INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 14, No. 1, p. 533-542, 2019

REVIEW PAPER OPEN ACCESS

Overview of quality protein maize and molecular breeding approaches for its development

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Key words: Bio-fortification, Lysine, Malnutrition, Opaque-2, Tryptophan.

http://dx.doi.org/10.12692/ijb/14.1.533-542 Article published on January 31, 2019

Abstract

Cereals are a major source of dietary proteins and fibers. Maize is emerging as a third leading cereal after wheat and rice. It fulfills about 20-30% calorie requirements of human diet as a staple food in almost 21 countries. However most of the currently grown maize germplasm is deficient in essential amino acids i.e. lysine and tryptophan. It poses serious health hazards to people taking maize as a staple food and giving birth to serious issues i.e. reduced growth rate and kwashiorkor. Hence bio-fortification of maize is mandatory for maintenance of nutritional balance. Current review summarized the methods for improving nutritional profile of maize. Different methods i.e. dietary diversification, supplementation, fortification, fermentation and bio-fortification can be opted to overcome nutritional deficiency of lysine and tryptophan in maize. The last method is inexpensive and can be easily opted by majority of the maize growing nations. Opaque-2 gene mediated quality protein maize, its possible way of utilization in different breeding programs and its role in overcoming nutritional deficiency of maize crop was mainly focused in this review.

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Introduction

Maize is third major cereal crop after wheat and rice that is used as a food for human as well as feed for livestock (Prasanna *et al.,* 2001). It fulfills 15% of protein requirements and share 19% of calorie requirements from plant source (Vasal, 2002). Maize is a source of dietary proteins for millions of people around the globe especially in developing countries (Mbuya *et al.,* 2011). It is also an important component of livestock feed as in case of developing nations 78% of maize is used for livestock feed (Sofi *et al.,* 2009). In malnourished countries i.e. Africa maize is serving as rice source of daily calorie also it fulfill supply 17-60% of daily protein requirement to individuals who are at risk of protein and essential amino acid deficiencies (Krivanek *et al.,* 2007). Protein contents in maize can further be alleviated to as high as 18% by increasing the fraction of prolamine (zein) fractions in maize endosperm (Dudley *et al.,* 1969).

However maize is deficient in essential amino acids i.e. lysine and tryptophan (Abate *et al.,* 2015). Lysine is very essential amino acid for development of tissue as it helps in adsorption of calcium from intestinal mucosa. Tryptophan is also an essential amino acid as it serve as a precursor of B vitamin, niacin (Mpofu *et al.,* 2012). Efforts for development of quality protein maize (QPM) started in 1960s (Prasanna *et al.,* 2001) with the discovery of mutant (opaque-2 genes) which increased 70-100 % lysine and tryptophan as compared to ordinary cultivars (Bjarnason and Vasal, 1992). Protein quality in QPM maize approaches to the quality of protein obtained from cow's milk (Prasanna *et al.,* 2001). All these studies indicated that use of QPM maize in maize based economies will help in alleviation of malnutrition (Machida *et al.,* 2012).

Opqaue-2 (02) is a natural recessive mutation in the transcriptional activator conditioning negative expression of zein protein (Tripathy *et al.,* 2017). However lower yields of QPM maize versus non-QPM maize varieties as well as susceptibility of QPM varieties to different stresses i.e. ear rot which results in less lysine and tryptophan per unit area has limited the adaptation of QPM maize (Pixley, 2003). Scientists from CIMMYT has been successful to transfer opaque-2 genes in backgrounds with hard endosperm which gave comparative yield with non-QPM cultivars as well (Nuss and Tanumihardjo, 2011). Further evaluation of QPM maize under harsh climatic conditions also did not significantly affect the level of lysine and tryptophan which indicated that QPM maize is independent of climatic conditions (Zaidi *et al.,* 2008). This study was initiated to review research outputs and come up with some sort of conclusions and recommendations to exploit potential of QPM maize as food, feed and industrial raw materials for developing countries farmers whose livelihood is dependent largely on maize and feed processors.

