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Review Paper

Biofortification of maize: An Indian perspective

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Abstract

Micronutrient malnutrition particularly prevalent in resource poor families in the developing world has emerged as a major health challenge. Billions of people worldwide suffer from impaired growth and development owing to insufficient supply of essential amino acids, vitamins and minerals leading to significant economic losses. India is home to a large number of undernourished people warranting immediate interventions. Maize is a staple crop with diverse end uses; thus micronutrient enriched maize holds immense promise for sustainable and cost-effective solutions to overcome malnutrition. We present here a review on status, constraints and opportunities in developing biofortified maize cultivars with enhanced protein quality, provitamin A, and kernel -Fe and -Zn. Quality protein maize possessing higher lysine and tryptophan is a classical example of how its successful adoption has resulted in significantly reducing malnutrition. Novel genetic variants of *crtRB1* and *lcyE* genes have opened up new avenues for food-based solution to vitamin A deficiency. Availability of variation for kernel -Fe and -Zn and the possibility for manipulation of anti-nutritional- and promoting- factors offer distinct opportunity to deliver bioavailable minerals through diet. Development of multi-nutrient rich maize would help in achieving nutritional security in a more holistic way. Possible interventions to overcome the challenges of slow dissemination of biofortified crops have also been discussed.

Key words: Micronutrient malnutrition, maize, QPM, provitamin A, iron, zinc, biofortification

Introduction

The current global population of more than seven billion is likely to cross nine billion by 2050, requiring the world to feed two billion more people by then. We already have one billion people, mostly in developing countries, going to bed hungry every day (Khush et al. 2012). In addition, there is a stealthy form of hunger called “micronutrient malnutrition” or “hidden hunger” afflicting an estimated two billion people (Steur et al. 2015). Micronutrient deficiencies are particularly prevalent in rural population of the developing countries, where they mainly rely on cereal-based diet as staple food. India is home to a large number of undernourished people (17.5% of its population) in the world, where 42% of children (<3 years old) are underweight and 58% of them are stunted by two years of age (FAO/WFP/IFAD, 2012). It is estimated that India loses 2.5% of the national GDP on account of micronutrient deficiency (FAO, 2013). The challenge, therefore, is to deliver nutritious, safe and affordable food to reduce the impact of nutritional-insecurity. Though various interventions like ‘industrial food-fortification’, ‘supplementation’ and ‘dietary diversification’ have been tried worldwide to alleviate micronutrient deficiencies, none of these approaches has been found viable in the long run owing to ineffective distribution system, poor infrastructure and/or non-affordability (Tanumihardjo et al. 2007). On the other hand, the development of micronutrient-enriched staple plant foods through breeding approaches, a process popularly known as ‘biofortification’, holds promise for sustainable and cost-effective food-based solutions to combat micronutrient deficiencies (Pfeiffer and McClafferty, 2007). Biofortified crops would also serve as the logical vehicle for providing micronutrients in pure form in the diets (Bouis et al. 2011). Crop breeding will play a vital role in meeting this challenge by

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developing micronutrient-rich staple crop varieties to overcome the micronutrient deficiency.

Maize assumes worldwide significance owing to its utilization as a human food and livestock feed. Globally, annual maize production is 1016.73 million metric tonnes, of which Asia alone produces 304.31 million metric tonnes (FAOSTAT, 2013). India is the second-most important maize growing country in Asia, and is the world's sixth largest producer and the fifth-largest consumer of maize (Prasanna, 2014). During 2013-14, India produced 24.35 million metric tonnes of maize from nearly 9 million hectares (www.indiastat.com). Nearly 23% of the maize produced in India, is used for human food, while approximately 63% is utilized for poultry- and animal-feed (Yadav et al. 2014). The growing poultry industry is the biggest driver of growth in maize production, consuming more than half of the country's maize. By 2050, the demand for maize in the developing world will be doubled (Rosegrant et al. 2009). Considering the growing importance of maize as food and feed, biofortification of maize including enhancement of protein quality coupled with enrichment of micronutrients like provitamin A, Fe and Zn in grain assumes great significance.

Quality protein maize (QPM)

Nutritional quality of maize protein

Human beings require 0.66 g protein/kg body weight/day to meet the requirement for proper growth and development (WHO/FAO/UNU, 2007). Essential amino acids such as lysine and tryptophan are not synthesized in human body and other monogastric animals; thus are required to be provided through diet. The daily requirement of lysine is 30 mg/kg body weight/day for adults, while it is 35 mg/kg body weight/day for children of 3 to 10 years of age. Tryptophan is required at the rate of 4 mg/kg body weight/day and 4.8 mg/kg body weight/day in adults and children, respectively (WHO/FAO/UNU, 2007). Besides role in protein synthesis, lysine and tryptophan serve as precursors for several neuro-transmitters and metabolic regulators, and their deficiency leads to reduced appetite, delayed growth, impaired skeletal development and aberrant behavior (Tome and Bos, 2007; Moehn et al. 2012). A maize kernel generally contains 8-10% protein, but is deficient in essential amino acids like lysine and tryptophan. Maize protein contains 1.5-2.0% lysine, which is less than half of the recommended dose specified for human nutrition (Young et al. 1998). Further, maize protein is composed of high leucine-isoleucine ratio and possesses low biological value. About 60% maize storage protein comprises of prolamins/zeins, and the rest are non-zein proteins such as albumin (3%), globulins (3%) and glutelins (34%) (Vasal, 1999). Zeins composed of α-(19- and 22- kDa), β-(15-kDa), γ-(10- and 18-kDa) and δ- (16-, 27- and 50-kDa) fractions, are deficient in lysine and tryptophan (Olsen and Phillips, 2001; Wu and Messing, 2011).

Native genetic systems for enhancing protein quality in maize endosperm

Various kernel mutations, *viz*., *opaque2* (*o2*), *opaque6* (*o6*), *opaque7* (*o7*), *opaque11* (*o11*), *floury2* (*fl2*), *floury3* (*fl3*), Mucronate (*Mc*) and Defective endosperm (*De-B30*) possess significantly higher concentration of lysine and tryptophan in endosperm as compared to traditional maize (Boyer and Hannah, 2001). Among these stocks, recessive *o2* mutant has been utilized the most in the breeding programme for enhancement of kernel quality (Vivek et al. 2008). Mutant *o2* allele was discovered in the early 1920; and researchers at the Purdue University, USA later established that *o2* mutant caused nearly two fold increase in lysine and tryptophan compared to normal maize (Mertz et al. 1964). The *o2* gene located on chromosome 7L produces leucine-zipper (bZIP) protein that acts as a transcriptional factor for expression of zein family of storage protein genes, especially 22-kDa α-zeins (Ueda et al. 1992). The mutant protein causes reduction in synthesis of zein protein by 50-70% primarily due to its less affinity of binding to the promoter regions (Kodrzycki et al. 1989). The enhancement of nutritional quality in *o2* mutant is mainly due to reduction of lysine deficient zein proteins followed by enhanced synthesis of lysine-rich non-zein proteins (Habben et al. 1993). Recessive *o2* significantly reduces transcription of lysine keto-reductase (LKR), the enzyme that degrades lysine in maize endosperm, thereby enhancing the concentration of lysine (Kemper et al. 1999). Further, *o2* is involved in regulation of various metabolic pathways and causes enhanced synthesis of various lysine-rich proteins and enzymes (Jia et al. 2013). Lysine in maize endosperm possesses strong positive correlation with tryptophan, and normally the value of lysine is four times that of tryptophan (Vivek et al. 2008).

