मक्का के भ्रूणपोष में लायसीन एवं ट्रिप्टोफेन की बढ़ोतरी हेतु *ओपेक2* एवं *ओपेक16* जीन्स की चिह्नक की सहायता से पिरामिडिंग

Marker-aided pyramiding of *opaque2* and *opaque16* genes for enhancing lysine and tryptophan in maize endosperm

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Marker-aided pyramiding of *opaque2* and *opaque16* genes for enhancing lysine and tryptophan in maize endosperm

by

Konsam Sarika

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submitted to the Faculty of Post Graduate School,
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in
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CERTIFICATE

This is to certify that the thesis entitled **"Marker-aided pyramiding of *opaque2* and *opaque16* genes for enhancing lysine and tryptophan in maize endosperm"** submitted to the Faculty of Post Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of requirement of degree of **Doctor of Philosophy in GENETICS,** embodies the result of a bonafide research work carried out by **Mrs. Konsam Sarika** under my guidance and supervision. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that any help or source of information, as has been availed of in this work, has been duly acknowledged.

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To my beloved parents,

Shri. Konsam Shyam Singh

Smt. Konsam Swarnalata Devi

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ACRONYMS

FAO	:	Food and Agriculture Organization of the United Nations
WHO	:	World Health Organization
bZIP	:	Basic leucine zipper domain
eEF1A	:	Eukaryotic elongation factor 1A
kDa	:	kilo Dalton
CIMMYT	:	International Maize and Wheat Improvement Centre
PCR	:	Polymerase chain reaction
DNA	:	Deoxyribo Nucleic Acid
mm	:	millimeter
s	:	second
mg	:	milligram
h	:	hour
mM	:	millimolar
µg	:	microgram
µL	:	microliter
mL	:	milliliter
rpm	:	round per minute
SDS	:	Sodium Dodecyl Sulfate
PAGE	:	Polyacrylamide Gel Electrophoresis
CTAB	:	Cetyl Trimethyl Ammonium Bromide
DMR	:	Directorate of Maize Research
HCl	:	Hydrochloric acid
NaOH	:	Sodium hydroxide
GGT	:	Graphical Geno Type
UPLC	:	Ultra Performance Liquid Chromatography
IARI	:	Indian Agricultural Research Institute
ICAR	:	Indian Council of Agricultural Research
MABB	:	Marker-Assisted Backcross Breeding
MAS	:	Marker-Assisted Selection
QPM	:	Quality Protein Maize
SSR	:	Simple Sequence Repeats

Introduction

1 Introduction

"My great interest in QPM has always been as a nutritionally enriched food source for impoverished people in regions of the world where maize is a primary source of energy." - Norman Borlaug (2008)

With the current projections of continued global population growth until at least 2050, it is estimated that the number of people by 2040 would reach nine billion (www.census.gov). However, malnutrition caused by inadequate consumption of balanced diet is already pervasive in the under-developed and developing world afflicting an estimated three billion people (Stein, 2010). More than one-third of the world's children who are underweight due to protein-energy malnutrition live in India and it affects nearly 850 million people worldwide (Temba et al. 2016). Early days of the 21st century witnessed the mortality (up to 54%) of children in developing countries due to protein energy malnutrition (Bain et al. 2013). To alleviate such malnutrition especially in the developing countries, development of biofortified crop varieties through conventional breeding approaches provides a sustainable and cost-effective solution (Gupta et al. 2015).

Two-thirds of the world population depends entirely on three staple crops: rice, maize, and wheat. The rest depend on millets, tubers, and animal products. Maize is a preferred staple cereal food crop for people in Africa, South America and Asiaproviding 30% of the food-calorie intake of more than 4.5 billion people in as many as 94 developing countries (Gupta et al. 2015). It is a comfort staple food for the poor section of society whose income is less than 2 USD per day (Shiferaw et al. 2011). Maize is reputed to be a "poor man's nutricereal" due to its high content of carbohydrates, fats, protein, and some of the important vitamins and minerals (Prasanna et al. 2001). In the world, the production of maize is estimated to be the highest among cereals with approximately 1016 million tonnes (mt) from 184 million hectares (FAOSTAT, 2014). Apart from being a vital source of calories and proteins, a major portion of maize produced worldwide is used for animal consumption as feed and also as an industrial crop par excellence (Yadav et al. 2015). In India, maize occupies an important place as a source of animal feed (59%), human food (10%), industrial raw material (17%), export (10%) and other purposes (4%) (Kumar et al. 2013). Currently, the demand for maize is high in the developed world but by the year 2020 it is anticipated to be higher

in the developing countries (Rosegrant et al. 2009). It is estimated that by 2025, India would require 50 mt of maize grain, of which 32 mt would be required in the feed sector, 15 mt in the industrial sector, 2 mt as food, and 1 mt for seed and miscellaneous purposes (Yadav et al. 2015).

However, cereal proteins including maize have poor nutritional value due to reduced availability of essential amino acids such as lysine and tryptophan in the endosperm, which constitutes the bulk of the grains. Human, pig, and monogastric animals such as poultry birds cannot synthesize lysine and tryptophan, and are required to be supplemented through their diet (Prasanna et al. 2001). Therefore, healthy diets must include alternate sources of these amino acids (Bressani 1992; Knabe et al. 1992). Cereal protein contains 2% lysine on an average, which is less than half of the concentration recommended for human nutrition by FAO. The best sources of both lysine and tryptophan are meat, poultry, fish and dairy products, which are expensive and is neither affordable nor can it be made available to the poorer section of the population. Therefore, this warrants genetic amelioration of the major staple cereals to enhance the content of these essential amino acids in the grains. Agricultural research in general and crop breeding in particular will play a vital role in meeting this challenge. Low concentration of lysine and tryptophan in maize is due to the prolamin fraction that constitutes 70% of storage protein and is also known as zein (Gibbon and Larkins 2005; Bhan et al. 2003). The remaining fraction constitutes of other proteins such as albumins (3%), globulins (3%), and glutelins (34%), collectively called as non-zeins, possessing balanced amino acids (Vasal 2000).

Attempts to improve maize grain quality through focused efforts and painstaking research began at the end of the nineteenth century. In the 1960s, researchers found that the mutation, *opaque2* (*o2*), makes grain proteins nearly twice as nutritious as in normal maize endosperm (Mertz et al. 1964). However, in addition to a two-fold increase in lysine and tryptophan, *opaque2* mutation also has pleiotropic effects which make the endosperm soft and opaque that in turn causes enhanced susceptibility to insect-pest infestation, inferior food processing, and low yield, thus making the maize grains undesirable to the stakeholders (Bjarnason et al. 1992). Several other mutations such as *o1*, *o5*, *o9-11*, *o13*, *o17*, *fl1*, *fl2*, *fl3*, *Mucronate* and *Defective endosperm* B30 were discovered (Salamini et al. 1983). Several of these mutants have been experimentally tried singly or in combinations but resulted in severe yield losses

due to negative effects of the individual mutation (Huangs et al. 2004; Gibbon and Larkin 2005). The soft, chalky and opaque phenotype of *o2* kernel was modified by the accumulation of 'endosperm modifiers' leading to the birth of 'Quality Protein Maize' (QPM) (Vasal et al. 1980; Pandey et al. 2015). The deployment of *o2* along with the endosperm modifiers has led to the successful commercialization of diverse QPM hybrids worldwide with the enhancement of both lysine (from 0.15 to 0.37% in flour) and tryptophan (from 0.04 to 0.08% in flour) (Gupta et al. 2013).

The 3.7% increase in lysine in QPM still falls short of the 5% required for optimal human nutrition (Young et al. 1998). The search for a novel mutation that can be successfully utilized to develop high lysine maize continued in the new millennium till Yang et al. (2005) reported another recessive mutant from Robertson's Mutator stocks and named it as *opaque16* (*o16*). This recessive mutant allele is located on chromosome 8 and *o16,* along with *o2,* has been reported to increase lysine by 30% over *o2o2* or *o16o16* alone (Zhang et al. 2010; 2013). So far, the only research group that has reported the utilization of *o16* in their breeding programme is the one led by Dr. Wenpeng Yang at Guizhou Institute of Upland Food Crops, Guizhou Academy of Agricultural Sciences, China. There are no reports on the utilisation of this novel mutant elsewhere, including the Indian maize breeding programme. Moreover, the efforts on the improvement of nutritional quality with the help of *o16* are confined only to lysine accumulation and thus far no information is available on the effect of *o16* on the accumulation of tryptophan. Further, the effect of *o16* on grain phenotype and composition of zein has to be investigated to understand it better and gain valuable insights. Keeping this in view, the current investigation was undertaken with the following objectives:

1. To study the effects of *opaque16* on the accumulation of lysine and tryptophan, and attributes of endosperm modification

2. To introgress *opaque16* gene into *opaque2* genetic background using marker-assisted selection

Review of Literature

2.1 Protein-energy malnutrition (PEM)

A series of agricultural research and technological developments led to the Green Revolution in the 1960s with a marked increase in agriculture production around the world. Despite a quantum jump in the food grain productions, deficiencies in the dietary energy and protein popularly known as protein energy malnutrition (PEM) affects more than a billion people around the world. Even though significant advances have been made in genetic enhancement of crop plants for nutritional value, malnutrition still remains a widespread problem, and is particularly severe in developing countries with low per capita income. WHO defines malnutrition as the cellular imbalance between the supplies of nutrients and energy and the body's demand of them to ensure maintenance and growth (Blossner and de Onis 2005). For healthy growth and development, human beings require a supplement of balanced mineral elements, particularly essential amino acids and vitamins that can be met with diverse diets (White and Broadley 2009). However, human diets often lack one or more of these essential nutrients due to higher intake of staple foods alone. Apart from delivering enough calories, poor quality diets and low consumption of animal and fish products, fruits, and vegetables among the poor section of the society is also a major concern in the developing world as adequate essential nutrients are not met. During the early phases of the 21st century, the estimated mortality of children in developing countries due to PEM accounted for 54% (Bain et al. 2013). India is home to a large number of undernourished people (17.5% of its population) in the world, where 42% of children (<3 years) are underweight and 58% of them are stunted by two years of age (FAO/WFP/IFAD, 2012).

2.2 Maize and its importance

Maize (*Zea mays* L.) is an important staple cereal food crop for billions of people in Africa, South America, and Asia with an estimated world production of approximately 1016 million metric tonnes from 184 million hectares. In India, it is cultivated in 9.5 million hectares producing nearly 24.3 million metric tonnes (www.indiastat.com). Although a major portion of maize produced worldwide is used for animal consumption, more than 900 million poor consumers survive on it. It contributes nearly 35% of total cereal productions, and together with rice and wheat,

maize provides nearly 30% of the food calories to more than 4.5 billion people in as many as 94 developing countries (Shiferaw et al. 2011). It provides over 20% of total calories in human diets in 21 countries and over 30% in 12 countries that are home to a total of more than 310 million people (Nuss and Tanumihardjo 2011). Maize is processed and consumed in various ways greatly differing from country to country, with maize flour and meal being two of the most popular products.

Cereal proteins including maize, however, have poor nutritional value for humans due to reduced essential amino acids such as lysine and tryptophan. Cereal proteins contain on an average about 2% lysine, which is less than half of the concentration recommended for human nutrition by the Food and Agriculture Organization of the United Nations (FAO/WHO/UN 1985). Therefore, healthy diets for those who derive their major source of energy from maize must include alternate sources of these essential amino acids.

2.3 Importance of essential amino acids

Human beings require 0.66 g protein/kg body weight/day to meet the requirement for proper growth and development (WHO/FAO/UN 2007). Twenty per cent of our human body is made up of proteins that play crucial roles in almost all biological processes. Twenty different amino acids are required by our body and are usually incorporated into proteins (Lea and Azevedo 2003) and, therefore, amino acids serve as the building blocks of proteins. In the form of proteins, amino acids comprise the second-largest component; water being the largest in human muscles, cells, and other tissues (Latham and Michael 1997; Medici et al. 2004). Amino acids themselves are required to perform critical roles in processes such as neurotransmitter transport and biosynthesis. They are further classified into essential and non-essential amino acids. Humans and monogastric animals are not able to synthesize nine of the amino acids that are found in proteins. These nine amino acids (lysine, threonine, methionine, phenylalanine, tryptophan, isoleucine, leucine, valine, and histidine) are essential amino acids and must be acquired through the diet (Galili et al. 2002). Our body uses amino acids in a specific ratio to each other and lower levels of lysine or tryptophan affect the body's ability to use other amino acids. This is why these amino acids are called limiting amino acids and their deficiency lead to reduced appetite, delayed growth, impaired skeletal development and aberrant behavior (Tome and Bos 2007; Moehn et al. 2004).

