ala > Asp in *PSY* gene (Welsch et al., 2010) could be a potential solution for the future of provitamin A biofortification in maize. Gene editing tools may have wider public acceptability than genetic engineering technologies as they are closer to natural processes. Hence, gene editing may have a huge potential for genetic improvement of crop plants in general, and thus, this technology could be exploited in future for modifying the carotenoid biosynthesis pathway in maize for improvement of provitamin A carotenoids.

One of the challenges for marker-assisted selection (MAS) in provitamin A biofortification in maize, especially tropical maize is the limited high β-carotene resources. However, this can be overcome by the use of temperate or transgenic improved resources for backcrossing. The other challenge is that there are still very few favorable alleles of *PSY1, LcyE and crtRB1*genes that have been identified and manipulated in maize using MAS for enrichment of provitamin A. Moreover, such alleles are rare even in temperate maize. Therefore, association mapping and whole genome sequence analyses approaches could be used for rapid generation of selectable markers in diverse germplasm, and can enable breeders to find the favorable alleles in their locally adapted germplasm sources (Harjes et al 2008). In addition, the studies on identification of favorable alleles for other functional genes in the carotenoid biosynthetic pathways which increase the synthesis of total carotenoids and enhance the accumulation of β-carotene need to be carried out.

CONCLUSION

The application of genetic engineering and marker-assisted selection for provitamin A biofortification in maize has gained remarkable achievements. By gene technology, maize lines with hundreds fold higher β-carotene content compared to control have been produced. With marker-assisted selection, maize lines with β-carotene above the recommended level of 15 μg/mg dry weight and meeting 50% of vitamin A requirement for humans have also been reported. However, the release of provitamin A fortified transgenic plants is a long process. Marker-assisted selection (MAS) and Marker-Assisted Backcross Breeding (MABB) are viable approaches to develop provitamin A biofortified maize. However, due to the lack of natural genetic resources with high levels of β-carotene that can be used as donor materials and the number of potential functional genes and molecular markers associated with these genes being limited, MAS is still not fully effective. With the development of environmental friendly gene technology (use of plant-derived genes and antibiotic-free selection), gene editing and mutation producing by gene editing tools like Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), Clustered Regularly Interspaced Short Palindromic Repeats/Cas (CRISPR/Cas), or applying whole genome sequencing and Genome Wide Association Study (GWAS) for rapid identification of functional genes and generation of selectable markers for MAS, the provitamin A biofortification in maize and other staple crops will be more accurate, fast and efficient.

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