Transgenic approach for enhancing protein quality

Since *o2* is recessive, it is required to be introgressed into both the parents of the hybrid to harness the benefit of enhanced nutritional quality. However, development

of suitable dominant mutant that can potentially increase lysine and tryptophan in the endosperm, would be desirable as conversion of one parent of the hybrid would harness the nutritional benefit to a great extent. Dominant genetic system developed through transgenic approach holds promise in enhancing protein quality in maize. Unger et al. (1993) created two mutant forms of the *O2* gene that possessed deletions encoding (i) the basic domain and (ii) the first 175 N-terminal residues. When either of these mutant *O2* genes was co-expressed with wild-type *O2*, it inhibited expression of 22-kDa α-zein by ~10-fold in maize endosperm suspension cells. Since, 22-kDa α-zein is deficient in lysine and tryptophan, its reduction causes concurrent increase in non-zein proteins that are rich in these essential amino acids. Further, Segal et al. (2003) developed RNA interference (RNAi) constructs derived from a 22-kDa α-zein, and produced a dominant opaque phenotype. Antisense RNA for 22-kDa α-zein was also tried and it reduced the expression of 22-kDa α-zein, however it failed to produce an opaque phenotype. Wu and Messing (2011) used a novel genetic system in which RNAi construct was directed against both 22- and 19- kDa α-zeins. The transgenic plants showed significant reduction in synthesis of zeins and recorded high lysine concentration. However, these transgenic maize lines with great promise are yet to be utilized for commercial production of maize hybrids with enhanced lysine and tryptophan.

Utilization of native o2 and development of QPM

The nutritional benefits of native *o2* prompted the breeders across the world to introgress the mutant allele in different genetic backgrounds (Vasal et al. 1980). However, the nutritionally enriched maize thus developed could not be accepted due to negative pleiotropic effects of *o2*, *viz*., (i) soft and opaque endosperm, (ii) low kernel density, (iii) slow dry down, (iv) increased susceptibility to insect-pests and diseases, and (v) problem in mechanical processing due to softness in the kernel (Wessel-Beaver and Lambert, 1982). To circumvent these problems, especially soft endosperm associated with *o2*, different double mutant combinations such as *o2/fl2* and *o2/su2* were tried, but they could not provide the desirable solution (Vasal, 1999). The breakthrough, however came with the appearance of some proportion of vitreous/hard kernel in some of the backcross derived *o2*-based progenies especially with Carribean, Cuban, and flint backgrounds. The chromosomal regions effecting the conversion of soft into vitreous kernel, are referred to as 'endosperm modifier genes' (Vasal et al. 1980).

Endosperm modifiers are under the control of complex genetic system (Vasal et al. 1980). It possesses (i) various types of gene actions, *viz*., additive, dominant, recessive, semi-dominant inheritance, (ii) effects of background genome and environments, (iii) reciprocal and dosage effects, (iv) incomplete penetrance, (v) variable expressivity and (vi) xenia effects (Wessel-Beaver and Lambert, 1982; Belousov, 1987; Lopes and Larkins, 1991, 1995; Hossain et al. 2008a, b). Though mechanism of endosperm modification in QPM is not fully understood, 2 to 3 fold increase in 27-kDa γ-zein has been identified as the major factor of endosperm modification (Wu et al. 2010). In *o2*, reduction of α-zein synthesis causes severe reduction in size of protein bodies (PBs) that in turn causes loose packaging with air spaces in between, leading to opaque- and soft-kernel (Wu et al. 2010). However, elevated synthesis of 27-kDa γ-zein in presence of modifier loci results in more PBs that is smaller in size (Dannehoffer et al. 1995; Lopes and Larkins, 1995). Cystein residues in the periphery are engaged in formation of disulphide bonds with the neighbouring PBs that result in formation of compact packing, thus vitreousness is restored (Wu et al. 2010). Besides, amorphous, non-crystalline amylopectin molecules at the surface of starch granules in the modified kernel interact and form contacts that link starch granules together (Gibbon et al. 2003). Starch granules in normal maize do not form cohesive contacts with each other. Amylopectin in modified kernel has reduced levels of intermediate-length of α-1, 4-linked glucose chain that is associated with increased swelling in water and formation of tight contacts between starch granules. The physical contacts in QPM restore hard and vitreous phenotype. Alteration of amylopectin branching patterns in starch in *o2* genotype could contribute to soft- and starchy-endosperm (Jia et al. 2013). Several loci for kernel hardness have been identified by various researchers (Lopes and Larkins, 1995; Lopes et al. 1995; Dannehoffer et al. 1995; Gutierrez-Rojas et al. 2010; Holding et al. 2008, 2011). Sustained breeding efforts at CIMMYT, Mexico (Villegas et al. 1992) and University of Natal, South Africa (Geevers and Lake, 1992) could successfully accumulate desirable endosperm modifiers in *o2* genetic background that finally led to the development of nutritionally enriched vitreous maize, popularly phrased as quality protein maize (QPM) (Vasal et al. 1980).

Development of QPM cultivars

Apart from increasing lysine and tryptophan, *o2*-based genotypes possess 80% biological value as compared to 45% in normal maize. Further, based on nitrogen balance index protein quality of QPM is 90% to that of milk (Graham et al. 1980). Decreased leucine-isoleucine ratio in QPM is helpful in liberating more tryptophan for niacin biosynthesis, and thus, helps to combat pellagra (Vasal, 1999). Besides, beneficial effects of QPM over normal maize on chickens and pigs in increasing their body weight and overall growth and development are well documented (Burgoon et al. 1992; Osei et al. 1999).

The nutritional benefits of QPM prompted the breeders to develop suitable QPM germplasm adapted to different agro-ecologies. Large number of open pollinated varieties and hybrids in QPM genetic background has been released worldwide; and many of them are quite popular especially in Africa, Asia and Latin America (Vasal, 1999; Prasanna et al. 2001; Nuss and Tanumihardjo, 2011). In 1992, 'Obatanpa' an open-pollinated variety of QPM was introduced in Ghana, and by 2005, it comprised >90% of the maize seed sale. During 2008, Ghana grew QPM on 350,000 hectares of land that accounts one of the largest areas in the world (www.cimmyt.org). In India, the effort to enrich maize with high lysine and tryptophan began in 1960s, which subsequently led to the release of three *o2*-based maize composites, *viz*., Shakti, Rattan and Protina in 1971 (Table 1). These are the first biofortified maize cultivars developed through targeted breeding in the country. However, due to soft endosperm and other associated problems, these high lysine maize composites could not become popular. Breeders through accumulation of endosperm modifiers successfully developed first vitreous endosperm-based QPM composite, Shakti-1 during 1997. With the availability of diverse QPM inbreds, focus was gradually shifted to develop QPM hybrids for different agro-ecologies of the country. In 2001, the first QPM hybrid, 'Shaktiman-1' (a white kernel-based three way cross) was released for commercial cultivation in the country. During 2004, 'Shaktiman-2', a white kernel-based single-cross hybrid was released. 'Shaktiman-3' and 'Shaktiman-4', the first yellow-kernel based single cross hybrids, were released together during 2006. Later, a number of single cross QPM hybrids (with yellow kernel), *viz*., HQPM-1, HQPM-4, HQPM-5, HQPM-7, Pratap QPM Hybrid-1 and Shaktiman-5 have been released (Table 1). However, majority of the QPM hybrids released in India are developed from inbreds of CIMMYT-origin. Thus, there is an urgent need to diversify the QPM germplasm in the country and develop new QPM inbreds in different maturity groups. Maize breeding programme at Chaudhary Charan Singh Haryana Agricultural University (CCSHAU), Uchani; Rajendra Agricultural University (RAU), Dholi, Bihar; ICAR-Vivekananda Parvatiya Krishi Anusandhan Sansthan (VPKAS), Almora; ICAR-Indian Institute of Maize Research (IIMR), New Delhi; ICAR-Indian Agricultural Research Institute (IARI), New Delhi; Acharya N.G. Ranga Agricultural University (ANGRAU), Hyderabad; and Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya (CSK-HPKV), Bajaura are actively involved in generating diverse QPM inbreds (Hossain et al. 2007; Hossain et al. 2008a; Dass et al. 2012; Kumar et al. 2012). Experimental hybrids with high lysine and tryptophan, coupled with higher -endosperm modification and -grain yield have been identified, and currently are under various stages of testing.