2.3.1 Lysine

Lysine is one of the most important essential amino acids that our body can only get from food. It is the first limiting amino acid needed to digest food proteins; and the lack of it hinders protein metabolism. 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 (WHO/FAO/UN 2007). Lysine builds proteins of muscle and collagen—the connective tissue component—and promotes the absorption of calcium and its incorporation into bone tissue. Being a limiting amino acid in monogastric animals, it is an important additive to animal feed when optimizing the growth of certain animals such as pigs and chickens for the production of meat. Lysine supplementation allows the use of lower-cost plant protein source for feed derived from maize, for instance, rather than feeding soy while maintaining high growth rates. Lysine is found in abundance in red meat, chicken, turkey, and dairy products but not in important cereals such as wheat and corn (White and Broadley 2009).

2.3.2 Tryptophan

Tryptophan is a unique amino acid that is an essential component of the human diet. It 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/UN 2007). Although it has the lowest concentration in the human body relative to the other 19 primary amino acids, tryptophan is a key component in the synthesis of numerous metabolites such as kynurenine and serotonin (Harper and Yoshimura 1993). Ninety per cent of tryptophan is used in kynurenine synthesis and a further 3% in the synthesis of serotonin, a neurotransmitter. It is an important determinant of mood, cognition, and behaviour, and also in improving depression symptoms by increasing the level of serotonin in the brain (Riedel et al. 2003)

2.4 Maize storage protein

2.4.1 Zein proteins

The maize grain, as in other cereal grains, constitutes of pericarp (6%), endosperm (82%), and germ (12%). The main structural component of the endosperm is starch; it constitutes on an average 71% of the grain. Maize grains contain 8-10% protein, of which 70% of protein is composed of prolamin that are soluble in 70 % alcohol (Prasanna et al. 2001). Prolamins are a group of plant storage proteins having

high proline and glutamine content, and are known under different names in diverse crops viz., gliadin (wheat), kafirin (sorghum), zein (maize), hordein (barley), secalin (rye), and avenin (oats). The remaining fraction of protein, collectively called non-zeins, comprised of globulins (3%), glutelins (34%), and albumins (3%) that are balanced in essential amino acids (Vasal 2000). In normal maize endosperm, zeins contain higher proportion of leucine (18.7%), phenylalanine (5.2%), isoleucine (3.8%), valine (3.6%), and tyrosine (3.5%). However, deficiencies of key limiting essential amino acids such as lysine and tryptophan in prolamins result in relatively poor grain protein quality. An inverse relationship between prolamin accumulation and protein quality in endosperm is generally observed. As a point of comparison, normal maize protein has a biological nutritional value of 40% of that of milk (Bressani 1991) and therefore, needs to be eaten with complementary protein sources such as legumes or animal products.

Zeins consist of one major class: α-zein and three minor classes: γ, β and δ-zein, based on difference in aqueous solubility and ability to form disulfide bands (Coleman and Larkins 1999). Table 2.1 depicts the proportion of different classes of zein out of the total zein present in the endosperm. The most abundant (22- and 19-kDa α-zeins) are encoded by four different gene sub-families consisting of 40 genes across six chromosomal locations (Feng et al. 2009). The β-zein (15-kDa), γ-zein (16- and 27-kDa), and δ-zein (10-kDa), on the other hand, are encoded by single-copy genes rather than highly duplicated gene families as α-zeins (Xu and Messing 2009). However, the expression of the genes varies across different maize backgrounds. The synthesis of zeins starts about 12 days after pollination and is most active between 16 and 35 days, and can occur up to 50 days after pollination (Wall and Bietz 1987). Due to their high levels of expression and complexity, zein synthesis serves as a model system to analyze coordinated genetic regulation of several genes expressed at very high levels at a specific developmental stage (Soave and Salamini 1984).

The synthesis of zein is regulated by a basic leucine zipper (bZIP) transcription factor (TF), *Opaque2* (*O2*), which is specific to the maize grain (Schmidt et al. 1990). Cloned by transposon tagging (Schmidt et al. 1987), *O2* recognizes many motifs in the promoters of 22-kDa α-zeins and also regulates other α-zeins (19-kDa α-zeins), as well as 10-kDa γ-zein, 14-kDa β-zein, 27-kDa and 50-kDa γ-zeins. Besides zeins, *O2* also has non-zein transcriptional targets and also influences DNA methylation and histone

modification states of zein genes showing its role in the *O2*-mediated activation of zeins (Locatelli et al. 2009). Studies also revealed other regulatory factors influencing the expression of zein genes such as Dof-type *Prolamine-Box Binding Factor* (PBF) of 27-kDa γ-zein expression (Zhang et al. 2015); MADS-box protein (*ZmMADS47*) of α-zeins and the 50-kDa γ-zein gene (Qiao et al. 2016).

2.4.3 Protein bodies (*PB*)

All storage proteins are synthesized initially on the polyribosomes of the rough endoplasmic reticulum (RER) and are subsequently translocated into the lumen of the RER where they are directly assembled into PB accretions that remain within the ER, or may be transported through the endomembrane system to protein storage vacuoles (PSVs) (Vitale and Denecke 1999; Lending and Larkins 1992). In the case of maize, PB accretions are retained within the ER lumen (Choi et al. 2000). The abundant accumulation of zeins in endoplasmic reticulum localized PB is achieved through efficient packaging and is a key determinant of hard grain texture phenotype in the mature seed; an essential characteristic of cereal grains like maize (Holding 2014). Maize grain has a high proportion of glassy or vitreous endosperm at the periphery of the mature grain and a central opaque region. The vitreous outer region of the endosperm contains much more zein in a certain ratio of starch grains, PB, and viscous cytoplasm than the soft, opaque interior, where zein protein bodies are smaller and less abundant (Tsai et al. 1978). A typical protein body at 18 to 20 DAP is spherical, discrete, and has a highly ordered architecture of α- and δ-zeins which are deposited in the centre of the PB, while γ- and β-zeins are located in the peripheral layer (Lending and Larkins 1989). Disturbance in the architecture of accumulation of zeins results in irregular PB shapes, weak packaging, and opaque seed phenotypes (Kim et al. 2006), which are observed in the opaque mutants where the central opaque region extends to the periphery of the endosperm. However, several recent studies have shown that other factors are also involved which results in opaque phenotypes without affecting the zein synthesis and protein body structure (Holding 2014).

Table 2.1 Per cent proportion of each class of zein out of the total zein content

Zein fraction	**Protein**	**% of total zein**
α-zein	22, 19 kDa	60 -70
γ-zein	50, 27, 16 kDa	20 - 25
β-zein	15 kDa	5 - 10
δ-zein	18, 10 kDa	< 5

2.5 High lysine mutants

2.5.1 Discovery of opaque2 (*o2*)

From the human nutrition point of view, lysine, followed by tryptophan, is the most important limiting amino acid in the maize endosperm protein (Keis et al. 1965; Bressani 1975). The need for adopting a genetic enhancement strategy for the poor nutritious cereal grains such as maize has been recognized for a long time. Until 1960s, efforts were limited only to screening elite maize germplasm and the improvement strategy such as recurrent selection could not be easily implemented in the absence of specific genes enhancing lysine and tryptophan (Prasanna 2001). The discovery of the *o2* mutant by Jones and Singleton in the 1920s provided a significant breakthrough as it caused two-fold enhanced accumulation of lysine and tryptophan in the endosperm (Mertz et al. 1964). The recessive *o2* is the most important mutant and one of the first to be characterized. It is been mapped on the chromosome 7L. *O2* gene apart from encoding a bZIP transcriptional activators which regulates α-zeins synthesis, also down regulates the synthesis of lysine ketoglutarate reductase (LKR) (Schmidt et al. 1990). The *o2* mutant induces a general reduction in the accumulation of 22- and to a lesser extent 19-kDa α-zeins, with an overall reduction of 50-70% in zeins. Reduction in the α-zeins results in PBs with abnormal morphology, size and number; it leads to opaque grains with soft and starchy texture. The mutant *o2* is generally accompanied by an increase in non-zeins such as cytoskeleton-associated carbohydrate metabolizing enzymes (Azama et al. 2003) and eEF1A (Lopez-Valenzuela et al. 2004), which are relatively rich in lysine and tryptophan. Furthermore, the loss of LKR activity results in increased levels of free lysine.

2.5.2 Discovery of other mutants

The search for new mutants that could alter the amino acid profile and increase the concentration of lysine and tryptophan of maize endosperm led to the discovery of several other genetic mutations such as *floury1* (*fl1*), *floury2* (*fl2*), *floury3* (*fl3*), *opaque5* (*o5*), *opaque6* (*o6*), *opaque7* (*o7*), *opaque15* (*o15*), *Defective endosperm* (*Def-B30*), and *Mucronate* (*Mc*) (Balconi et al. 2007). While all the other mutants confer an opaque phenotype to the endosperm with an increase in the overall lysine content, *o2*, *fl2*, and *Def-B30* affect different aspects of storage protein synthesis and alter zein content and has ~35-55% of the wild type level of storage protein (Morton et al. 2016).

Reduction of only ~10-20% is observed in mutants *o9*, *o11*, and *Mc* (Hunter et al. 2002). *fl2* encodes a 22-kDa α-zein gene with a defective signal peptide that causes the mutant polypeptide to accumulate in the ER membrane (Coleman and Larkins 1999). Similarly, the *Def-B30* mutant was recently shown to encode a 19-kDa α-zein with a serine to proline substitution in 15th amino acid mutation in the signal peptide; transgenic expression of this gene was sufficient to cause opacity (Kim et al. 2004). In contrast to these two examples of mutations in the signal peptide, the *Mc* gene encodes a 16-kDa γ-zein with a frameshift mutation. Precisely how these mutations, which affect a relatively small fraction of the zein polypeptides, disrupt the formation of vitreous endosperm is unclear (Soave and Salamani 1984). The *o1* mutation appears to have little effect on zein synthesis (Nelson et al. 1965). The *o15* mutation exerts its effect primarily on the 27-kDa γ-zeins (Dannenhoffer et al. 1995). The *fl1* mutation is a result of the abnormal placement of α-zeins within the protein bodies. *Fl1* encodes a trans-membrane protein that is located in the ER membrane (Holding et al. 2007). Similarly, *o5* mutant phenotype is caused by a reduction in the galactolipid content of the maize endosperm, with no change in zein proteins (Myers et al. 2011).

An alternative approach to understand the relationship between zein synthesis and the origin of the opaque endosperm phenotype is to perturb zein accumulation using transgenic approach. Two groups reported the knockdown of 22-kDa and 19-kDa α-zeins by RNA interference (RNAi) (Segal et al. 2003; Huang et al. 2004). An important observation from these studies was that the lysine content was increased in the transgenic lines by 15-20%. Notably, this increase is well below the 100% increase that is often observed in *o2* genotypes. Nevertheless, these experiments showed that transgenic approaches could be useful to increase grain lysine content. The second major finding of these studies was that the reduction of α-zein protein synthesis was sufficient to induce an opaque endosperm phenotype. It is noteworthy that the 22-kDa α-zein RNAi lines showed a more pronounced opaque phenotype than the 19-kDa α-zein RNAi lines. The interaction of 22-kDa α-zein with the γ- and β-zeins, which coat the exterior surface of protein bodies, is stronger than that of the 19-kDa α-zein,. Therefore, the absence of the 22-kDa α-zein could perturb the insertion of 19-kDa α-zein into the centre of protein bodies, or might enable direct contact of the 19-kDa α-zein with the γ- and β-zeins. Such abnormal associations could disrupt PB formation leading to the opaque endosperm phenotype. Several of these mutants in different

combinations were used to enhance lysine and tryptophan but were not successful because of large negative pleiotropic effects.

2.5.3 Discovery of opaque16 (*o16*)

However, recently, a recessive *opaque16* (*o16*) mutant isolated from Robertson's Mutator (Mu) stock was found to be associated with higher nutritional value in maize (Yang *et al.* 2005). The recessive *o16* located on chromosome 8 possesses higher lysine content compared to normal maize. The o*16* in *o2o2* genetic background increases lysine by ~30% over *o2o2* or *o16o16* alone. Further, genotypes with *o16o16* possessed nearly two-fold more lysine (0.247% in mutants; 0.125% in wild types) and tryptophan (0.072% in mutants; 0.035% in wild types) compared to normal maize (lysine: 0.125% and tryptophan: 0.035%) (Sarika et al. 2016). The study also showed that *o16* alone can be as good as *o2* for improving the nutritional quality of maize. The markers linked to *o16* have been identified offering additional advantage of adopting marker-assisted breeding along with *o2* in maize breeding programmes (Yang et al. 2005; Zhang et al. 2010, 2013).