Food Security

Starvation is common problem of people living in 3rd world countries. One out of nine persons in developing countries and 805 million people universally are suffering from starvation. In developing countries 13.5% people are malnourished due to lack of diversity in their daily calorie intake. Majority of dietary intake comes from cereals (plant source) which lacks balance in terms of protein, fats, minerals and micro nutrients (Truswell and Mann, 2012). Protein malnutrition causes number of health problems including stunted growth, renal failure, lack of immunity and weakened intellectual development especially among the children of less than 5 years. Approximately 200 million children younger than five are suffering from protein malnutrition and related disease (Prasanna *et al.,* 2001).

Malnutrition

Malnutrition is defined as "a state of nutrition in which a deficiency or excess amount of energy, protein and micronutrients causes significant negative effects on tissue/body form (body shape, size and composition), body function and clinical outcome" (Stratton *et al.*, 2003). Protein energy malnutrition causes diseases like Kwashiorkor and Marasmus. To overcome macro and micro nutrients deficiencies including protein following strategies could be adopted: dietary diversification, supplementation, food fortification, enrichment, fermentation and bio-fortification (Vasconcelos *et al.,* 2017). These approaches are discussed here in detail:

Strategies to Overcome Malnutrition

Dietary diversification

Dietary diversification with protein containing foods such as milk, eggs and meat, which are good sources of essential amino acids (Vivek *et al.*, 2008) is a one of approach to address malnutrition. Animal based protein diets are expensive hence have limited excess in developing countries. Consequently there is need to encourage the use of essential food sources to add worth to such foods to meet nutritional necessities of target population. Despite that, dynamic ingestion behavior of communities look exhausted when there is lack of awareness due to dependence on limited food resources. Moreover socioeconomic conditions and illiteracy are major hurdles of poor population in diversifying their food (Keatinge *et al.,* 2010).

Supplementation

In a systematic randomized studies of protein supplementation, there is increased in growth weight and anthropometric measurements in the individuals of all groups. It further suggests that use of protein supplements decreases mortality rates among high risk individuals. Although these commercially manufactured nutritional supplements are alternative to high calories protein rich foods that are expensive (Potter *et al.,* 1998). Supplementation also required good health status that is often lacking in developing countries. As oral protein supplementation is not tolerated by dialysis patients additionally side effects were reported in patients (Jeloka *et al.,* 2013).

Food enrichment

Food fortification is addition of missing nutrients which are absent or present in minimum amounts in food. Whereas food enrichment is addition of a particular nutrient to increase its amount (Maberly *et al.,* 1994). Food fortification is more beneficial than supplementation in terms of cost benefit ratio or cost per life saved (Hunt, 2002). Although both these strategies provide quick solution to overcome malnutrition but due to limited distribution to large population, they have not been successful in third world countries. Political issues in some developing countries are major hurdles for distribution of fortified foods. Despite of fact, that dietary diversification, supplementation and food fortification/enrichment are most desirable approaches for alleviation of malnutrition but these are expensive and out of reach for poor farming communities who left with option to eat staple food (Zhu *et al.,* 2007).

Food fermentation

Fermentation is a process of increasing nutrient availability in food items by preventing losses after harvesting, detoxifying the raw material and increasing uptake of nutrients. During fermentation of foods presence of probiotics causes good effects on health (Holzapfel *et al.,* 2002). There is an increase in availability of essential amino acids His, Lys, Leu and Try when quality protein maize was passed through solid state fermentation process. Although nutritional profile of QPM maize was relatively higher than non QPM maize that can further be increased by fermentation. Solid-state fermentation technology has broad usage in increasing nutrition, texture and aroma of soya bean and now equally effective on QPM maize temph flour for increasing nutritional quality. It has been observed that gelatinization temperature was higher in QPM temph flour (fermented) than untreated QPM temph flour (Cuevas-Rodriguez *et al.,* 2006).