The beginning of 21st century marked with the enhanced application of molecular markers in crop improvement, and maize in India was no more an exception. Availability of gene-based co-dominant markers (SSRs, *viz*., *umc1066* and *phi057*) coupled with densely mapped SSRs throughout the genome provides opportunity to undertake marker-assisted selection (MAS) for *o2* allele (Agrawal and Gupta, 2010; Prasanna et al. 2010). The first example of 'proof of concept' on application of molecular markers for improvement of nutritional quality in India, was the introgression of *o2* allele from a white kernel QPM inbred, CML176 into an elite yellow inbred, V25 using marker-assisted backcross breeding (MABB) strategy (Babu et al. 2005). This research effort at VPKAS led to the increase of tryptophan in endosperm protein from 0.41% to as high as 0.82% in the selected MAS-derived inbreds. Background selection employed was effective to recover >90% of recurrent parent genome in just two generations of backcross. Utilizing the same strategy at VPKAS, parental inbreds (CM145 and CM212) of Vivek Hybrid-9 were improved for endosperm lysine and tryptophan; and it led to the development and commercial release of Vivek QPM-9 (Gupta et al. 2009; Gupta et al. 2013). Vivek QPM-9 possesses 41% more tryptophan and 30% more lysine over the original hybrid, with similar grain yield potential of Vivek Hybrid-9. Stringent selection of endosperm modification helped in having high degree of vitreous kernels in the reconstituted version of parental inbreds and hybrids. Vivek QPM-9 earned

Table 1. Details of the maize cultivars in India with enhanced endosperm lysine and tryptophan[$]

S.No.	Name	Type	Parentage	Kernel colour	Year	Centre[#]	Area of adaptation
Soft endosperm							
1.	Shakti	Composite	JLo2, Cuba 1Jo2, Antigua 2Do2	Yellow	1971	Delhi	MP, Rajasthan and Tarai belt of UP
2.	Rattan	Composite	J1o2	Yellow	1971	Ludhiana	Punjab and Rajasthan
3.	Protina	Composite	(Jowatigua x Antigua car II) o2 x (Doeto × G.C.C.) o2	Yellow	1971	Pantnagar	Punjab, Rajasthan, Mysore and Tarai belt of UP
Hard endosperm/QPM							
4.	Shakti-1	Composite	Antigua, Ver 181 HEo2, Amarillo crstallino HEo2, Ant Rep Dom, HEo2, temperate HEo2	Yellow	1997	Delhi	Across the country
5.	Shaktiman-1	Three way cross	(CML142 x CML150) x CML186	White	2001	Dholi	Bihar
6.	Shaktiman-2	Single cross	CML176 × CML186	White	2004	Dholi	Bihar
7.	HQPM-1	Single cross	HKI193-1 × HKI163	Yellow	2005	Uchani	Across the country
8.	Shaktiman-3	Single cross	CML161 × CML163	Yellow	2006	Dholi	Bihar
9.	Shaktiman-4	Single cross	CML161 × CML169	Yellow	2006	Dholi	Bihar
10.	HQPM-5	Single cross	HKI163 × HKI 161	Yellow	2007	Uchani	Across the country
11.	HQPM-7	Single cross	HKI193-1 × HKI161	Yellow	2008	Uchani	Karnataka, AP, TN and Maharashtra
12.	Vivek QPM-9[*]	Single cross	VQL1 × VQL2	Yellow	2008	Almora	J & K, Uttarakhand, HP, AP, TN, Karnataka and Maharashtra
13.	HQPM-4	Single cross	HKI-193-2 × HKI 161	Yellow	2010	Uchani	Across the country
14.	Vivek QPM-21[*]	Single cross	VQL1 × VQL17	Yellow	2012	Almora	Uttarakhand
15.	Pratap QPM Hybrid-1	Single cross	DMRQPM-106 × HKI-193-1	Yellow	2013	Udaipur	Gujarat, Rajasthan, MP and Chattisgarh
16.	Shaktiman-5	Single cross	CML161 × CML165	Yellow	2013	Dholi	Orissa, Bihar, Jharkhand, West Bengal and Eastern UP

[$]As per DMR, 2011 and ICAR-Annual Report 1971-72; [#]AICMIP-centre for maize improvement; [*]Hybrids developed through MAS

the distinction of being the first MAS-based maize cultivar released for commercial cultivation in India. Vivek QPM-21, developed through marker-assisted introgression of *o2* allele into Vivek Hybrid-21 was yet another QPM hybrid released in 2012 for Uttarakhand state. With the success of this technology, *o2*-based SSRs are now being routinely utilized for accelerated development of QPM hybrids at different breeding centres of India. Several institutions under the Indian Council of Agricultural Research (ICAR) and State Agricultural Universities (SAUs) have targeted enhancement of lysine and tryptophan in selected normal maize hybrids using accelerated breeding strategy (Gupta, 2014). Research efforts at IARI have led the development of QPM version of five commercial hybrids, *viz*., HM-4, HM-8, HM-9, HM-10 and HM-11 (Hossain et al. 2014). DHM-117 and Palam Sankar Makka-2 from ANGRAU and CSK-HPKV, respectively have been improved for protein quality (Lata et al. 2014). Further, single cross hybrids, *viz*.,

Buland and PMH-1 have been targeted for conversion to QPM using MAS (Vikal et al. 2014a). These experimental MAS-derived QPM hybrids are currently under different stages of multi-location testing. Several QPM inbreds have also been developed using MAS strategy at ICAR-Research Complex for NEH Region (ICAR-RC-NEH), Barapani; Jawaharlal Nehru Krishi Vishwa Vidyalaya (JNKVV), Jabalpur; Banaras Hindu University (BHU), Varanasi and G.B. Pant University of Agriculture and Technology (GBPUAT), Pantnagar (Pattanayak et al. 2014; Tiwari et al. 2014; Tufchi and Singh, 2014). Several research efforts worldwide have also successfully deployed MAS for developing QPM genotypes (Danson et al. 2006; Manna et al. 2006; Jompuk et al. 2011).

Further enhancement of protein quality in QPM

Though introgression of *o2* causes drastic enhancement of lysine and tryptophan in normal maize genotypes, accumulation of favourable amino acid modifiers in the genetic background plays a vital role in further increasing the level of amino acids (Pandey et al. 2014). Lysine (2.7-4.5% of total protein in whole grain flour) and tryptophan (0.5-1.1% of total protein in whole grain flour) exhibit wide range of variation among QPM genotypes (Vivek et al. 2008). Wang and Larkins (2001) observed that free amino acid (FAA) content in Oh545o2 was 10 times higher than in Oh51Ao2 and W64Ao2. QTL mapping using $F_{2:3}$ mapping population generated between two *o2* inbreds identified four significant loci that account ~46% of the phenotypic variance (Wang and Larkins, 2001). Although FAA represents 1-3% of the total non-protein N in Oh545o2, free lysine accounts one-third of the total lysine, indicating specific loci influencing the FAA accumulation. Pineda-Hidalgo et al. (2011) identified QTLs that are responsible for accumulation of higher FAA in the *o2* genetic background. Gutierrez-Rojas et al. (2010) also localized QTLs for accumulation of lysine and tryptophan in QPM genotypes. Recently, Babu et al. (2015) developed $F_{2:3}$ mapping population from a cross between VQL2 and VQL8 (two isogenic QPM inbreds) that significantly differed in tryptophan, and identified five significant QTLs on chromosomes 5, 7 and 9 that together explained 38.6% of the total phenotypic variance. Accumulation of these amino acid modifier loci through MAS coupled with stringent biochemical analyses would tremendously help in increase of lysine and tryptophan in *o2* genotypes (Babu et al. 2012). At VPKAS, MAS-derived QPM inbreds, *viz*., VQL1, VQL2 and VQL17 have been targeted for accumulation of amino acid modifiers from new QPM donors with high tryptophan (Dr. P.K. Agrawal, Personal communication).