2.6 Quality Protein Maize (QPM)

2.6.1 Endosperm modifiers

The elation over the discovery of *o2* and its utility in breeding programmes was short-lived with the discovery of pleiotropic effects such as soft endosperm resulting in damaged grains, increased susceptibility to pests and fungal diseases, and inferior food processing (Shewry and Thatam 1990). Since grain weight is reduced due to less density per unit volume as starch is loosely packed with larger air spaces, there is a corresponding decline in the yield which can be almost to the tune of 10% or above (Singh and Venkatesh 2006). This grain phenotype or appearance was also a major barrier to their acceptance in the developing countries where hard/vitreous grain is preferred.

In an effort to overcome the problems associated with original, soft *o2* materials, researchers at CIMMYT, Mexico, and at the University of Natal, South Africa, started carefully examining the nature of inherent problems, and came out with viable strategies to overcome the problems (Geevers and Lake 1992; Villegas et al. 1992). The inferior quality of *o2* can be overcome with the accumulation of *o2* endosperm modifiers without affecting the improved lysine content from 1.5% to 3.7% and

tryptophan from 0.4% to 0.8% on average (Krivanek et al. 2007); this finding led to the development of QPM. The mechanism of *o2* endosperm modification in QPM is not clear and inheritance of these modifier genes is complex and is likely to involve several loci, which complicates the genetic analysis of QPM. The identity of genes controlling the development of vitreous endosperm in QPM has remained elusive, but promising candidates have been identified by biochemical analysis. A possible role of 27-kDa γ-zein in recovering the vitreous phenotype has been put forward. The most prominent biochemical feature observed in QPM endosperm is the accumulation of the 27-kDa γ-zein at 2-3 fold higher levels than in wild type and *o2* (Wallace et al. 1990; Geetha et al. 1991; Lopes and Larkins 1995). Though the mechanism of this increase is unknown, the degree of QPM endosperm vitreousness closely correlates with the level of 27-kDa γ - zein protein (Lopes and Larkins 1995). The 27-kDa γ-zein appears to initiate and facilitate the formation of PBs, and endosperms with increased amounts of this protein contain more protein bodies because the 27-kD γ-zein becomes cross linked by disulfide bonds during grain desiccation. Thus, the level of 27-kD γ-zein could be an important component of endosperm modification by modifiers in QPM (Gibbon et al. 2003). Genetic mapping of *o2* endosperm modifiers in QPM was found to be linked with the locus encoding 27-kDa γ-zein storage proteins in chromosome 7. It was supported by Wu et al. (2010) demonstrating the hypothesis of γ-zeins over *o2* endosperm modification by silencing 27- and 16-kDa γ-zein genes which resulted in clumping of PB and opacity of QPM seeds.

Endosperm modifier genes have been used in breeding programmes worldwide for development of QPM genotypes that are vitreous/semi-vitreous in grain phenotype. Selection was carried out for grain modifiers in *opaque2* genetic background that led to a gradual increase of endosperm modification from the crown of the grain towards the tip. Various aspects of grain modification were reviewed by scientists at Purdue University and CIMMYT (Bauman 1975; Vasal et al. 1980, 1984; Glover and Mertz 1987; Bjarnason et al. 1988; Glover 1988; Prasanna et al. 2001). QPM is essentially interchangeable with normal maize in cultivation and grain phenotype, and has become a popular choice among the farming community (Gupta et al. 2013; Babu and Prasanna 2014; Pandey et al. 2015).

2.6.2 Development of QPM

In order to ameliorate the negative pleiotropic effects of *o2,* genotypes having a portion of hard endosperm in *o2* background were recombined to increase the frequency of modifiers. Four tropical populations viz., (i) Composite K (H.E.*o2*), (ii) Ver.181-Ant.gp *o2* × Venzuela-1 *o2*, (iii) Thai *o2* Composite and (iv) PD (MS6) H.E.*o2*, and one highland population (Composite I) were used with controlled full-sib pollination in the initial cycle followed by modified ear-to-row system (Bjarnason and Vasal 1992). Alternatively, yellow and white families possessing some degree of hard endosperm were recombined separately to develop (i) Yellow H.E.*o2* composite and (ii) 'White H.E.*o2*' composite, respectively. The repeated selection of modified ears for 3-4 cycles, led to the development of a series of QPM donor stocks by the mid-1970s (Prasanna et al. 2001).

Large-scale efforts were employed to develop diverse QPM germplasm in tropical-, subtropical- and highland-genetic background with different maturity groups and grain colour and texture. Considering the complexity and nature of grain modification trait, an innovative breeding procedure, designated as 'modified backcrossing-cum-recurrent selection', was designed in place of simple backcross programme, to develop diverse QPM germplasm (Vasal et al. 1980; Vasal et al. 1984). A number of advanced maize populations in CIMMYT were successfully converted to QPM populations using this procedure. During conversion, emphasis was placed on yield, grain modification and appearance, reduced ear rot incidence, rapid drying, and other desirable agronomic attributes. Besides the conversion programme, considerable resource allocation and research efforts were also devoted to the development of broad-based QPM gene pools (Bjarnason and Vasal 1992).

The conversion programme in CIMMYT led to the development of several QPM populations, pools, inbreds, and hybrids adapted to subtropical and tropical environments, which are widely used in the development of QPM cultivars in several countries including Brazil, China, Ghana, India, and several Latin American countries (Vasal 2001; Gupta et al. 2009). In India, a nutritionally superior *o2* composite with hard grain texture, 'Shakti-1', was released in 1997. Since 1998, intensive efforts were made in different breeding centres in the country, resulting in the release of a series of QPM hybrids. The first white-grained QPM hybrid 'Shaktiman-1' (a three-way cross hybrid using CIMMYT-QPM lines) was released during 2001 by RAU, Pusa, Bihar. 'Shaktiman-2' (a single-cross hybrid with white grain) was released by the same

institution during 2004. The first yellow-grained single-cross hybrid 'HQPM-1' was released by CCSHAU, Karnal, during 2005, followed by the release of 'Shaktiman-3' and 'Shaktiman-4' (2006), 'HQPM-5' (2007), 'HQPM-7' (2008), Vivek QPM-9 (2008), HQPM-4 (2010), Vivek QPM-21 (2012), Pratap QPM Hybrid-1 (2013), and Shaktiman-5 (2013). *2.6.3 Nutritional quality of QPM*

The data demonstrating the nutritional superiority of QPM over normal maize is overwhelming. QPM genotypes were found to have 2-3-fold increase of lysine and tryptophan in the endosperm proteins (Vasal 2001; Prasanna et al. 2001). The lysine value for *o2* was 3.3 to 4.0 mg/100 mg of protein, which was more than twice that of endosperm of normal maize, i.e. 1.3mg lysine/100mg of protein (Mertz et al. 1964; Habben et al. 1993). The decreased leucine content in QPM produces a favourable leucine-isoleucine ratio, which liberates more tryptophan for niacin biosynthesis. For this reason, QPM reduces pellagra significantly, even though QPM has the same niacin content as normal corn (Vasal 2001).

Many animal feed trials were conducted to evaluate the nutritional value of QPM as animal feed. Paes and Bicudo (1994) reported an increment of 50% in white and 40% in yellow cultivars in lysine and corresponding 40% to 35% in tryptophan for QPM compared with the normal ones. Studies on rats found that rats fed with QPM diet weighed more, were thicker, longer, denser, and stronger than those fed on normal maize (Serna-Saldivar et al. 1992). Burgoon et al. (1992) found that pigs raised on QPM have double the weight-gaining rate as compared to pigs fed on only normal maize. Use of QPM in animal feed is especially profitable for small-holder farmers who cannot afford synthetic lysine in animal feed. QPM in poultry diet improves the growth performance of broilers and resulted in higher weight gains when replaced with normal maize (Nyanamba et al. 2003; Onimisi et al. 2008). The protein of QPM is found to be equivalent to 90% of the milk protein as compared to the 40% in normal maize (Prasanna et al. 2001; Bressani 1992). The nutritional and biological superiority of QPM has been well established in model systems such as rats, pigs, infants, and small children as well as adults (Prasanna et al. 2001). In Guatemala, it was demonstrated that *o2* maize has 90% of the nutritive value of milk protein in young children. Children in Colombia suffering from kwashiorkar, a severe protein deficiency disease, were brought back to normalcy on a diet containing only *o2* (Bresanni 1994; Prasanna et al.

2001). However, the increased 3.7% lysine in QPM is still short against the 5% required for optimal human nutrition (Young et al. 1998).

2.7 Marker-assisted backcross breeding (MABB) for improving lysine and tryptophan

To provide an appropriate level of food security will be challenging in the coming decades while enhancing the sustainability of agricultural practices. With the global change in the climate, it will be even more intimidating with the declining availability of irrigation water and increasing cost of fertilizers (FAO 2011). Meeting the fast increasing demand in maize grain and feed will require an acceleration of the selection process so far achieved. The recent developments in plant biotechnology including molecular mapping and MAS offer a choice of options for introgression of the target gene(s), enhancing selection efficiency, and development of new cultivars with higher yield potential (Varshney et al. 2012). Prior to this advancement, conventional breeding procedures were used to convert commercial lines to QPM forms, though the procedure is tedious and time consuming. Introgression of a recessive gene such as *o2* through conventional backcrossing is not an easy procedure because of these major reasons viz., (i) identification of *o2* recessive gene from each selfed generation of each backcross generation (ii) requirement of a minimum six backcross generations to recover satisfactory levels of recurrent parent genome (iii) simultaneous selection of multiple modifiers, and (iv) rigorous biochemical tests of lysine and tryptophan levels in the selected materials in each breeding generation; it requires enormous labour, time, and material resources.

MABB has enormous potential to improve the efficiency and accuracy of conventional plant breeding (Collard et al. 2005). It significantly accelerates the breeding process (Ribaut et al. 2003) involving two steps: (1) foreground selection: marker-assisted selection of targeted gene through marker and (2) background selection: marker-assisted recovery of the recurrent parent genome (Hospital et al. 1992); it is the most effective way of transferring specific gene(s) to an otherwise agronomically superior variety or parental lines. Whilst identifying the gene of interest through foreground selection, background selection expedites significantly the rate of genetic gain/recovery of recurrent parent genome by just two backcrossings (Young and Tanksley 1989; Hospital et al. 1992; Visscher et al. 1996; Frisch et al. 1999). Easy access and availability of reliable PCR-based gene-specific markers have made MAS an

attractive option. Among the different types of PCR-based DNA markers available, the microsatellite or Simple Sequence Repeat (SSR) markers are often preferred for reasons of cost, simplicity, and effectiveness. SSR markers are robust, codominant, hypervariable, abundant, and uniformly dispersed in plant genomes (Powell et al. 1996). In maize, several thousand mapped SSR markers are available in the public domain (www.maizegdb.org).

The cloning and characterization of the *O2* gene (Schmidt et al. 1987; Motto et al. 1988), followed by detection of three SSR markers (*phi057*, *phi112* and *umc1066*) within the gene (Lin et al. 1997), led to effective differentiation of the *O2* and *o2* alleles (Kassahun and Prasanna 2003; Babu et al. 2004). These *o2*-specific SSR markers offer tremendous advantages in molecular marker-assisted conversion of non-QPM lines into their QPM versions (Babu et al. 2004). In India, a MAS-derived QPM hybrid, 'Vivek QPM 9', was released during 2008 by the Vivekananda Parvatiya Krishi Anusadhan Sansthan (VPKAS) in Almora (ICAR). It utilizes the *o2*-specific SSR markers for foreground selection of the *o2* allele and phenotypic selection for endosperm modifiers in the parental lines: CM145 and CM212) (Gupta et al. 2014). 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 grains in the reconstituted version of parental inbreds and hybrids. Vivek QPM-9 earned 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, Hyderabad, and CSK-HPKV, Palampur, respectively have been improved for protein quality. Further, single-cross hybrids, viz., Buland and PMH-1 have been targeted for conversion to QPM using

MAS. These experimental MAS-derived QPM hybrids are currently under different stages of multi-location testing (Gupta et al. 2015).

For further enhancing the grain nutritional quality attributes, especially lysine and tryptophan, in the endosperm protein, the availability of suitable linked SSR markers has offered a promising option of marker-assisted introgression of *o16* in QPM (*o2o2*) genetic background. . In this context, linked SSR markers, *umc1141* and *umc1149,* were successfully utilized for introgression of *o16* (Yang et al. 2005, 2013; Zhang et al. 2010, 2013). So far, the only research group that has reported the utilization of *o16* in their breeding programme is led by Dr. Wenpeng Yang at Guizhou Institute of Upland Food Crops, Guizhou Academy of Agricultural Sciences, China.. However, the efforts are confined to the effects of *o16* only on lysine accumulation in Chinese genetic background, and no information on its effect on tryptophan accumulation is available. For the first time, the *o16* mutation has been used in the maize breeding programme in India, and in the present investigation, attempts have been made to introgress *o16* on to the genetic background of four elite parental lines of four QPM hybrids.