Bio-fortification

Bio-fortification is an approach in which nutritional quality of food crops is increased by agronomic practices, conventional plant breeding and modern biotechnology. It is used to overcome hidden hunger by providing increased quantity of micronutrients to large population (Harvest Plus 2008). Bio-fortification is subdivided to two broad categories i.e. agronomic bio-fortification and genetic bio-fortification (Yilmaz *et al.,* 1997). Agronomic bio-fortification deals with application of mineral nutrition or fertilizer (especially micronutrients along with NPK) to soil or to leaves to increase micronutrient contents in edible part of plant (De Valença *et al.,* 2017). It was observed that protein contents in both QPM and non QPM maize cultivar showed dependency on nitrogen application either as organic nitrogen or nitrogen fertilizer (Kniep and Mason, 1991).

Protein contents were higher (9%) in maize fertilized with nitrogen @ 200Kg/ha in comparison to 6.5% protein without nitrogen application.

Genetic bio-fortification

Genetic bio-fortification deals with breeding crops to increase their nutritional value. This can be done either through conventional selective breeding or through genetic engineering. Genes that controls high level of essential amino acids should be used in breeding program for genetic bio-fortification of maize crop. The mutant floury 2 gene produces changes in amino acid profile of maize protein by increasing concentration of essential amino acids like lysine and methionine (Nelson *et al.,* 1965).

Countries where staple foods are cereals like wheat, rice and maize are deficient in macro and micro nutrients as they cannot buy variety of foods to fulfill their daily nutrients requirement. Cereals are leading crops in agriculture but are deficient in balanced proteins. Despite of having poor protein cereals fulfills 50% of protein requirements in world, whereas in developing countries 74% of protein requirements are accomplished by cereals. Cereals provide three times more protein nutrition to human and animals than legumes which accounts more than 200 million tons of protein (Shewry and Halford, 2002). In countries where maize is used as a staple food people are at more risk of protein deficiency specifically poor people that are unable to buy variety of protein foods like eggs, milk and meat (Byerlee and Eicher, 1997).

Maize fulfills about 20-30% calorie requirements of human diet as a staple food in almost 21 countries around the globe (Shewry and Halford, 2002). Protein profile of maize is not a balanced one as it is deficient in two essential amino acids lysine and tryptophan (Vivek, 2008). A breakthrough occurred during 1960s when some maize mutants i.e. opaque 2 and floury 2 with quality protein, increased level of essential amino (acids lysine and tryptophan) and reduction in the fraction of prolamin proteins were introduced (Vivek, 2008). From 1960s to onwards extensive work was done at CIMMYT for development of maize lines having opaque 2 gene with good agronomic characteristics and twice level of essential amino acids lysine and tryptophan (Bjarnason and Vasal, 1992; Vivek, 2008).

Genetics of QPM

Opaque-2 as a genetic base of quality protein maize

Gene coding for increased lysine and tryptophan contents in maize protein was first identified in the Connecticut, United States. The gene was responsible for soft and opaque color endosperm phenotype (Singleton, 1939). In 1960 it was discovered that maize homozygous for recessive opaque 2 mutation has almost double amount of lysine and tryptophan (Mertz *et al.*, 1964). Opaque 2 is a regulatory mutation which regulates the expression of α zein protein by binding to its specific promoter sequence. Opaque 2 protein is a 47- KD protein that regulate 22-KD α zein protein (Hartings *et al.* 1989; Schmidt *et al.* 1990). The structure of opaque 2 protein is similar to transcriptional activator (Hartings *et al.*, 1989), zein regulatory putative protein consists of 454 amino acids having a domain similar to leucine zipper motif present in DNA binding protein of animals like fos, jun, mys and in transcriptional activator of GCN4 and C/EBP. Other than opaque 2, many loci in maize are also diagnosed that influence regulation of zein protein developing endosperm (Motto *et al.*, 1989).