Further, a recessive *opaque16* (*o16*) mutant was also found to be associated with higher nutritional value in maize (Yang et al. 2005). Mutant combination of *o2* and *o16* offers possibility of enhancement of lysine by 40-80% over *o2* genotype alone. *O16* has been mapped on chromosome 8L, and *umc1141* and *umc1149* were identified as closely linked markers. Several researchers have successfully pyramided *o2* and *o16* in diverse genetic background using MAS, and reported higher concentration of lysine and tryptophan (Zhang et al. 2013; Yang et al. 2013). Breeders at IARI have recently initiated the introgression of *o16* into commercially available QPM hybrids (Gupta, 2014). The F_1s with *o16* allele in heterozygous conditions have been obtained from Guizhou Institute of Upland Food Crops, Guizhou Academy of Agricultural Sciences, China; and are being used as donors in the MABB programme.

Though availability of SSR markers for *o2* and *o16* has effectively accelerated the introgression of the target allele(s) into elite normal maize, accumulation of both endosperm- and amino acid-modifiers with desirable effects in *o2* background poses major challenge as these modifiers are polygenic and their expression is highly influenced by genetic background. Despite availability of QTLs and closely linked markers for many such modifier loci, standard 'light box test' for endosperm modification and biochemical analyses for lysine and tryptophan; are still followed. Hence, introgression of endosperm- and amino acid-modifiers loci are essential to achieve desirable level of modification in early segregating generations.

Provitamin A rich maize

Vitamin A in human nutrition

Vitamin A (retinol) is essentially required by humans for the normal functioning of the visual system, growth and development, and maintenance of epithelial cell integrity, immune system and reproduction (Olson, 1999). World Health Organization (WHO) recommends an estimated average requirement of 250 and 500 RE (Retinol Equivalents) per day for children and adults, respectively, for normal growth and development (Bouis and Welch, 2010). Since humans and animals cannot synthesize vitamin A in their body, needs for vitamin A are provided through dietary means. Preformed vitamin A is found in animal products such as human milk, glandular meats, fish liver oil, egg

yolk and other dairy products. Dietary provitamin A is obtained from a number of fruits and vegetables such as apricots, papaya, carrots, spinach, peaches and sweet potatoes. In resource poor developing world, plant foods containing provitamin A carotenoids are much affordable than animal products. β-carotene-rich maize is efficacious when consumed as a staple food as compared to vitamin A supplementation and commercial fortification; and the risks of hyper-vitaminosis A from provitamin A rich foods are almost non-existent (Gannon et al. 2014).

Vitamin A deficiency (VAD) results in blindness, and affects nearly 190 million preschool-age children and 19 million pregnant women, mostly in Africa and South Asia (WHO, 2009). VAD has emerged as a global challenge and is particularly present in developing countries like India, where one-third of our 120 million pre-school children are vitamin A deficient (Akhtar et al. 2013). Besides blindness, VAD also results in predisposition of several diseases like anaemia, diarrhoea, measles, malaria and respiratory infections (West, 2000). It further contributes to maternal death, malnourished pregnancy and lactation making the young children, pregnant women and lactating mothers most vulnerable (Akhtar et al. 2013).

Genetic and molecular bases of accumulation of carotenoids

White maize lacks carotenoids in the endosperm due to the presence of recessive *y1* (*phytoene synthase1* or *psy1*), the key gene that controls the first step in the carotenoid biosynthesis pathway (Buckner et al. 1990). Among the staple cereals, yellow maize by virtue of functional *Y1* gene, possesses natural genetic variation for carotenoids thereby showing promise for provitamin A biofortification through breeding approaches. The yellow kernel maize contains several carotenoid isoforms, including two carotenes (α-carotene and β-carotene) and three xanthophylls (β-cryptoxanthin, zeaxanthin and lutein) (Watson, 1962). Various research efforts worldwide have reported the existence of wide genetic variation for carotenoids (Ortiz-Monasterio et al. 2007; Chander et al. 2008; Menkir et al. 2008). The extent of variability for maize kernel carotenoids observed in various studies conducted in India is reported in Table 2. Studies conducted on genetic variability for kernel carotenoids in Indian maize germplasm are mostly limited to the estimation of total carotenoids. Very few reports are available on variation of kernel β-carotene in maize. Though yellow maize genotypes contain enough carotenoids, the fraction with provitamin A activity (β-carotene, α-carotene and β-cryptoxanthin which can be converted to vitamin A) is less compared to zeaxanthin and lutein (Muthusamy et al. 2015).

Table 2. Variation for different carotenoids in maize germplasm studied in India

Carotenoid concentration (μg/g)		Reference
TC	: 0.03-25.8	Mishra and Singh, 2010
TC	: 0.94-38.25	Das and Singh, 2012
TC	: 12.2-30.10	Tiwari et al. 2012
TC	: 6.5-67.3	Sivaranjani et al. 2013
TC β-carotene	: 3.3-27.4; : 0.0-4.81	Rashmi and Singh, 2014
β-carotene	: 4.5-7.92	Selvi et al. 2014
TC	: 0.1-11.4	Vikal et al. 2014b
β-carotene	: 1.1-18.8	Chaudhary et al. 2015
Lutein Zeaxanthin β-cryptoxanthin β-carotene	: 1.3-11.3; : 1.7-20.0; : 0.1-3.3; : 0.0-1.8	Muthusamy et al. 2015

TC = Total carotenoids

The carotenoids in maize are reported to have high heritability (Egesel et al. 2003; Menkir et al. 2008; Muthusamy et al. 2015). Preponderance of additive genetic variance for carotenoids in maize further offers possibility of higher response to selection in developing carotenoid-rich maize genotypes (Suwarno et al. 2014; Muthusamy et al. 2015). Many reports have also suggested that the influence G × E interaction is very less and the carotenoids are stable across locations (Menkir et al. 2008; Muthusamy et al. 2015).

Based on the factors such as bioconversion ratio of 12:1, nutrient status of the host, type of carotenoid, food matrix and amount of food consumed in the meal, nutritionists have estimated a goal of 15 μg/g of provitamin A in maize kernel (Bouis et al. 2011). Though wide variations for various carotenoids are observed, the proportion of β-carotene, the major provitamin A carotenoid is quite low to meet the target level. Besides, quantification of carotenoids in maize genotypes using high performance liquid chromatography (HPLC) is expensive. Hence, quantification of provitamin A carotenoids in a large breeding population is often a challenging task. The poor correlation among kernel colour and β-carotene makes the selection of genotypes for higher kernel

provitamin A based on grain colour quite unreliable (Muthusamy et al. 2015). Thus, an effective and rapid selection strategy would help in accelerating the breeding programmes. Efforts have been undertaken to biofortify maize especially with provitamin A carotenoids by exploiting the natural variants of key genes (Harjes et al. 2008; Yan et al. 2010).