Materials and Methods

3 Materials and Methods

3.1 Analyzing the effects of o16 on endosperm modification

3.1.1 Plant materials

The plant material for the study consisted of four F_1s obtained from China in the year 2013. They were derived from the crosses CML161 × QCL3024, CML193 × QCL3024, CML533 × QCL3024 and CML537 × QCL3024 (Table 3.1). CML161 and CML193 are CIMMYT-based *o2* inbred lines, while CML533 and CML537 are CIMMYT-based normal inbred lines.

3.1.2 Generation of F_3 seeds from F_2 populations

The F_1s were grown in *Kharif* 2014 in New Delhi, and were tested for its true hybridity by using *o2* gene-based (*phi057, phi112* and *umc1066*) and *o16*-linked markers (*umc1121, umc1141* and *1149*). Strategy for deriving genetic materials for analysing the endosperm modification is presented in Fig 3.1. The sequence information of the primers is presented in the Table 3.2. The positive plants were selfed to derive F_2 populations which were raised during *Rabi* 2014-15 at the Maize Winter Nursery Centre, Hyderabad (Table 3.3).

3.1.3 Grain hardness

Grain hardness studies were performed withthe F_3 seeds of the selected progenies from CML161 × QCL3024, CML193 × QCL3024, CML533 × QCL3024, and CML537 × QCL3024. Five randomly selected grains per line were used for measuring grain hardness (GH) using Texture Analyzer (Scientific Microsystem, UK). The hardness of grains was measured at a moisture content of ~14%. For measurement of hardness, a cylindrical probe of 75 mm diameter (P75 mm compression platen) was used. Individual seeds were placed centrally beneath the probe with the embryo facing down. The test speed of the probe was fixed at 2 mm/s and the compression distance at 70% with a trigger load cell of 500 kg. The first peak force (N: newton) in the force deformation curve was noted as GH of the seeds (Mohsenin 1986). T-test was performed to test the significant differences between the two different classes of *O2O2/O16O16, O2O2/o16o16, o2o2/O16O16,* and *o2o2/o16o16* in each population by using the R statistical package. As control, a normal and an *o2* line were used for the

comparative study. For comparison, CML543 (Normal: *O2O2*), MGUQ-102 (full opaque-*o2o2*) and a QPM line (HKI193-1: *o2o2*-hard endosperm) were used as checks.

3.1.4 Scanning electron microscopy

Maize grains were decapped and degermed with a razor blade and cut through the centre of the grain giving a fracture with rough surface rather than a clean cut. A small piece from the central region of endosperm was used for the study and was coated with an alloy of gold and palladium and documented in Zeiss EVO MA 10 Scanning electron microscope at 20kV/EHT and 80 Pa with a magnification of 1.50 Kx. CML543 (Normal: *O2O2*), MGUQ-102 (full opaque-*o2o2*) and a QPM line (HKI193-1: *o2o2*-hard endosperm) were used as checks for comparison.

3.1.5 Protein profiling

The α, β, γ, and δ zein fractions of different samples were extracted in accordance with Yue et al. (2014). Fifty milligram of maize flour of each sample was defatted by treating it with 1 mL hexane for 1 h, with one repetition. Proteins were extracted from skimmed flour with 1 mL of extraction buffer (12.5 mM sodium borate (pH 10.0), 1% SDS and 2% β-mercaptoethanol) and incubated overnight at 37°C with shaking. The samples were centrifuged at room temperature for 15 min at 13,000 rpm. The supernatant consisted of total protein extract. Zein fraction was extracted by adding 700 μL of ethanol into 300 μL of total protein extract in a 1.5 mL microcentrifuge tube. It was mixed well and incubated for 2 h. It was centrifuged again at room temperature for 15 min at 13,000 rpm. The derived supernatant consisted of only total zein fraction, while the pellet consisted of non-zein proteins. For non-zein fractionation, the pellets were dissolved again in sodium borate buffer. The extracted alcohol soluble zein and non-zein protein fractions were profiled by loading 10 μL samples in 15% SDS-PAGE and documented in visible white light. For comparison, CML543 (Normal: *O2O2*), MGUQ-102 (full opaque-*o2o2*), and a QPM line (HKI193-1: *o2o2*-hard endosperm) were used as checks.

3.2 DNA isolation and PCR analysis

Leaf samples were collected from three-week-old seedlings. Genomic DNA was isolated from the leaves by using standard CTAB procedure (Murray and Thompson, 1980). Polymerase chain reaction (PCR) (Bio-Rad, California, USA) was carried out for 15 μLreaction mixture using REDtaq ReadyMix^{TM} PCR Reaction Mix (SIGMA-

ALDRICH). The PCR amplification was carried out with a "touch-down" procedure standardised at Maize Genetics Unit, IARI. This 'touch down' procedure consisted of three steps. The first step was set for 12 cycles: denaturation at 94˚C for 30 s, annealing at 62˚C for 30 s (reducing the annealing temperature subsequently by 0.5˚C per cycle), and extension at 72˚C for 45 s. The second step was set for 45 cycles: denaturation at 94˚C for 30 s, annealing at 58˚C for 45 s, and extension at 72˚C for 45s. The final extension was carried out at 72˚C for 7 min. The resulting PCR amplicons were resolved in 4% agarose gel and 8% poly-acrylamide gel electrophoresis (PAGE) at 120 V for 4 and 3 hours, respectively. The resolved amplified products were visualised using a gel documentation system (AlphaInnotech, Califronia, USA).

3.3 Testing of opacity and endosperm modification

One hundred random seeds from all individual homozygous plants from selfed generations were selected and analysed for the degree of opaqueness by using light box testing. For analysis of endosperm modification, the grains were rated on a scale of 0 to 100, with '100', '75', '50', '25' indicating 100%, 75%, 50%, 25% opaqueness, respectively, and '0' indicated 100% vitreous/hard. Endosperm modification scores were calculated by the formula: Degree of opaqueness = $(N_{100} \times 100 + N_{75} \times 75 + N_{50} \times 50 + N_{25} \times 25 + N_0 \times 0)/100$, where N_{100}, N_{75}, N_{50}, N_{25} and N_0 are the numbers of seeds with 100%, 75%, 50%, 25% and 0% opaqueness, respectively.

3.4 Estimation of lysine

Lysine content was estimated by using UHPLC (Dionex MLtimate 3000, Thermo Scientific). Five milligram of degermed endosperm flour per sample was acid hydrolysed overnight for 16 h at 110˚C with 800 µL of 6 N HCl, 100 µL 0.1 N HCl, 100 µL of nor-leucine and 10 µL of phenol. The hydrolysed sample was transferred to a 10 mL volumetric flask. Exactly 500µL of 12.5M NaOH solution was added and the volume was made up to 10 mL with 0.1 N HCl. The solution was filtered by using 0.22µm nylon filters (Chromatopak, CNW^R technologies). For derivatization process, 100 µL of filtered sample was transferred into a Falcon tube and 900 µL of boric acid and 1mL FMOC were added to it. The mixture was vortexed slightly and kept for 5 min. Again 4 mL of n-pentane was added and vortexed continuously for 45 s. The upper layer was discarded and the lower layer

Table 3.1 Genetic material used to study the effects of *o16*

S.No.	Genotypes	Pedigree	Kernel colour
1.	F_2 & F_3	CML161 × QCL3024	Yellow
2.	F_2 & F_3	CML193 × QCL3024	Yellow
3.	F_2 & F_3	CML533 × QCL3024	Yellow
4.	F_2 & F_3	CML537 × QCL3024	Mix of white and yellow

Table 3.2 Details of SSRs used in foreground selection

S. No.	Markers	Sequence	Primer
opaque2			
1.	*phi057*	CTCATCAGTGCCGTCGTCCAT	Forward
		CAGTCGCAAGAAACCGTTGCC	Reverse
2.	*phi112*	TGCCCTGCAGGTTCACATTGAGT	Forward
		AGGAGTACGCTTGGATGCTCTTC	Reverse
3.	*umc1066*	ATGGAGCACGTCATCTCAATGG	Forward
		AGCAGCAGCAACGTCTATGACACT	Reverse
opaque16			
4.	*umc1141*	AGAGGAGAAAGAGACAGACAGGCA	Forward
		CAGGAACTGAATGAAAGCAACTCA	Reverse
5.	*umc1149*	TACAGTAGGGATTCTTGCAGCCTC	Forward
		GTGGGACCTTGTTGCTTCCTTT	Reverse

Table 3.3 Details of populations generated for studying the effects of *o16*

S. No.	Generations	Seasons	Experimental location for raising populations
1.	F_1	*Kharif* 2014	IARI Experimental Farm, New Delhi
2.	F_2	*Rabi* 2014-15	Maize Winter Nursery Centre, Hyderabad

was used for lysine estimation after transferring it to a 2 mL vial. The mobile phases A and B have two components: buffer and organic phase. Buffer phase has tetra-methyl ammonium chloride and sodium acetate trihydrate with a pH of 3.5. The organic phase consisted of acetonitrile and methanol in the ratio 49:1. The mobile phases A and B have the buffer and organic phase in the ratio of 9:1 and 1:9, respectively. The samples were eluted through Acclaim™ 120 C18 column (5µm, 120 angstrom, 4.6*150mm, Thermo Scientific) and detected with a RS photodiode array detector (PDA) with absorbance in 265nm wavelength. The injected volume was fixed at 25 µL with a flow rate of 1 mL min^{-1}. Eight dilutions of standard amino acids (HiMedia) were made for deriving the standard curve. Thus the concentration of the amino acids in each sample was estimated by standard regression using external standards.

3.5 Estimation of tryptophan

Tryptophan content was also estimated by using UHPLC (Dionex MLtimate 3000, Thermo Scientific). Twenty milligram of degermed endosperm flour per sample was hydrolysed with 2 mL of 4M NaOH. Exactly 200 µL of 0.1% ascorbic acid was added and mixed properly and kept overnight for 16 h at 120˚C. The hydrolysed sample was cooled down and neutralized with 6N HCl to maintain a pH of 7-8. Then the volume was made up to 10 mL with water. It was then filtered with Whatman filter paper and the volume was made up to 25 mL with water. About 1 mL of the sample was transferred into a UHPLC 2 mL vial and used for injecting. Water and acetonitrile were used as buffer. The samples were eluted through Acclaim™ 120 C18 column (5 µm, 120 angstrom, 4.6*150 mm, Thermo Scientific) and detected with a RS photodiode array detector (PDA) with absorbance in 280 nm wavelength. The injected volume was fixed at 40 µL with a flow rate of 1 mL min^{-1}. Eight dilutions of standard amino acids (AAS 18-5ML, SIGMA ALDRICH) were used for constructing the standard curve. Thus the concentration of amino acids in each sample was estimated by standard regression using external standards.

3.6 Marker-assisted backcross breeding (MABB)

3.6.1 Plant material

The experimental material for the present study consisted of four elite inbred lines viz. HKI161, HKI163, HKI193-1, and HKI193-2. These inbreds are parents of four commercial QPM maize hybrids (HQPM1, HQPM4, HQPM5 and HQPM7), which

have been adopted all across the country (Table 3.4). All the recurrent parental inbred lines are QPM genotypes with *o2* allele and have enhanced lysine and tryptophan content in the endosperm. The *o16* donor line, QCL3024, is a high lysine line isolated from a population derived from Robertson's Mutator stocks and was reported from China (Yang et al. 2005). The donor inbred, QCL3024 having *o16* could not be obtained as such, however F_1s derived from crosses of two CIMMYT *o2* inbred lines, CML161 and CML193 and the donor QCL3024 were obtained from Institute of Upland Food Crops, Guizhou Academy of Agricultural Sciences, Guizhou, China, and used as donor parents for introgressing the targeted *o16* allele into the four QPM parental inbred lines. For HKI161 and HKI163, F_1 of cross CML161 × QCL3024 and for HKI193-1 and HKI193-2, F_1 of CML193 × QCL3024 were used as donor lines, respectively (Table 3.5).