Presence of opaque 2 mutant allele in recessive homozygous condition (o2o2) reduced zein protein up to 60%, especially 22KD component of zein protein was almost missing (Jones *et al.*, 1977) and led to high accumulation of lysine and tryptophan (Crow and kermicile, 2002). Reduction of zein protein in opaque 2 mutant was due to reduction of zein mRNA indicating that zein protein is transcriptionally regulated by opaque 2. Opaque 2 work as trans-acting transcriptional activator in regulating zein gene transcription (Hartings *et al.*, 1989) as opaque 2 lies at short arm of chromosome 7 and genes for the 22kD zein protein are present on chromosome 4.

Maize opaque 2 protein has nuclear localization signals that redirect opaque 2 (47-KD) protein to nucleus. This protein localizes both in maize and tobacco that was transformed with opaque 2 gene.

Experiment was conducted by partitioning opaque 2 in three parts (A, B and C) and fusion a GUS (reporter protein) with each part for confirmation of localization. It was observed that A and B parts were localized in nucleus. Two localization signals were identified in start 101 and 135 residues and in middle 223 and 254 residues of protein. This nuclear localization of opaque 2 protein was also confirmed in transiently transformed onion cells (Varagona *et al.*, 1992).

Modifying gene action of Opaque 2

Hard endosperm texture in normal maize genotypes is due to association of zein protein with starch granules. Presence of opaque-2 genes renders pleotropic effects making kernel soft and reduces association of less concentration of zein protein (Schmidt *et al.*, 1990). These pleotropic effects result in post-harvest losses as shown in Fig 1. Therefore development of maize high in quality protein required the exploitation of genetic system for overcoming pleotropic effects of opaque 2 mutation.

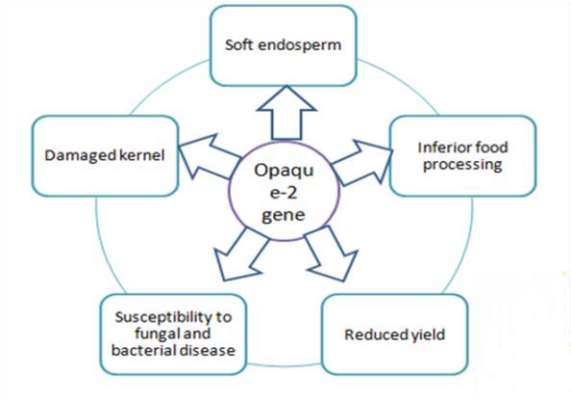

Fig. 1. Pleotropic effects of opaque 2 mutation.

Determination of Lysine and Tryptophan

Genes responsible for increasing lysine and tryptophan contents should be selected in breeding population (Nurit *et al.*, 2009). There are numbers of alleles that are responsible for higher level of lysine and tryptophan. Range of lysine and tryptophan in QPM cultivar ranges between 2.6-5% developed from normal cultivar having range of 1.5-2.8% (Moro *et al.*, 1996). There are no molecular markers available for selection of these alleles in breeding populations; phenotypic selection is therefore necessary (Nurit *et al.*, 2009). An association of lysine and tryptophan was worked out and it was observed that ratio of lysine and tryptophan is 3:1 ratio (Bjarnason and Vasal, 1992). As protocol for lysine determination is very costly and time consuming so it is rarely measured directly rather it is estimated from given ration. Hence measurement of only tryptophan is done for estimation of both amino acids (Nurit *et al.*, 2009). There are following procedures for the determination of tryptophan contents in maize:

Colorimetric method

For determination of tryptophan concentration in maize flour Nurit *et al.* (2009) developed a reliable and inexpensive colorimetric method on the principle of a colorimetric reaction of tryptophan with oxalic acid in presence of sulfuric acid and iron chloride that produces a colored compound absorbed at 560 nm.

Near infra-red reflectance spectroscopy (NIRS)

Colorimetric method is time consuming as more than 20 hours are needed for reaction and become more laborious when large numbers of samples are to be examined (Rosales *et al.*, 2011). NIRS is a technology that combines spectroscopy and mathematics for determination of CH-, OH-, NH- and SH groups containing compounds required less time for sample preparation and many traits can be examined simultaneously (Melchinger *et al.*, 1986). Rosales *et al.* (2011) determined tryptophan and lysine contents in QPM breeding population ranges between 0.005 to 0.85 for tryptophan and 0.02 to 0.5 for lysine showing a potential of use of this technique in the QPM breeding program.