Native genetic system for provitamin A enrichment in maize

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

Using allele mining strategy, four natural *lcyE* polymorphisms, *viz*., *lcyE* 5'TE [Transposable Element; in 5'-untranslated region (UTR)], *lcyE* SNP216 (in exon 1), *lcyE* SNP2238 (in intron 4) and *lcyE* 3'InDel (in 3'-UTR) were identified, of which, the favourable allele of *lcyE* 5'TE causes more increase in provitamin A in the endosperm (Harjes et al. 2008). Yan et al. (2010) through association mapping approach, detected three polymorphisms, *viz*., 5'TE (in the 5'-UTR), InDel4 (in the coding region) and 3'TE (spanning the sixth exon and 3'-UTR) in *crtRB1* that were significantly associated with conversion of β-carotene to β-cryptoxanthin and zeaxanthin in maize kernels. Of which, *crtRB1* 3'TE favourable allele alone causes two to ten-fold variation in the β-carotene concentration among the inbreds (Babu et al. 2013; Muthusamy et al. 2014). The favourable allele with reduced transcript expression causes enhanced accumulation of β-carotene. Transcript expression of these two key genes is tissue specific; where the difference in expression of wild and mutant alleles is very high in endosperm, while it is not much in embryos, and similar in leaves (Babu et al. 2013). PCR-based co-dominant markers have been designed for both *lcyE* and *crtRB1* based polymorphisms which can pave way for rapid improvement of provitamin A in maize through MAS (Babu et al. 2013).

Since these favourable alleles are reported to cause higher accumulation of β-carotene to other carotenoids, identification of genotypes with the favourable allele of these two key genes thus can help in identification of provitamin A-rich genotypes without intensive HPLC assay. Vignesh (2012) reported very low frequency of favourable allele for both *lcyE* (3.38%) and *crtRB1* (3.90%) while screening large set of maize inbreds in India. Similar results of nil to low frequency of the favourable alleles were also reported (Rashmi and Singh, 2014; Selvi et al. 2014; Vikal et al. 2014b). Inbreds specifically bred under the CIMMYT-HarvestPlus Programme possess ~15 µg/g of β-carotene (Vignesh et al. 2012). Interestingly, the Indian genotypes with the favourable allele of these genes were quite low in β-carotene, in contrast to the CIMMYT-HarvestPlus genotypes. This phenotypic variation could be attributed to the effect of the genetic background as the concentration of β-carotene is regulated by various genes other than *lcyE* and *crtRB1* in the carotenoid biosynthesis pathway. This could also be attributed to the presence of nucleotide variation within the favourable allele thereby leading to phenotypic variation. Vignesh et al. (2013) while comparing a set of high- and low-β-carotene inbreds, identified SNPs and InDels in the 3'UTR region of the *crtRB1*-favourable allele.

Development of provitamin-A rich maize using natural mutants

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

hybrids into β-carotene-rich version using MABB approach. Improved version of Vivek QPM-9 possesses high β-carotene coupled with higher lysine and tryptophan, thereby providing multi-nutrients through maize-based diet. This is the first successful example of combination of nutrients, *viz*., provitamin A and QPM (Muthusamy et al. 2014). Parental lines of the hybrids HM-4 and HM-8 are also targeted for improvement of lysine and tryptophan in a separate breeding programme (Hossain et al. 2014) and the efforts are on to combine QPM and provitamin A. MAS is also being used to pyramid favourable alleles, *viz*., *lcyE* and *crtRB1* to further enhance kernel β-carotene in QPM hybrids. Currently, maize breeding programme at IARI, VPKAS, CSK-HPKV, ANGRAU, GBPUAT, and Tamil Nadu Agricultural University (TNAU), Coimbatore are actively involved in generating/ selecting diverse inbreds with high β-carotene. A diverse set of inbreds with favourable alleles of *lcyE* and/or *crtRB1* have been characterized for their effective utilization in the breeding programme (Choudhary et al. 2014, 2015). Globally, three maize hybrids from Zambia (GV662A, GV664A, GV665A), two hybrids (Ife maize hyb-3, Ife maize hyb-4) and two synthetics (Sammaz 38, Sammaz 39) from Nigeria and one synthetic from Ghana (CSIR-CRI Honampa) were released that contain 6 to 8 µg/g of provitamin A (www.harvestplus.org).

Transgenic approach for enrichment of provitamin A

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

The major challenge in breeding for enhanced provitamin A in maize is the loss of β-carotene during post-harvest/processing stages (De-Moura et al. 2013). Since, carotenoids are highly heat labile, it is essential to develop biofortified maize to sustain the carotenoid level during the post-harvest handling and processing. Studies at CIMMYT have shown that loss of provitamin A is higher at initial stages of storage and becomes stable after 6-8 weeks. However, degradation is also influenced by genetic background and few inbreds with lesser degradation during the storage have been identified (De-Moura et al. 2013; Suwarno et al. 2015). A native variant of *CCD1* (*carotenoid cleavage dioxygenase 1*) that causes reduced loss of provitamin A during storage has been recently identified (Suwarno et al. 2015). Thus, research efforts need to be directed to develop maize genotypes that retain higher levels of provitamin A for a longer period of time while storage.

Kernel -Fe and -Zn

Fe and Zn in human nutrition

Iron (Fe) is required in humans for basic cellular functions, proper functioning of muscle, brain, red blood cells, and is an integral part of various enzymes (Hallberg, 1982). Zinc (Zn) is an essential mineral for many biological functions and is part of more than 300 enzymes responsible for the synthesis and degradation of various biomolecules, *viz*., carbohydrates, proteins and lipids (Sandstorm, 1997). Over 60% and 30% of the world's populations are deficient in Fe and Zn, respectively (White and Broadley, 2009). These deficiencies affect people of all ages but are more prominent in pregnant women and children. Fe-deficiency anaemia leads to one-fifth of perinatal mortality and one-tenth of maternal mortality particularly in developing countries like Africa and Asia. Besides, it causes mental retardation, goiter and eye problems, reduction in reproductive performance and work productivity (Scrimshaw, 1984). Zn-deficiency leads to growth retardation, delayed sexual- and bone-maturation, increased susceptibility to infections and behavioural changes like depression and psychosis (Prasad, 1996). It further results in short stature, hypogonadism, impaired immunity, skin disorders, cognitive dysfunction, anorexia, altered reproductive biology and gastrointestinal problems (Solomons, 2003).

Genetic and molecular bases of Fe- and Zn-accumulation

In maize, concentration of minerals such as Fe is reported to be highest in the pericarp and scutellum,

however due to the greater proportion of endosperm in the grain, the total content of them is higher in the endosperm (60-80%), followed by scutellum (15-35%) and seed coat (8-12%) (Bityutskii et al. 2002). Major proportion of Fe and Zn is deposited in the aleurone, which in maize kernel is made up of single layered cells. Multiple aleurone layers (MAL; 2.0-3.7 layers per kernel) due to occasional doubling of individual aleurone layer have been reported in maize (Wolf et al. 1972). MAL genotypes are reported to have 19% and 39% higher -Fe and -Zn, respectively, over the single aleurone layer genotypes (Welch et al. 1993). Thus, MAL mutants can be explored for enhancement of kernel -Fe and -Zn in maize.

As per estimated average requirement (EAR), human requires 1460 µg/day of Fe, while it is 1860 µg/day for Zn. Considering bioavailability (5% for Fe; 25% for Zn) and 90% retention after processing, 60 µg/g of Fe and 38 µg/g of Zn (on dry weight basis) have been fixed as the target level in maize (Bouis and Welch, 2010). Sufficient genetic variation for kernel -Fe and -Zn in maize has been reported worldwide (Banziger and Long, 2000; Pixley et al. 2011). In India, Chakraborti et al. (2009), Chakraborti et al. (2011a, b), Prasanna et al. (2011), Agrawal et al. (2012), Guleria et al. (2013), Goswami et al. (2014), Mallikarjuna et al. (2014) and Pandey et al. (2015) reported wide genetic variation for kernel -Fe and -Zn in diverse set of normal- and QPM- inbreds (Table 3). The presence of ample variability for kernel -Fe and -Zn indicates the possibility of genetic enhancement of these micronutrients in maize.