3.6.2 Generation of backcross populations under MABB

The field experiments were undertaken at two places: IARI-Experimental Farm, New Delhi, and Maize Winter Nursery Centre, IIMR, Hyderabad, during *Kharif* and *Rabi* seasons, respectively. All the standard agronomic practices recommended for maize from sowing till harvest were followed. The geographical coordinates of the two experimental locations are, Delhi: 29°41'52.13"N and 77°0'24.95"E; Hyderabad: 17°21'50.39"N and 78°29'42.31"E. The details of the various generations derived during the conversion are presented in Table 3.4 and depicted in Fig 3.2. The donor F_1s were tested for their true hybridity by using gene-specific marker for *o2* and linked marker for *o16.* The two inbreds, HKI161 and HKI163, were crossed as female parents with the Chinese donor F_1 derived from CML161 × QCL3024. In the case of HKI193-1 and HKI193-2, F_1 from CML193 × QCL3024 was used as donor. The BC_1F_1 crosses were attempted during *Rabi* 2013-14 at IARI, New Delhi (Table 3.6). The BC_1F_1 progenies of the four crosses were grown during *Kharif* 2014. Foreground selection for *o2* and *o16* were carried by using their respective markers. Plants from each BC_1F_1 population with higher genome recovery of respective recurrent parent and maximum phenotypic similarity were selected and backcrossed to the respective recurrent parent to generate BC_2F_1 seeds. BC_2F_1 plants were grown in Maize Winter Nursery Centre, Hyderabad during *Rabi* 2014-15, and were subjected to foreground and background selection. The selected BC_2F_1 plants were self-pollinated to generate BC_2F_2 plants which were raised during *Kharif* 2015 to generate BC_2F_3 seeds (Table 3.6).

All the four populations except the HKI163-based population could be generated as per the strategy. In the case of HKI163, due to insect damage and non-synchrony between BC_1F_1 and HKI163, two foreground positive plants having high genome recovery were crossed to generate modified-BC_1F_2 seeds at IARI, New Delhi (Table 3.6 & Fig 3.3). The modified-BC_1F_2 population was raised during *Rabi* 2014-15 at Maize Winter Nursery Centre, Hyderabad. Modified-BC_1F_3 plants homozygous for *o2* and *o16* were further backcrossed to HKI163 at IARI, New Delhi during *Kharif* 2015. During *Rabi* 2015-16, the modified-BC_2F_1 population was raised at the Maize Winter Nursery Centre, Hyderabad (Table 3.5). Each of the plants in modified-BC_2F_1 that were heterozygous for *o16* but homozygous for *o2* was selfed. In each and every generation, foreground selection was carried out and only positive plants were forwarded and subjected to background selection. The strategy adopted for MABB for introgressing *o16* into the genetic background of QPM inbred lines is depicted in Fig 3.1.

3.6.3 Marker-assisted foreground selection

Polymerase chain reaction (PCR) was performed for the markers to be used in foreground selection. The markers were tested for polymorphism between the recurrent parents and the respective F_1s. For *o2* locus, gene-based markers *phi057, phi112,* and *umc1066*, while linked markers *umc1141* and *1149* were used for the *o16* locus. The sequence information of the primers is presented in the Table 3.2. The corresponding polymorphic markers of each locus were used for the foreground selection in each of the backcross- and selfed- generations for selecting the target genes. The PCR amplicons were resolved in agarose gel and native PAGE acrylamide gel. The amplified fragments were scored for the presence of favourable *o2* and *o16* alleles. Chi-square test was performed using the standard procedure for testing the goodness-of-fit of the observed segregation pattern at the *o2* and *o16* loci in each of the generations.

3.6.4 Marker-assisted background selection

A set of 300 SSR markers covering uniformly all the 10 chromosomes of maize was used for polymorphic survey between recurrent parents and the respective donor F_1s. The primer sequences for the SSR primers were adapted from the maize genome database (www.maizegdb.org) and were custom synthesized (SIGMA Tech., USA). The primers which were polymorphic between the recurrent parents and donors were

employed for recovering the recurrent parental genome (RPG) in viz., BC_1F_1, BC_2F_1 and BC_2F_2 populations. In case of HKI163, the same was performed in BC_1F_1, modified-BC_1F_2, modified-BC_1F_3 and modified-BC_2F_1 populations. Recovery of background genome among the backcross progenies was ascertained using Graphical Geno Types (GGT) version 3.0 (Van-Berloo 1999).

3.6.5 Selection for phenotypic characteristics

Stringent phenotypic selection was performed in each backcross- and selfed-generations for the similarity in plant-, ear- and grain-characteristics of the recurrent parent. Plant height, ear height, days to 50% anthesis, days to 50% silking, cob length, cob girth, number of grain rows, number of grains per row, and 100-grain weight of the selected progenies were recorded.

Table 3.4 List of QPM hybrids targeted in the study with their parentage and their characteristics

S. No.	Hybrid	Parentage	Maturity group	Area of adaptation	Grain Yield (tonnes/ha)
1	HQPM-1	HKI193-1 × HKI163	Late	NWPZ, NEPZ, CWZ, PZ	6.2
2	HQPM-4	HKI193-2 × HKI161	Late	NWPZ, NEPZ, CWZ, PZ	6.0
3	HQPM-5	HKI163 × HKI161	Late	NWPZ, NEPZ, CWZ, PZ	5.8
4	HQPM-7	HKI193-1 × HKI161	Late	PZ	7.2

Table 3.5 Genetic material used in MABB

S.No.	Genotypes	Pedigree	Kernel colour
Recurrent parents			
1.	HKI161	Selection from CML161	Yellow
2.	HKI163	Selection from CML163	Yellow
3.	HKI193-1	Selection from CML193	Yellow
4.	HKI193-2	Selection from CML193	Yellow
Donor genotypes			
1.	F_1	CML161 × QCL3024	Yellow
2.	F_1	CML193 × QCL3024	Yellow

Table 3.6 Details of populations generated under MABB

S.No.	Generations	Seasons	Experimental location for raising populations
HKI161, HKI193-1, HKI193-2			
1	F_1	*Rabi* 2013-14	IARI Experimental Farm, New Delhi
2	BC_1F_1	*Kharif* 2014	IARI Experimental Farm, New Delhi
3	BC_2F_1	*Rabi* 2014-15	Winter Maize Nursery, Hyderabad
4	BC_2F_2	*Kharif* 2015	IARI Experimental Farm, New Delhi
5	BC_2F_3	*Rabi* 2015-16	Winter Maize Nursery, Hyderabad
HKI163			
1	F_1	*Rabi* 2013-14	IARI Experimental Farm, New Delhi
2	BC_1F_1	*Kharif* 2014	IARI Experimental Farm, New Delhi
3	Modified-BC_1F_2	*Rabi* 2014-15	Winter Maize Nursery, Hyderabad
4	Modified BC_1F_3	*Kharif* 2015	IARI Experimental Farm, New Delhi
5	Modified BC_2F_1	*Rabi* 2015-2016	Winter Maize Nursery, Hyderabad

Results

4.1 Identification of polymorphic SSR markers of *o2* and *o16*

Testing of polymorphism for the targeted genes viz., *o2* and *o16* was carried out in the F_1s of all four crosses (CML161 × QCL3024, CML191 × QCL3024, CML533 × QCL3024 and CML537 × QCL3024) obtained from Guizhou Institute of Upland Food Crops, Guizhou Academy of Agricultural Sciences, China. CML161 and CML193 are the *o2*-based QPM inbreds, while CML533 and CML537 are the normal inbreds. As the *o16* donor used in the crosses was unavailable, and the three lines viz., CML161, CML193 and CML537 (obtained from CIMMYT) were used as the reference for wild type (*O16*) allele. Since the recessive *o16* mutant allele is known to be absent from the CIMMYT-derived genetic materials and is currently available only in Chinese genetic background, the mutants were easily identified by comparing the amplified alleles of the targeted markers among these inbreds and F_1s. For *o2* alleles, the three reported gene-based SSR markers viz., *phi112*, *phi057* and *umc1066* were used to test for polymorphism (Lin et al. 1997). With *umc1066,* polymorphism was observed in 4% agarose gel in both the crosses CML161 × QCL3024 and CML191 × QCL3024 (Fig 4.1A). For *o16*, the three SSR linked markers *viz. umc1121*, *umc1141* and *umc1149* were used for testing polymorphism (Yang et al. 2005). In three out of the four crosses *viz.* CML161 × QCL3024, CML533 × QCL3024 and CML537 × QCL3024, *umc1149* showed a distinct polymorphism in 4% agarose and was used for genotyping (Fig 4.1B). However, in CML191 × QCL3024, polymorphism was detected in 8% acrylamide native PAGE using *umc1141*. Therefore, the identification of the mutant alleles and true hybridity of all the F_1(s) were carried out using *umc1066* in the case of *o2* and *umc1141* and *umc1149* for *o16.* These three polymorphic markers were further used for genotyping the segregating populations.

4.2 Segregation pattern of identified markers

From among the true F_1s of all crosses, 5-7 plants were selfed to derive F_2 populations. Two F_2 populations of 119 and 150 plants from CML161 × QCL3024 and CML191 × QCL3024, respectively segregated in the ratio of 1:2:1 for both the *o2* and o16 loci (*p*-values > 0.05). In the other two crosses viz., CML533 × QCL3024 (population size: 159) and CML537 × QCL3024 (population size: 143) segregating for

only *o16* locus also did not show any significant deviation from the normal Mendelian segregation ratio of 1:2:1 (Table 4.1).

4.3 Effect of *o16* on endosperm modification

Phenotypic screening of the individual seed endosperm opacity with a light box was carried out on both F_2 and F_3 seeds. The light box testing showed the degree of opaqueness in each individual seed very clearly as the well modified portion looked translucent, while the opaque region was black in colour (Hossain et al. 2008b). One hundred randomly selected seeds per individual line were grouped into five classes based on scores that describe degree of opaqueness at 100%, 75%, 50%, 25%, and 0% (Vasal et al., 1980). In the crosses, CML161 × QCL3024 and CML191 × QCL3024 the degree of opaqueness in F_2 seeds was found to be 26.09 and 28.98, respectively (Table 4.2). A mere 2.25% was observed in the case of CML533 × QCL3024 and zero in CML537 × QCL3024 (Fig. 4.2). The degree of opaqueness in F_2-derived F_3 seeds was analysed further and found to be negligible in CML533 × QCL3024 and CML537 × QCL3024 segregating only for *o16* (Table 4.3). However, significant degree of endosperm opaqueness was observed in CML161 × QCL3024 and CML191 × QCL3024, where both *o2* and *o16* were segregating. F_3 seeds from individual lines positive for both *o2* and *o16,* or *o2* alone in crosses, showed 95.81 to 98.24 % opacity (Fig. 4.3). The ratio of central soft and outer periphery vitreous region of *O2O2/o16o16* was showing similar pattern with that of normal wild line but distinctly different from *o2o2*-soft and *o2o2*-QPM (Fig. 4.4).

4.4 Effect of *o16* grain hardness

Nine genotypic classes could be obtained in F_2-derived F_3 seeds of CML161 × QCL3024 and CML191 × QCL3024, since the progenies were segregating for both *o2* and *o16*. For CML533 × QCL3024 and CML537 × QCL3024, where only *o16* was segregating, three classes could be obtained in F_2 populations. F_3 seeds only from F_2 homozygous plants *viz. o2o2/o16o16, o2o2/O16O16, O2O2/o16o16,* and *O2O2/O16O16* were only used for the grain hardness analysis. In case of CML161 × QCL3024 and CML193 × QCL3024, F_3 seeds derived from F_2 plant of genotypes *o2o2/o16o16, o2o2/O16O16* were showing similar hardness index with the check MGUQ-102 (*o2o2*-fully opaque, 188.19N) line, whereas seeds of *O2O2/o16o16*

Table 4.1 Segregation pattern of *o2* and *o16* in different F_2 populations

F_2 generation	No. of plants genotyped	*o2o2*	*O2o2*	*O2O2*	χ^2	*P* value	*o16o16*	*O16o16*	*O16O16*	χ^2	*P* value
CML161 × QCL3024	119	28	58	33	0.495	0.780 ^{ns}	30	56	33	0.563	0.754 ^{ns}
CML191 × QCL3024	150	32	81	37	1.293	0.523 ^{ns}	39	76	35	0.306	0.858 ^{ns}
CML533 × QCL3024	159	-	-	-	-	-	41	81	37	0.257	0.879 ^{ns}
CML537 × QCL3024	143	-	-	-	-	-	40	69	34	0.678	0.71 ^{ns}

ns - non-significant

Table 4.2 Degree of opacity in randomly selected 100 F_2 seeds of different crosses

Pedigree (F_2 seeds)	Parental genotypes	0%	25%	50%	75%	100%	Opaqueness (%)
CML161 × QCL3024	*o2o2/O16O16×O2O2/o16o16*	67	4	7	0	22	**26.09**
CML191 × QCL3024		66	0	5	11	18	**28.98**
CML533 × QCL3024	*O16O16×o16o16*	91	5	2	0	0	**2.25**
CML537 × QCL3024		100	0	0	0	0	**0**

Table 4.3 Mean degree of opacity (%) in F_3 seeds of the four crosses

F_3 seeds (Pedigree)	***o2o2/o16o16***	***o2o2/O16O16***	***O2O2/o16o16***	***O2O2/O16O16***
CML161 × QCL3024	98.24	97.65	2.15	1.23
CML191 × QCL3024	96.34	95.81	3.55	1.72
CML533 × QCL3024	-	-	4.30	2.03
CML537 × QCL3024	-	-	0.35	1.49