Conversion of soft endosperm to hard endosperm

Above two mentioned component are not sufficient for development of QPM cultivar. A third component of maize genetic system should be needed for conversion of o2o2 induced soft endosperm to hard endosperm. Third component of maize genetic system consists of minor loci which modify the opaque endosperm to vitreous (Vivek *et al.*, 2008). Opaque color of maize can be visualized on the light table for easy selection of soft and hard endosperm texture. For development of QPM lines there must be an essential procedure for determination of all three

systems during a breeding program. Marker assisted selection protocol are only available for o202 gene. Other two method still requires the morphological screening of germplasm (Nurit *et al*., 2009).

Breeding approaches for QPM development

Exploitation of genetic variability for parent selection

The major objective of any breeding program is to increase yield through various conventional and non-conventional breeding techniques. Depending on objective, first priority of breeding program is to exploit genetic material of concerned crop for trait of interest along with various agro-morphological traits that play important role in plant breeding as these traits can be positively or negatively correlated with trait of interest and yield. Correlations between traits of plants determine the choice of most efficient selection procedure that varied according to trait, population (inbred lines, hybrids of open pollinated varieties) and location (Abadassi, 2016). Direct selection of parents for variety development could be of no use as inter-relationship between agro-morphological traits may lead to selection for those traits that may be negatively correlated with yield or environmental conditions (Azad *et al*., 2012). As diversity is prerequisite for plant improvement therefore, breeders have to exploit genetic diversity among genotypes before starting any breeding program. No doubt variability can be created through hybridization and mutation, rather evaluation of genetic material is necessary even for development of hybrids that need cross between divergent parents. For analyzing diversity principal component analysis is mostly used that analyzed contribution of total diversity, helpful for selecting desired parents (Ranawat *et al*., 2013). Prakash *et al.* (2017) characterized 40 QPM and non QPM lines on basis of molecular, biochemical and morphological analysis to determine best lines for conversion of normal lines to QPM by using principal component and cluster analysis.

Marker assisted backcross breeding

For foreground selection of opaque 2 gene in germplasm three SSRs (phi 57, phi 112 and umc 1066) are used. These sequences are located as internal repetitive sequences within the opaque 2 gene. Phi 112 a dominant marker, identifies O2O2 and O202, whereas phi 57 and umc 1066 are co-dominant markers which can identify all three genotypes either dominant homozygous, dominant heterozygous or recessive (Danson *et al*., 2006). Three specific SSR primers phi (57, phi 112 and umc 1066) detect lines having opaque 2 gene, six SSR primers for gene that modify the amino acids contents (bnlg 2248, phi 072, bnlg 1633, bnlg 1382, phi 75 and mmc 0241) and one primer umc 1216 for the genes that are responsible for the endosperm hardness.

Three specific SSR primers phi 57, phi 112 and umc 1066 were identified in maize genome that were used by CYMMT for developing QPM hybrid (Dreher *et al*., 2003). Parental polymorphism was detected between normal and QPM lines by Babu *et al.* (2005); Ignjatovic-Micic *et al.* (2009) and Magulama and Emma (2009). They found co dominant nature of phi 57 showing parental polymorphism with amplification of 160 bp fragment in normal and 170 bp in QPM lines. Umc 1066 showed parental polymorphism by amplifying 150 bp in QPM and 170 bp in normal lines. Dominant nature of phi 112 was observed that amplifies 150 bp fragments in non QPM inbred lines only. SSR markers (phi 57, umc 1066 and phi 112) were used by Magulama and Sales (2009) in screening of maize lines having opaque 2 gene to accelerate breeding procedure by selecting best lines for marker assisted back cross breeding. At BC3F2 7 lines were converted to QPM having tryptophan and lysine increased to 1% and 4% by using the phi 57 marker.