Kernel -Fe and -Zn are also reported to be significantly influenced by the environments (Chakraborti et al. 2009; Chakraborti et al. 2011a). They are mainly affected by soil type, soil fertility, soil moisture, and interactions among nutrients (Arnold and Bauman, 1976; Prasanna et al. 2011; Agrawal et al. 2012; Qin et al. 2012; Guleria et al. 2013). Despite significant G × E interactions, many studies have reported that the major proportion of variation is due to genetic factors, thus it is possible to identify Fe- and Zn- rich maize genotypes (Prasanna et al. 2011; Agrawal et al. 2012; Guleria et al. 2013). Accumulation of Fe and Zn in maize kernel is governed by polygenes (Gorsline et al. 1964; Arnold and Bauman, 1976). QTLs governing the accumulation of these micronutrients in maize have been reported (Lungaho et al. 2011; Simic et al. 2011; Qin et al. 2012; Baxter et al. 2013). Genome-wide analyses of transporters could also provide insight into the key genes that are responsible for accumulation of minerals in maize kernel (Mondal et al. 2013). Nature of genetic variance governing these traits is important to select appropriate breeding procedures for genetic improvement. Many studies have suggested the preponderance of additive gene action for kernel Fe and Zn (Gorsline et al. 1964; Arnold and Bauman, 1976; Pixley et al. 2011; Simic et al. 2011). In contrast, Chakraborti et al. (2010) observed preponderance of dominance gene action for kernel-Zn. The heritability of these micronutrients are reported to be moderate to high with 0.46 to 0.73 for kernel-Fe and 0.59 to 0.70 for kernel-Zn (Lungaho et al. 2011; Simic et al. 2011; Pixley et al. 2011; Qin et al. 2012; Baxter et al. 2013). These results indicate that performance of inbreds will be a good indicator of performance of hybrids; thus best inbred lines with high kernel -Fe and -Zn can be used as parents to increase the concentration in the hybrids (Menkir, 2008).

Positive correlation between kernel -Fe and -Zn has been reported in many studies (Arnold et al. 1977; Chakraborti et al. 2009; Lungaho et al. 2011). This could be due to the pleiotropic effect and/or linkage among the genes governing kernel -Fe and -Zn accumulation. A large number of genes encode metal transporter proteins and some of which may transport multiple metals together (Qin et al. 2012). Further, some of QTLs for both these traits been found to be co-localized in the same region of the chromosome

Table 3. Variation for kernel -Fe and -Zn in maize germplasm studied in India

S.No.	Range		References
	Fe (mg/kg)	Zn (mg/kg)	
1.	13.23-40.09	13.44-46.39	Chakraborti et al. 2009
2.	12.02-38.46	17.57-49.14	Chakraborti et al. 2011a
3.	13.95-39.31	21.85-40.91	Chakraborti et al. 2011b
4.	11.28-60.11	15.14-52.95	Prasanna et al. 2011
5.	20.38-54.29	7.01-29.88	Agrawal et al. 2012
6.	-	3.81-35.83	Guleria et al. 2013
7.	12.1-37.8	10.6-21.3	Goswami et al. 2014
8.	16.6-83.4	16.4-53.2	Mallikarjuna et al. 2014
9.	23.8-42.7	12.6-39.4	Pandey et al. 2015

(Qin et al. 2012). This finding suggests the possibility of simultaneous improvement of kernel -Fe and -Zn. Contrasting reports are also available showing weak association (Simic et al. 2009) or no association between Fe and Zn in maize (Arnold and Bauman, 1976; Prasanna et al. 2011; Agrawal et al. 2012). Existence of unique QTLs for kernel -Fe and -Zn suggests the possibility of independent improvement of these traits (Simic et al. 2011). Negative association between yield and micronutrients has been reported in various studies (Banziger and Long, 2000; Long et al. 2004; Simic et al. 2009; Pixley et al. 2011). Brkic et al. (2003), Lungaho et al. (2011), Menkir (2008) and Simic et al. (2009) detected no association between grain yield with that of kernel -Fe and -Zn. In contrast, Chakraborti et al. (2009) observed positive correlation between grain yield and kernel-Zn. This variable trend could be attributed to the inherent nature of the specific germplasm used in these studies (Gupta et al. 2015); and is therefore possible to develop high yielding maize cultivar with enhanced kernel -Fe and -Zn. QPM genotypes with *o2* allele are reported to have higher concentration of kernel -Fe and -Zn. Higher accumulation of kernel -Fe and -Zn in *o2* genotypes were reported as compared to normal maize (Arnold et al. 1977; Welch et al. 1993). Chakraborti et al. (2009) also reported higher concentration of Zn in QPM inbreds compared to normal inbreds, whereas no significant difference was observed for kernel-Fe. This association of micronutrients with essential amino acids offers possibility to develop multinutrient-rich maize (Gupta et al. 2015; Pandey et al. 2015).

Enhancement of Fe- and Zn-bioavailability

Various factors, *viz*., type of food, physiological status of the body, level of anti-nutritional- and promoting-factors play a vital role in making -Fe and -Zn bioavailable to the humans (Gupta et al. 2015). Of which the anti-nutritional component, phytic acid/ phytate plays a major role in reducing the bioavailability of -Fe and -Zn, thus an important target for biofortification in maize. Nearly 80% of the total phosphorus in the maize grain is present as phytic acid (Raboy et al. 2000) and the primary function of the phytate in the seed is to store phosphorus as energy source and antioxidants essentially required for the germinating seeds. But the negative charge of the phytic acid chelates the positively charged minerals like Fe and Zn, and makes them unavailable in the animal gut (Raboy, 2001). Phytic acid in grains reduces the availability of phosphorus to poultry since monogastric animals cannot digest it, and the undigested phytic acid when released into environment causes environmental pollution (Cromwell and Coffey, 1991). Thus, breeding for low phytate maize offers several advantages both as food and feed. On the other hand, lysine, carotenoids and vitamin C are reported to enhance the absorption of micronutrients in human (Ortiz-Monasterio et al. 2007; Welch and Graham, 2004). Therefore, besides increasing the *per se* concentration of kernel -Fe and -Zn, breeding approaches must also be undertaken to reduce the anti-nutritional factors and/or to enhance the promoting factors to increase bioavailability.

Genetic and molecular bases of low phytate in maize

Various low-phytic acid (*lpa*) mutations have been isolated in maize (Raboy et al. 2000, Pilu et al. 2003, Shi et al. 2005). The *lpa1* mutation is caused by variation in a gene downstream of the pathway that encodes transmembrane transporter protein (MRP), which transports phytic acid into protein storage vacuoles of the seed. The *lpa2* mutation is caused by variation in *inositol phosphate kinase* (ITPK), located upstream to the MRP and leads to the synthesis of the phytic acid along with other kinases. The *lpa2-1* mutation is caused by genomic sequence rearrangement in the ZmITPK, while *lpa2-2* is due to a nucleotide transition mutation (C to T) at position 158, that generates a stop codon in the N-terminal region of *ZmITPK* open reading frame (Shi et al. 2003). The *lpa3* is resulted by a mutation in a gene that encodes *myo-inosital kinase* (MIK), which catalyzes the production of Ins(3)P1 in maize seed. Mutants like *lpa241* is due to the variation in the *myo-inositol(3)P1 synthase* (*MIPS*) and, can cause a reduction of phytic acid up to 90% (Pilu et al. 2003; Raboy, 2001). Mutant *lpa1* reduces phytic acid by 66%, while *lpa2* and *lpa3* result in 50% reduction each in phytate.