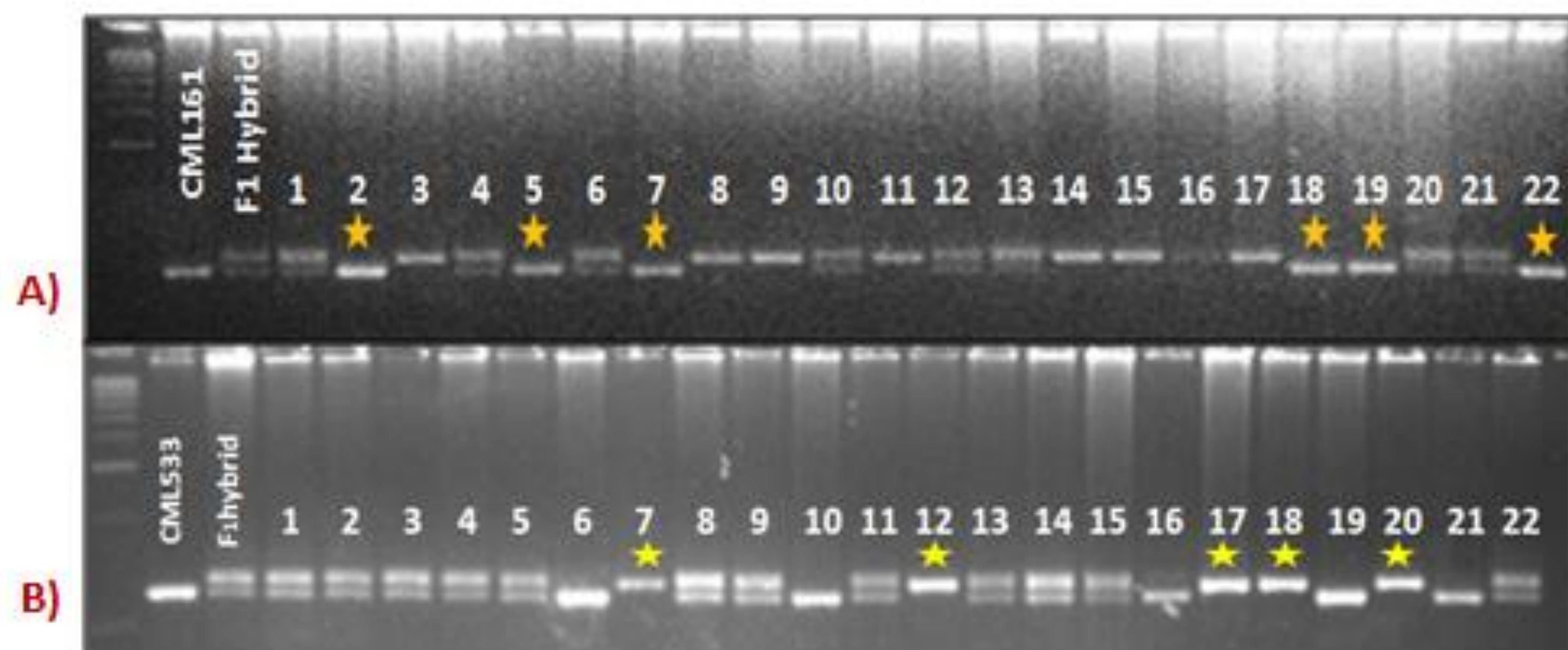


Figure 4.1 Segregation in F_2 populations A) ***o2-based*** **marker,** ***umc1066*** **in CML161 x QCL3024 B)** ***o16*** **linked marker,** ***umc1149*** **in CML533 x QCL3024**

★ **Favourable homozygous genotypes**

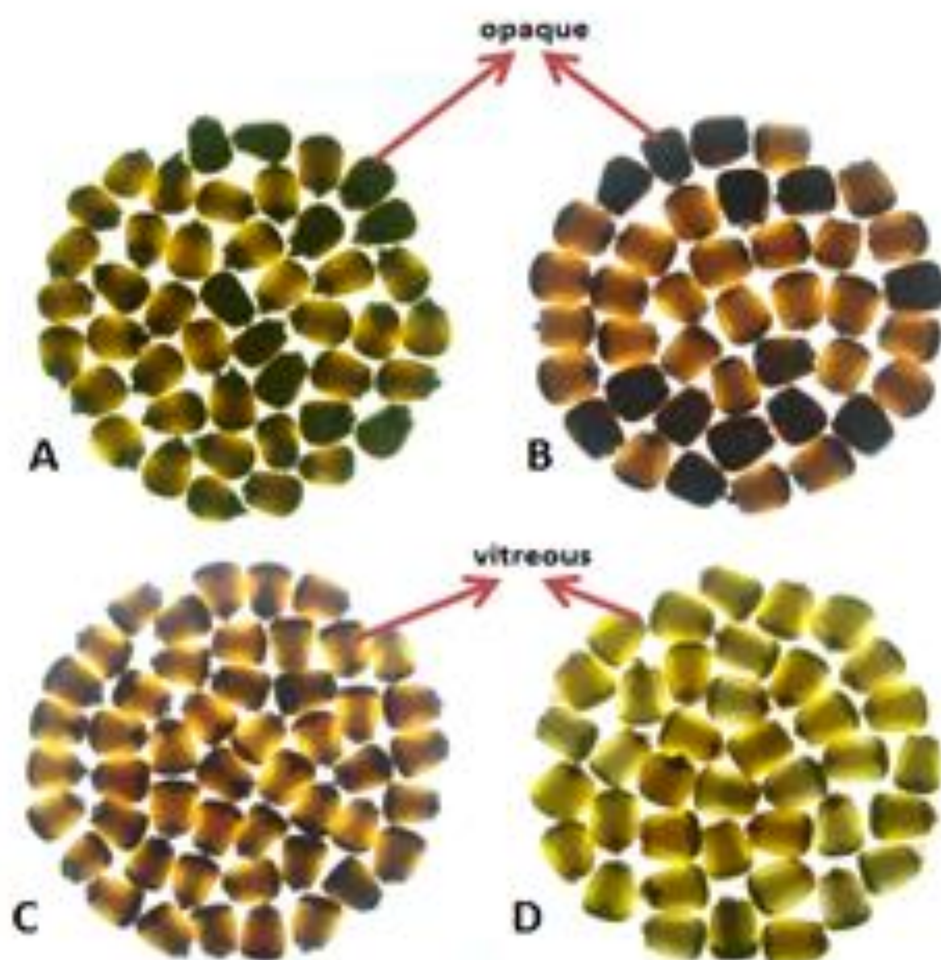


Figure 4.2 Light box testing of F_2 seeds from (A) CML161 × QCL3024 (B) CML193 × QCL3024 (C) CML533 × QCL3024 and (D) CML537 × QCL3024

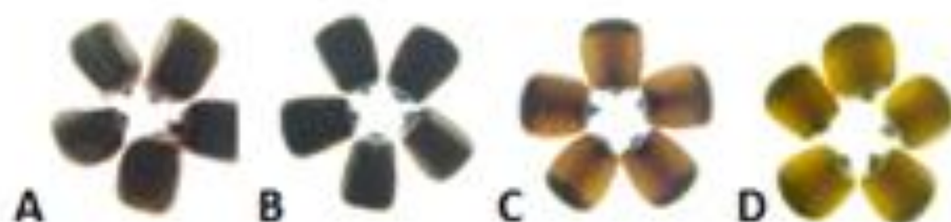


Figure 4.3 Light box testing of different F_2 families seeds of the cross CML161 × QCL3024

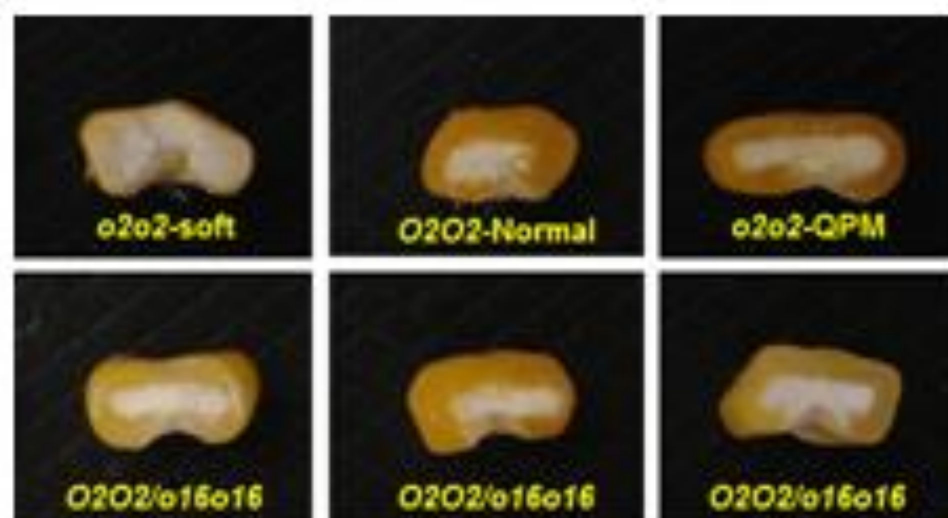


Figure 4.4 Comparison of the ratio of middle soft and outer periphery hard region of endosperm among *soft-opaque2*, normal, QPM and *o16o16* genotypes

(399.73N) showed similar force required to break the corresponding wild genotypes *O2O2/O16O16* (414.97N) as well as with the normal line, CML543 (426.45N)

(Table 4.4). Similar trend could be observed in CML191 × QCL3024, where both the loci were segregating. The F_3-seed derived from *O2O2/o16o16* (332.89N) had similar degree of hardness as that of wild genotypes viz., *O2O2/O16O16* (337.18N) of the population.

In the F_2 populations of CML533 × QCL3024 and CML537 × QCL3024, where only *o16* was segregating, a similar observation could be observed where *O2O2/o16o16* genotypes (312.25N and 372.98N, respectively) required a similar force index as that of the corresponding *O2O2/O16O16* genotypes (378.34N and 423.12N, respectively). Thus, *O2O2/o16o16* possessed hard endosperm, as reasonably higher degree of force was required to break the F_3 seeds in all the populations which were observed to be *at par* with the wild CML543 line (Table 4.4).

4.5 Microscopic arrangement of protein bodies and starch granules in endosperm

Scanning electron microscopy (SEM) was performed to analyze the microscopic structure and organization of starch granules and protein bodies in different genotypes for a comparative study on effect induced by *o16*. The micrographs were taken with a magnification of 1500X. It revealed that the starch granules of normal inbred (*O2O2/O16O16*) were large, uniform in size and angular polygonal shape with proteinaceous matrix surrounding them characterizing with many contacts and interconnections forming between the adjacent starch granules (Fig. 4.5 A). But we observed a significant reduction, almost negligible amount of proteinaceous matrix adhering to the starch granules in the case of MGUQ-102 possessing *o2o2/O16O16* (Fig. 4.5 B). The starch granules were round smooth, relatively smaller and were loosely packed with large intergranular space. But in QPM inbred viz., HKI193-1 (*o2o2/O16O16*), though the starch granules were spherical and smooth, a relatively more proteinaceous matrix was observed adhering to the starch granules which revealed a tighter interaction with lesser air space (Fig. 4.5 C). However, in *O2O2/o16o16* genotypes, the starch granules were angular polygonal shape with tighter organization with no intergranular space. *O2O2/o16o16* was observed to be almost similar in microscopic organization with that of *O2O2/O16O16* (Fig. 4.5 D). In *o2o2/o16o16* genotype, the starch granules were not exactly round in shape but with similar amount

of proteinaceous matrix and inter granular space as that of the *o2o2/O16O16* and QPM inbred, HKI193-1 (Fig. 4.5 E).

4.6 Comparison of zein protein profiles

SDS-PAGE was used to compare qualitatively and to some extent quantitatively for prolamin fraction in the lines (Hunter et al. 2002). To obtain a standardized comparison, equal amount of endosperm flour were subjected for extracting prolamin fraction. The variation in zein protein profile between the two mutants *o2* and *o16* and the wild could be observed in the Fig. 4.6. The total zein fraction of each sample had been profiled in two replicates. We could observe significant qualitative as well as quantitative differences in the zein fractions among the genotypes. The fully opaque-*o2o2* (MGUQ-102) showed a reduced amount of all zein fractions. We could also observe a 2-3 fold increase in the expression of 27 kDa γ-zein, and also increase in the expression of 16-kDa γ-zein in modified vitreous QPM inbred (HKI193-1) than the fully opaque-*o2o2* line. The *o16o16* genotype showed a very similar profile with that of the normal line, CML543 but with a slight reduction of 50 kDa γ-zein. However, it showed a completely different pattern from *o2o2* lines with higher level of expression in all zein fractions except 27-kDa γ-zein. The double mutant *o2o2*/*o16o16* showed an intermediate pattern of profile with that of *o2o2* and *o16o16* having semi-polygonal starch granules and proteinaceous matrix being present but less packed compared to *o1616*.