Hybrid and Synthetic development in QPM

QPM Hybrid yield potential is 13% more than open pollinated cultivars (OPCs) of QPM while protein contents of QPM are less than OPCs. OPCs had 2% higher protein contents than hybrid but quality of protein in terms of tryptophan and endosperm modification is same for all QPM OPCs and hybrid (Pixley and Bjarnaso, 2002). Selection of pollen parent also imparts significant differences on protein quality and quantity. In a study pollen from different sources were used and it was determined that when normal parent is used as a pollen source there was 37% reduction in the tryptophan concentration of grain when

non QPM parent was used. There are no differences in total protein contents either pollinated by normal, QPM or selfed (Pixley and Bjarnason, 1994). Duarte *et al.* (2004) found that there is no difference in performance of QPM and non QPM hybrid developing from same inbred lines. Ranawat (2013) determined yield contributing traits in QPM and non QPM lines and also examined mutual relationship among morphological traits. So difference of opinion exist whether QPM and non QPM parents and their putative hybrids differ in yield potential or not.

Assessment of QPM performance

Before release of any crop variety developed, its yield potential and susceptibility must be evaluated across wide locations. Akandi and Lamidi (2006) assessed agronomic performance and susceptibility to diseases of three hybrids and five open pollinated varities (OPVs) during two years. There were considerable differences in yield potential among hybrids and OPVs, hybrid yield was more than OPVs, showing all QPM varieties were susceptible to southern leaf blight, curvelaria leaf spot and maize rust fungal diseases. QPM varieties were also found susceptible to *Fusarium* spp (Ignjatovic-micic *et al.*, 2009).

Major achievements in QPM

Nutritional benefits from QPM consumption on both young and adults after meta-analysis was determined on nine community based studies with 9% increase in rate of growth in height and 12% increase in rate of growth in weight in under nourished young children (Gunaratna *et al.*, 2010). Children suffered with kwashiorkor disease in Columbia were also recovered to normal health by feeding them on QPM maize (Vivek *et al.*, 2008). A study on infants and small children recovered from malnutrition shown that when QPM used as only source of protein causes 45% more retention of N than normal maize (Graham *et al.*, 1989). In another study Graham *et al.* (1989) ascertained that growth rate of young children was equivalent for both groups either given QPM or modified cow's milk formula as a diet. These young malnourished children received 100% protein and 90% their energy diet from QPM. QPM also improves source of protein for adults human when given OPM as only protein source in diet but lysine deficiency cannot be accomplished by mere intake of QPM in adults (Kies *et al.*, 1972). Hence QPM has a wide variety of health benefits to mankind as well.

Apart from effects of QPM on human health it has wide variety of positive effects on animal and poultry health as well. Nutritional aspects of QPM on animal feed were also examined i.e. broilers feed on QPM instead of normal maize causes 5% reduction in cost of poultry feed as there is no need of expensive protein sources (De Groote *et al.*, 2010). Beneficial impacts of QPM were observed on animal health too (Krivanek *et al.*, 2007).

Conclusion

QPM maize offers better way to overcome malnutrition in maize eating areas around the globe. For the foreground selection of opaque 2 gene in germplasm three SSRs (phi 57, phi 112 and umc 1066) are used. These markers are located as internal repetitive sequences within the opaque 2 gene. Phi 112 a dominant marker, identifies O2O2 and O202, whereas phi 57 and umc 1066 are co-dominant markers which can identify all three genotypes either dominant homozygous, dominant heterozygous or recessive. Six SSR markers (bnlg 2248, phi 072, bnlg 1633, bnlg 1382, phi 75 and mmc 0241) are used as identifier of gene that modify amino acids contents and umc 1216 is used to identify genes that are responsible for endosperm hardness. This marker system can effectively be used for the marker assisted selection of QPM maize and development of quality protein maize hybrids to overcome food security and malnutrition issues simultaneously.

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