Development of low phytate maize

In India, *lpa2-2* allele has been successfully introgressed into elite inbreds using MABB approach at TNAU. SSR marker '*umc2230'* closely located at 0.4 cM from *lpa2-2* (on chromosome 1) has been identified (Sureshkumar et al. 2014). Using this marker the mutant allele was transferred to 'UMI395', an elite maize inbred thereby validating the potentiality of this marker in MAS for low phytic acid (Sureshkumar et al. 2014). Tamilkumar et al. (2014) introgressed the *lpa2-2* allele to an elite inbred 'UMI285', which serves as one of the parents in multiple commercial maize hybrids. Marker-assisted introgression of *lpa1* and *lpa2*

mutants in early maturing inbreds, *viz*., CM145 and V334, respectively has also been recently carried out at VPKAS (Dr. P.K. Agrawal, Personal communication). Recent advances in genetic engineering has led to development of transgenic lines with low phytic acid by expressing exogenous phytase genes in maize (Chen et al. 2008). It is worth mentioning that phytic acid is part of plant metabolism, and plays a major role in plants' response to different abiotic stresses, besides possessing positive effects on imparting resistance against pathogens and insect-pests (Graham et al. 2001; Welch and Graham, 2004). It is also required for higher seedling vigour and reduced aflatoxin development in grain (Morris, 1995). Besides, phytate has been found to protect seeds against oxidative stress during the seed's life span (Doria et al. 2009). Thus, development of agronomically suitable high yielding genotypes with sufficiently low phytic acid is a challenge for the researchers.

Factors promoting enhancement of bioavailability of Fe and Zn

Dietary substances that promote/enhance the absorption of kernel -Fe and -Zn, can be increased in maize by combining these genes through suitable breeding strategy (Graham et al. 2001; Bouis and Welch, 2010). The *o2* allele is reported to enhance the concentration of micronutrients especially Zn (Arnold et al. 1977; Welch et al. 1993; Chakraborti et al. 2009). Zn-deficient rats showed an increase in absorption of Zn from 64% to 69% with the supplementation of lysine (House et al. 1996). Thus, breeding for QPM has immense potential to enhance the kernel-Zn concentration and its absorption in digestive system. Yellow/orange maize contains higher amount of carotenoids and plays a vital role in enhancing bioavailability of minerals. β-carotene increases the Fe-absorption level up to 1.8 fold (Garcia-Casal et al. 1998). Besides, addition of lutein in maize based diet increased the bioavailability of Fe by two fold (Garcia-Casal, 2006). Thus addressing anti-nutrients and promoter substances in crops could be an effective approach for biofortification of Fe and Zn, as fewer genes with profound effects have been found to operate in their biosynthesis and metabolism as compared to the complex mechanism of uptake, transport, and deposition of Fe and Zn in kernels, where it involves large number genes with minor effects. An increase of bioavailable-Fe from 5 to 20% would in turn relate to four fold increase in total Fe (Bouis and Welch, 2010). Already available QPM- and recently developed β-carotene-rich- maize hybrids thus possess great potential to enhance the bioavailability of kernel -Fe and -Zn.

Challenges in dissemination of biofortified maize

Biofortified maize with enhanced lysine, tryptophan, provitamin A, Fe and Zn possesses enormous potential to alleviate micronutrient malnutrition. Maize cultivars with improved protein quality and provitamin A have been developed; and available genetic- and genomic-resources coupled with advanced tools and techniques holds promise for developing high -Fe and -Zn rich maize cultivar. However, the successful adoption of biofortified maize cultivars depends on various factors. We discuss here some of the important factors, which once effectively addressed, could likely to pave the way for rapid dissemination of biofortified maize.

Apprehension of low yield potential

The general perception of low yielding potential of nutritionally-rich crops in general could play a crucial role in slowing down the pace of its dissemination. Similar apprehension of low yield potential of QPM also exists. A comparison of the yield performance of two QPM hybrids (CZH04034 and CZH04025) with a non-QPM commercial check (SC635) across 25 locations in eastern- and southern-Africa, revealed the possibility of developing better QPM cultivars than the normal check (Vivek et al. 2008). MAS-derived QPM version, Vivek QPM-9 possesses similar grain yield potential as that of the original hybrid, Vivek Hybrid-9 (Gupta et al. 2013). Muthusamy et al. (2014) evaluated MAS-derived versions of HM-4, HM-8, Vivek QPM-9 and Vivek Hybrid-27 at two diverse locations of India, and concluded that β-carotene-rich hybrids were similar to their respective original hybrids for grain yield potential. Farmers' own evaluation of the agronomic performance of QPM cultivar *vis-a-vis* normal maize is the most important factor for adoption of QPM (Groote et al. 2010). Thus, it is important to develop high yielding biofortified maize cultivars with desirable traits required by the end users. It is noteworthy that the genetic base of nutritionally improved inbreds is not as wide as normal maize, since only a few breeding centres have active quality breeding programme in the country. Thus, strengthening the breeding programme through developing diverse heterotic pools for nutritional quality traits, and deriving promising inbreds with high *per se* productivity are the way forward.

Difficulty in phenotyping

Estimating micronutrients among large number of segregating progenies/inbreds often acts as a deterrent to successful implementation in the breeding programme. Establishment of a high throughput laboratory in each of the breeding centres is therefore essential for estimation of nutritional quality traits. Further the effects of micronutrients such as lysine, tryptophan, provitamin A, Fe and Zn are invisible, and farmers would face difficulty in convincing the trader regarding the extent of quality of his produce while selling in the market. Development of a portable device that rapidly determines the quality of the produce would be of great help to the farmers. Brix meter is one such example, where sugar concentration in sweet corn is analyzed rapidly. Creation of trained human resources for precise estimation of the nutritional quality is key to the success of biofortification programme. Formulation of separate 'biofortification' trial in the All India Coordinated Maize Improvement Project (AICMIP) could aid in precise evaluation of micronutrient-rich entries, as additional precautions during pollination, harvesting and storage are required for biochemical estimation.

Dilution of nutrition quality

Majority of the genes that enhance nutritional quality are recessive in nature, and strong xenia effects dilute the nutritional benefits of biofortified maize especially for QPM (Hossain et al. 2008a, b), provitamin A and low phytate. In a situation, where some farmers grow biofortified maize while others grow traditional maize in nearby fields, the level of contamination is high in the border area with the contamination decreasing towards the middle of the field. In an experiment, 'Obatanpa', a white grained QPM variety was grown in a large field completely surrounded by a yellow-grained normal maize cultivar. Xenia effects caused contamination of QPM harvest to an extent of 11% of the total harvest (Ahenkora et al. 1999; Twumasi-Afriyie et al. 1996). Though the quality attributes of the grains may not be completely lost, contamination by foreign pollen would likely to cause significant decrease in the nutritional value of the produce. The extent of contamination in QPM cultivar depends on the proximity of the normal maize, synchrony of flowering between QPM and normal maize cultivars, direction of wind during pollination; and competitiveness of the normal- and QPM-pollen for fertilization (Vivek et al. 2008). In India, a majority of the farm holdings are small which further contribute to higher extent of contamination once normal maize is grown in the vicinity of QPM. Adoption of an entire village for the cultivation of biofortified maize with specific quality trait is therefore, ideal to harness the complete nutritional benefit. For example, sweet corn grains are due to recessive genes, *viz*., *sugary1* and *shrunken2*; and contamination by normal pollen from maize results in reduction of kernel sweetness drastically, thereby causing decline in market price (Khanduri et al. 2010, 2011; Hossain et al. 2013). 'Manoli' village in Haryana is a successful example, where farmers in the entire area grow only sweet corn cultivars to harness the benefits of grain quality and desirable market price. Mechanical mixture of normal maize grains with biofortified grains during threshing and packaging may also lead to the reduction of nutritional benefits. Ahenkora et al. (1999) reported that QPM benefits are lost when 20% or more of normal grains are mixed with QPM grains. Thus, the requirement of separate storage arrangements for biofortified maize grains is an essentiality to avoid contamination from normal maize grains.