4.7 Effect of *o16* on accumulation of lysine and tryptophan

The concentration of the amino acids in the each sample was estimated by standard regression from the standard curve established with six different concentration of amino acids standard for both lysine and tryptophan (Fig. 4.7) and Fig. 4.8 depicts the peaks of lysine and tryptophan in samples. CML533 × QCL3024 and CML537 × QCL3024 segregating for *o16*, were used for quality analyses. Since grains of *o2o2* and *o2o2/o161o16* of CML161 × QCL3024 and CML193 × QCL3024 possessed 90-100% opaqueness (due to absence of favourable endosperm modifier loci) and soft endosperm, they do not possess any direct significance in the breeding programme. The estimation of nutritional quality for these two populations thus was not considered owing to high cost involved in UPLC analyses. However, the nutritional status of *o2o2/o16o16* genotypes was evaluated in MABB-derived lines.

Table4.4 Force (N) required to break the F_3 seeds of four different crosses

F_3 families	Genotypes	Mean (Newton, N)
CML161 × QCL3024	*o2o2/o16o16*	213.65
	o2o2/O16O16	267.85
	O2O2/o16o16	399.73
	O2O2/O16O16	414.97
CML191 × QCL3024	*o2o2/o16o16*	205.52
	o2o2/O16O16	246.96
	O2O2/o16o16	332.89
	O2O2/O16O16	337.18
CML533 × QCL3024	*O2O2/O16O16*	312.25
	O2O2/o16o16	378.34
CML537 × QCL3024	*O2O2/O16O16*	372.98
	O2O2/o16o16	423.12
CML 543	*O2O2/O16O16*	426.45
MGUQ-102 (*o2o2- fullopaque* line)	*o2o2/O16O16*	188.19
QPM (HKI193-1)	*o2o2/O16O16*	301.06

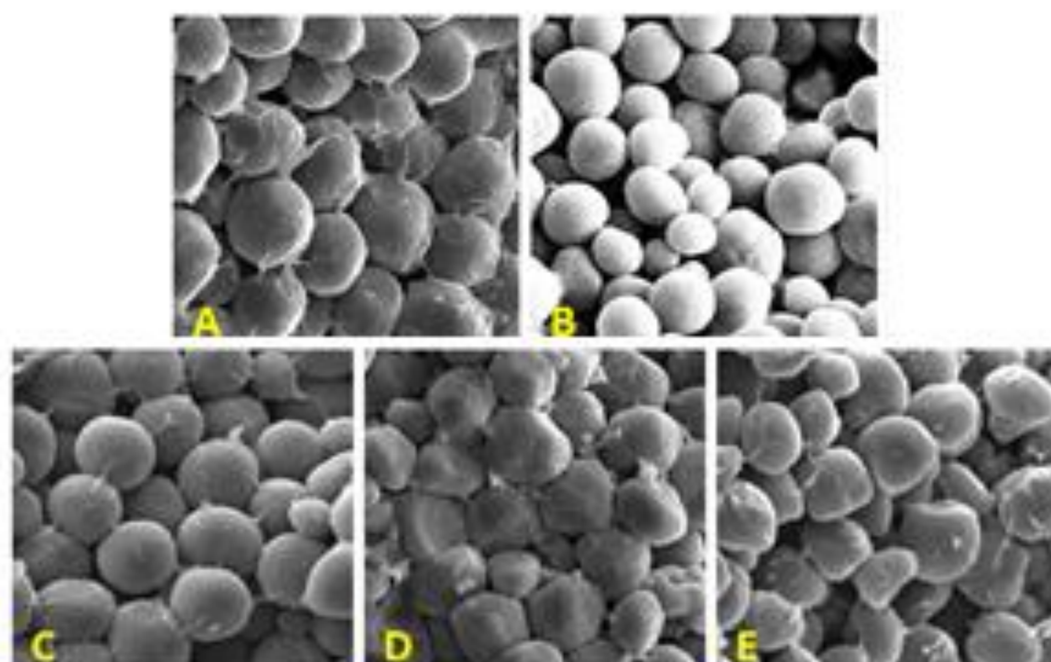


Figure 4.5 Microscopic view of protein bodies and starch granules organization under SEM. (A) a normal line, CML543 (B) fully opaque line, MGUQ-102 (C) a QPM, HKI193-1 (D) an *opaque16* line, (E) a double mutant (*o2o2/o16o16*) line

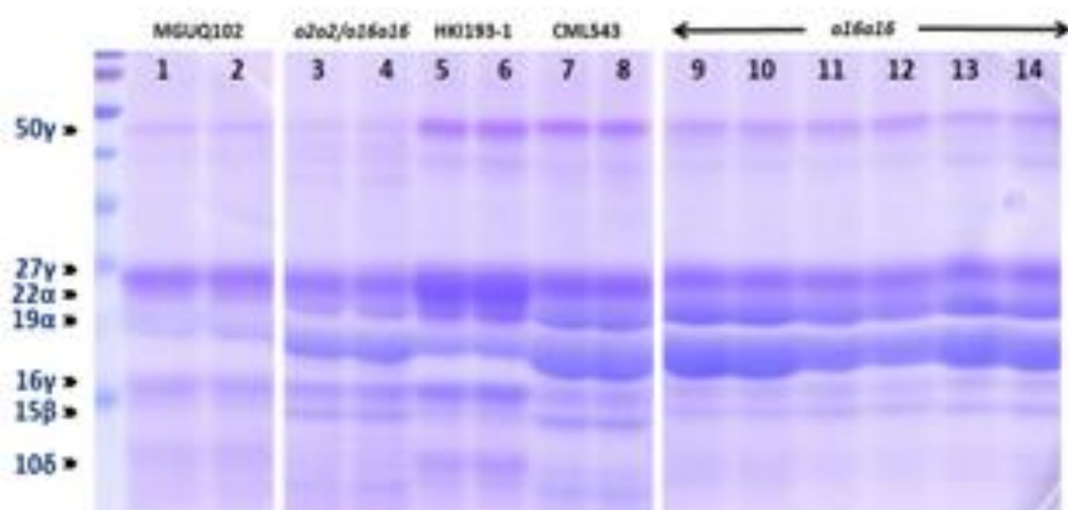
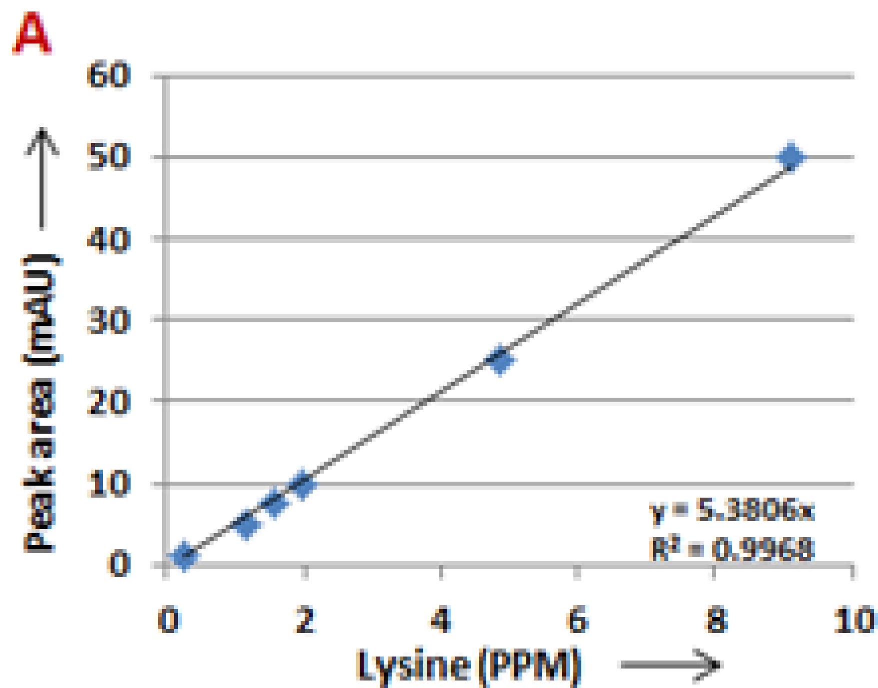


Figure 4.6 SDS-PAGE analysis of endosperm zein proteins in MGUQ-102: a fully opaque *o2o2* line (1 & 2 lane); *o2o2/o16o16* (3 & 4 lane), QPM: HKI193-1 (5 & 6 lane); a normal line: CML543 (7 & 8 lane) and different *o16o16* lines (9-14 lane)