Resistance in accepting nutritious foods with altered appearance

Change of appearance in crop produce and food due to nutritional quality enhancement may also prove to be a deterrent to the easy acceptability of biofortified grains among consumers. This would further add to the unwillingness of farmers to grow biofortified crops in their fields. In Africa, traditional maize grains have white kernel, and yellow/orange maize is not liked for consumption, despite having more provitamin A as compared to that in white maize. In India, white maize is preferred for consumption purpose at many places. Strong extension activities may play a major role in the popularization of biofortified crops. For example, in many of the Sub-Saharan African countries, people traditionally consume white and yellow sweet potatoes that are deficient in β-carotene (2 μg/g). Once β-carotene-rich (30-100 μg/g) orange sweet potato varieties were introduced in their diet, the prevalence of VAD was reduced considerably. Extension and policy supports were specifically designed to popularize orange flesh sweet potato in Mozambique and Uganda (HarvestPlus, 2012). Four dimensional strategies, *viz*., (i) providing knowledge on nutritional benefits of vitamin A to women, (ii) developing an orange sweet potato vine distribution system including subsidized vines to households, (iii) providing extension to men and women in farm households on orange sweet potato production practices and

marketing opportunities, and (iv) developing markets for roots and processed products of orange sweet potato, were adopted. Further, extension workers and volunteers were specifically trained, and awareness among villagers was created through community drama, radio broadcasts, and other activities such as field days, training for grandmothers and community leaders, and market promotion events. These efforts led to an increase in cultivated area under orange sweet potato from 9% to 56% in Mozambique and from 1% to 44% in Uganda with significant reduction in the occurrence of VAD among both children and women (HarvestPlus, 2012).

Lack of awareness on health benefits

It is interesting to note that despite well documented health benefits, QPM cultivars accounts for only 1% or less of 90 million hectares grown in Mexico, Latin America, Sub-Saharan Africa and Asia (CIMMYT, 2012). India is also not an exception in this regard, despite the availability of dozens of diverse QPM hybrids. Groote et al. (2010) reported that the adoption of QPM cultivars by the farmers varied a lot among East African countries with 70% adoption in Uganda and 30% adoption in Tanzania while Kenya reported none. Besides the knowledge of nutritional benefit of QPM, the response of farmers' participation in extension activities and reliable supply of good quality seeds were the important factors for the successful adoption. Gregoy and Sewando (2013) undertook a study on various determinants of QPM adoption in Tanzania. Among various factors, *viz*., (i) education of household head, (ii) farmers' participation in demonstrated trial, (iii) attendance in field day, and (iv) number of livestocks owned by the farmer were important for the adoption of QPM. A study in Zimbabwe by Stevens et al. (2008) revealed that ~94% of the respondent agreed to consume yellow maize instead of traditional white maize, if educated on health benefits.

Less organized food processing- and value-addition-industries

Steur et al. (2015) reported that the consumers are ready to pay 20-70% premium price for the biofortified foods developed through transgenic approach. Distinctive tag highlighting the health benefits on products made from biofortified maize would help the consumers to choose more nutritious foods over conventionally available ones. In India, RAU has taken a lead in producing various food products from QPM such as 'Pusa-Shakti', 'Kheer-mix/Dilkhush' Kadhi-mix/Proteino-H' (Singh and Chandra, 2014). The growing home and school (under the Mid-day Meal Programme) related market demands for these QPM-based products are a success story. Educating rural masses by Gram Panchayats and sensitization of health centres on the health benefits of biofortified maize could play a crucial role in increasing the popularity of biofortified maize. These value-added QPM-based products provide excellent opportunities to develop small-scale industry and in turn empower village women. For promoting of QPM as the nutritious food, several grain-based food processing industries producing flakes, chips, biscuits, etc. have also been established in India. The expansion of value-addition industry would enhance adoption of biofortified maize.

Inadequate policy support

Policy supports from the government are essential for the successful adoption of biofortified maize cultivars. Strengthening the seed chain to produce and supply good quality seeds is one of the first important steps for the popularization of biofortified maize. Providing subsidized seeds and other inputs would further contribute to the rapid dissemination of nutritionally improved cultivars among the farmers. Assurance of remunerative price through minimum support price and/or premium price for biofortified maize grains in the market will encourage the farmers to grow more biofortified maize. Easy loan and subsidy to village-level entrepreneurs to initiate small-scale enterprises for the development of biofortified maize-based processed food products would help in their greater dissemination. In India, a pilot programme on nutri-farms for introducing micronutrient-rich maize (QPM), Fe-rich pearl millet and Zn-rich rice, has been implemented to improve the nutritional status of malnourished people of 100 districts of nine states (Economic Survey, 2014). Such efforts from policy makers would definitely help in popularizing biofortified maize in India.

Future prospects

Breeding efforts have led to the successful development of QPM cultivars and more recently the provitamin A-rich maize. These 'first generation' biofortified maize cultivars have been improved for single micronutrient. Efforts should now be directed to develop 'second generation' biofortified maize genotypes with a combination of various micronutrients. The multinutrient-rich maize cultivars would contribute to nutritional security in a more holistic manner. Enhancement of kernel -Fe and -Zn in QPM

genetic background is of greater value as compared to improvement in normal maize. Considerable proportion of poultry feed is composed of maize grains, and the predominant portion of the maize produce in India is utilized for poultry industry. QPM grains due to enhanced lysine and tryptophan, thus hold immense promise as poultry feed. However, the level of methionine in maize grains is quite low, which necessitates addition of synthetic methionine in the diet that eventually leads to an increase in cost of the feed. Thus, development of QPM enriched with methionine holds great promise. Phillips et al. (2008) reported the availability of methionine-rich versions of A632, B73, and Mo17 with methionine level elevated as much as 12.5, 25.0 and 50.0%, respectively. These inbreds will serve as potential donors in the breeding programme. Further, reducing phytate content in the QPM genotypes would help in increasing the bioavailability of Fe and Zn in both human beings as well as poultry. Monogastric animals such as poultry birds poorly digest phytate, which eventually goes out of the body through excreta resulting in deficiency of phosphorus. Thus, low phytate-QPM would be a popular choice by the poultry industry. Though β-carotene-rich version of Vivek QPM-9 has been developed, concerted efforts should be made to generate diverse QPM hybrids with enhanced level of β-carotene for its wide spread dissemination. Dietary constituents particularly Fe and Zn increase the bioavailability of provitamin A, and it will be an effective strategy to pyramid genes/QTLs for β-carotene, Fe and Zn in a single genotype to maximize the benefits from all three nutrients. Selection of genotypes with high oil content also needs to be carried out simultaneously as a complementary approach as fat/ lipid increases the bioavailability of β-carotene upon consumption. Further, micronutrients such as vitamin E, folate and ascorbic acids also deserve attention for their improvement in maize. Availability of desirable alleles and QTL for nutritional quality traits coupled with closely linked/gene-based markers would aid in stacking multiple nutritional traits through accelerated breeding strategy.

Effective collaborations among various national- and international-research institutions are important to strengthen breeding programmes for developing more nutritious maize. The ICAR has recently initiated a Consortia Research Platform (CRP) on Crop Biofortification through which a set of crops including maize is being targeted for enrichment of nutritional quality. The Department of Biotechnology (DBT), Government of India has also been funding various research projects on biofortification in maize, where an array of hybrids has been improved for protein quality and kernel provitamin A. However, further strengthening of research collaborations among various national partners of the National Agricultural Research System (NARS) and international research organizations like CIMMYT and HarvestPlus would help in sharing novel germplasm and expertise for the development of biofortified maize. The recent establishment of the Borlaug Institute for South Asia (BISA) in India is expected to enhance regional cooperation among various institutions for the development and dissemination of biofortified maize, which will help in alleviating micronutrient deficiency to a great extent.

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