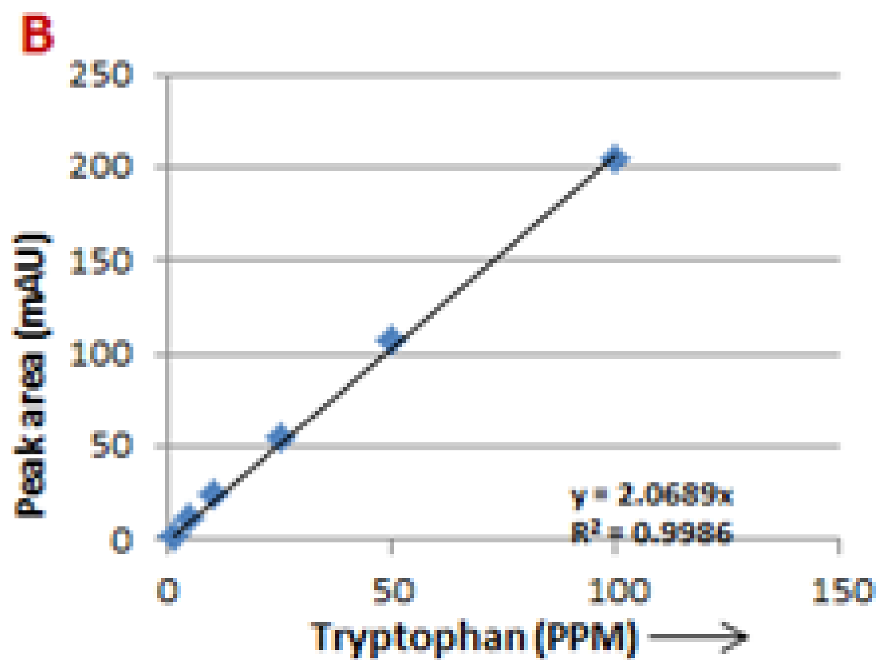


Figure 4.7 Standard curve of A) Lysine B) Tryptophan

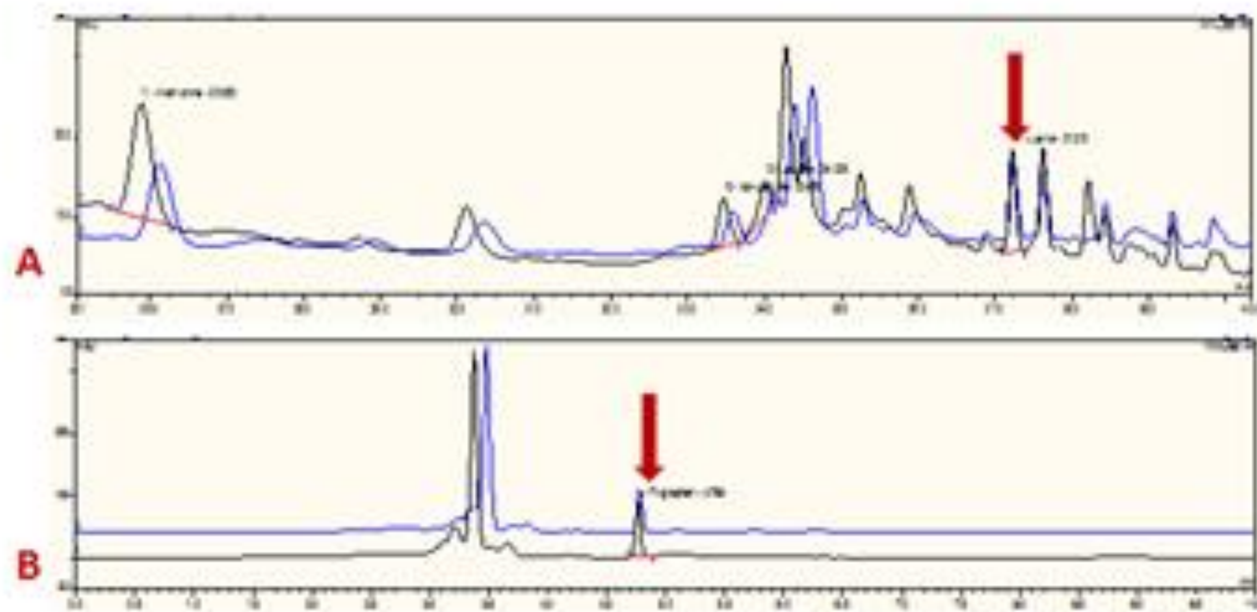


Figure 4.8 UPLC chromatogram showing peak of A) Lysine B) Tryptophan

All the harvested *o16o16* F_3 seeds, twenty nine in CML533 × QCL3024 and twenty one in CML537 × QCL3024 population, as detected by SSR marker *umc1149* in F_2 plants were subjected for lysine and tryptophan analysis using UPLC. Since, UPLC analysis involves high resource, five randomly selected individuals with wild type (*O16O16*) from each of the crosses, CML533 × QCL3024 and CML537 × QCL3024 were taken as control. Further, genotypes with *o2o2*-genetic constitution *viz*., MGUQ-102 and HKI163 (QPM inbred-*o2o2*) along with wild type *O2O2*/*O16O16* genotypes (HM4 and HM8: normal maize) were included as checks in the biochemical analyses. The distribution of the lysine and tryptophan in both F_2 population derived F_3 families, showed multiple peaks ($p < 0.05$) based on the normality Chi square testing (Fig. 4.9). The recessive *o16o16* possessed on an average 0.270% and 0.224% lysine as compared to 0.134% and 0.117% in CML533 × QCL3024 and CML537 × QCL3024 F_3 families, respectively (Table 4.5). The concentration of tryptophan also exhibited the similar pattern with *o16o16* having 0.075% and 0.070%, and dominant homozygotes possessing 0.044% and 0.026% tryptophan, respectively. *o16o16* families identified in the present study showed wide variation (Fig. 4.10) with lysine ranging from 0.111-0.376% and 0.128-0.317%, and tryptophan varying from 0.027-0.117% and 0.032-0.108% against the *O16O16* segregants where lysine varied from 0.097-0.197% and 0.107-0.124%, and tryptophan ranging from 0.034-0.055% and 0.010-0.045%, respectively in CML533 × QCL3024 and CML537 × QCL3024 (Table 4.6). The two wild *O16O16* genotypes, HM4 and HM8 were found to have 0.18% and 0.21% lysine, and 0.02% and 0.04% tryptophan in sample, respectively, whereas *o2o2* genotypes HKI163 (QPM-*o2o2*) and MGUQ-102 (fully opaque-*o2o2*) contained 0.34% and 0.38% lysine, and 0.08% and 0.083% tryptophan in sample, respectively.

The lysine and tryptophan in double mutant (*o2o2/o16o16*) genotypes were analysed among MABB-derived BC_2F_3 seeds in the genetic background of HKI161, HKI193-1, and HKI193-2 and in BC_1F_3 seeds for HKI163. The lysine ranges from 0.176-0.487%, 0.165-0.527%, 0.182-0.561%, 0.102-0.395 %, while tryptophan varied from 0.037-0.139%, 0.051-0.108%, 0.037-0.120%, 0.066-0.136% in sample, respectively among HKI161-, HKI193-1-, and HKI193-2- and HKI163-based MAS derived families. The respective lysine and tryptophan of the recipient parental lines (QPM-*o2o2*) are presented in Table 4.7.

4.8 MABB conversion programme

4.8.1 Parental polymorphism for foreground selection

The recurrent parent, HKI161 is derived from CML161; HKI93-1 and HKI193-1 are derivative of CML193. So for the selection of *o2,* the gene-based marker, *umc1066* was used, while for *o16, umc1141* (for HKI193-1 and HKI193-2) and *umc1149* (HKI161 and HKI163) were used for selecting the targeted loci. The F_1s were tested for their true hybridity based on these markers. After identifying, true F_1s of CML161 × QCL3024 were crossed with HKI161 and HKI163 whereas, true F_1s of cross CML191 × QCL3024 were crossed with HKI193-1 and HKI193-2, as recurrent parents. The segregation ratio for both the loci is presented in the Table 4.8. The *o2* gene-based SSR, *umc1066* segregated in the ratio of 1 (*o2o2*): 1 (*O2o2*) (p-values > 0.05) across all the BC_1F_1 populations, and *o2* mutant was fixed in the following generations by forwarding only *o2o2* progenies. For *o16, umc1141* and *umc1149* in their respective BC_1F_1 populations segregated in the ratio of 1 (*O16O16*) :1 (*O16o16*) (p-values > 0.05) without deviating from the Mendelian segregation (Fig. 4.11). The segregation of *o16* in BC_2F_1 across crosses was also in congruence with the expected Mendelian ratio. In BC_2F_2 populations of HKI161, HKI193-1, and HKI193-2, the segregation ratio *o16* was 1 (*O16O16*): 2 (*O16o16*):1 (*o16o16*) (p-values >0.05). As for HKI163, BC_2F_2 could not be derived due to non-synchronization of flowering time with the recurrent parent in the BC_1F_1 population, instead BC_1F_2 was derived and *o16* was found to be segregating in the same ratio of 1 (*O16O16*) :2 (*O16o16*) :1 (*o16o16*) (p-values >0.05) as per expectation. Therefore, *umc1066* for *o2* and *umc1141* and *umc1149* for *o16* were successfully employed for genotyping backcross generations and classifying the individual plant into different classes for further deriving desirable families having *o2o2/o16o16* genotypes.

4.8.2 Parental polymorphism for background selection

Out of the 350 SSR markers distributed uniformly across the ten chromosomes were screened for parental polymorphism between the parental inbreds and the F_1s (Fig. 4.12) the number of polymorphic markers varied from 138 (39.6%) in HKI193-1 × QCL3024 to 152 (43.5%) in HKI163 × QCL3024 (Table 4.9). The number of polymorphic markers in a chromosome ranged 9 to 23. These markers were used for background selection for recovering the recurrent parental genome (RPG) in the backcrosses populations (Fig. 4.13).

Table 4.5 Lysine and tryptophan content (% in sample) of *o16o16* and *O16O16* genotypes in F_3 seeds of crosses CML533 × QCL3024 and CML537 × QCL3024

CML533 × QCL3024			**CML537 × QCL3024**		
o16o16					
S. No.	**Lysine (%)**	**Tryptophan (%)**	**S. No.**	**Lysine (%)**	**Tryptophan (%)**
1	0.33 ± 0.024	0.12 ± 0.050	1	0.18 ± 0.025	0.04 ± 0.009
2	0.33 ± 0.006	0.09 ± 0.070	2	0.20 ± 0.018	0.05 ± 0.009
3	0.13 ± 0.008	0.10 ± 0.010	3	0.26 ± 0.02	0.06 ± 0.015
4	0.35 ± 0.008	0.12 ± 0.030	4	0.15 ± 0.012	0.08 ± 0.001
5	0.32 ± 0.008	0.10 ± 0.002	5	0.32 ± 0.023	0.08 ± 0.006
6	0.29 ± 0.011	0.10 ± 0.002	6	0.26 ± 0.037	0.10 ± 0.010
7	0.13 ± 0.032	0.12 ± 0.010	7	0.28 ± 0.035	0.07 ± 0.005
8	0.11 ± 0.005	0.10 ± 0.001	8	0.19 ± 0.022	0.09 ± 0.006
9	0.25 ± 0.036	0.10 ± 0.012	9	0.23 ± 0.020	0.04 ± 0.002
10	0.33 ± 0.020	0.11 ± 0.018	10	0.17 ± 0.011	0.06 ± 0.013
11	0.35 ± 0.010	0.06 ± 0.011	11	0.19 ± 0.007	0.06 ± 0.008
12	0.26 ± 0.018	0.05 ± 0.007	12	0.13 ± 0.009	0.08 ± 0.007
13	0.32 ± 0.023	0.06 ± 0.014	13	0.30 ± 0.038	0.09 ± 0.008
14	0.24 ± 0.040	0.05 ± 0.014	14	0.18 ± 0.023	0.10- ± 0.008
15	0.19 ± 0.007	0.03 ± 0.005	15	0.22 ± 0.021	0.10 ± 0.006
16	0.20 ± 0.011	0.04 ± 0.003	16	0.20 ± 0.041	0.11 ± 0.005
17	0.31 ± 0.012	0.05 ± 0.003	17	0.30 ± 0.037	0.06 ± 0.017
18	0.33 ± 0.020	0.07 ± 0.006	18	0.27 ± 0.043	0.04 ± 0.012
19	0.32 ± 0.020	0.06 ± 0.006	19	0.21 ± 0.027	0.07 ± 0.013
20	0.30 ± 0.013	0.10 ± 0.002	20	0.26 ± 0.038	0.09 ± 0.015
21	0.31 ± 0.006	0.08 ± 0.002	21	0.24 ± 0.014	0.03 ± 0.013
22	0.26 ± 0.030	0.06 ± 0.014	Mean	0.22 ± 0.011	0.07 ± 0.005
23	0.33 ± 0.009	0.07 ± 0.021	Range	0.13 – 0.32	0.03 – 0.11
24	0.18 ± 0.024	0.03 ± 0.003			
25	0.29 ± 0.040	0.06 ± 0.005			
26	0.33 ± 0.011	0.10 ± 0.006			
27	0.28 ± 0.042	0.07 ± 0.038			
28	0.38 ± 0.003	0.05 ± 0.004			
29	0.11 ± 0.012	0.05 ± 0.006			
Mean	0.27 ± 0.014	0.08 ± 0.005			
Range	0.11 – 0.38	0.03 – 0.12			
O16O16					
S. No.	**Lysine (%)**	**Tryptophan (%)**	**S. No.**	**Lysine (%)**	**Tryptophan (%)**
1	0.13 ± 0.019	0.05 ± 0.004	1	0.12 ± 0.001	0.02 ± 0.005
2	0.14 ± 0.014	0.05 ± 0.002	2	0.12 ± 0.001	0.05 ± 0.002
3	0.12 ± 0.028	0.04 ± 0.012	3	0.11 ± 0.003	0.01 ± 0.004
4	0.19 ± 0.003	0.03 ± 0.009	4	0.12 ± 0.006	0.02 ± 0.001
5	0.10 ± 0.022	0.06 ± 0.004	5	0.11 ± 0.006	0.04 ± 0.004
Mean	0.13 ± 0.013	0.05 ± 0.004	Mean	0.12 ± 0.003	0.03 ± 0.006
Range	0.10 - 0.20	0.03 - 0.06	Range	0.11–0.12	0.01 - 0.05

Table 4.6 Mean and range of lysine and tryptophan among *o16o16* and *O16O16* genotypes

S. No.	Pedigree	Genotype	No. of samples	% Lysine in sample		% Tryptophan in sample	
				Range	Mean	Range	Mean
1	CML533 × QCL3024	*O2O2/o16o16*	29	0.111-0.376	0.269	0.027-0.117	0.074
		O2O2/O16O16	5	0.097-0.197	0.134	0.034-0.055	0.044
2.	CML537 × QCL3024	*O2O2/o16o16*	21	0.128-0.317	0.223	0.032-0.108	0.069
		O2O2/O16O16	5	0.107-0.124	0.117	0.010-0.045	0.026
3.	HM4 (normal)	*O2O2/O16O16*	-	-	0.181	-	0.023
4.	HM8 (normal)	*O2O2/O16O16*	-	-	0.214	-	0.043
5.	HKI163 (QPM)	*o2o2/O16O16*	-	-	0.342		0.076
6.	MGUQ-102 (*o2o2*-full opaque line)	*o2o2/O16O16*	-	-	0.378	-	0.083

Table 4.7 Lysine and tryptophan content of recurrent parents

S. No.	Recurrent parent	Lysine (% in sample)	Tryptophan (% in sample)
1	HKI161	0.31	0.073
2	HKI193-1	0.37	0.082
3	HKI193-2	0.35	0.074
4	HKI163	0.34	0.076

Table 4.8 Segregation pattern of *o2*and *o16* in different backcross- and selfed- generations of four crosses

Cross	Generation	No. of plants genotyped	*o2o2*	*O2o2*	*O2O2*	χ^2	*P* value	*o16o16*	*O16o16*	*O16O16*	χ^2	*P* value
HKI161 × F_1-A	BC_1F_1	115	55	60	-	0.217	0.641^{ns}	-	51	64	1.469	0.225^{ns}
	BC_2F_1	223	223	-	-	-	-	-	114	109	0.112	0.737^{ns}
	BC_2F_2	168	168	-	-	-	-	39	84	45	0.428	0.807^{ns}
HKI193-1 × F_1-B	BC_1F_1	126	58	68	-	0.426	0.372^{ns}	-	59	67	0.507	0.476^{ns}
	BC_2F_1	235	235	-	-	-	-	-	114	121	0.208	0.647^{ns}
	BC_2F_2	212	212	-	-	-	-	55	98	59	1.358	0.506^{ns}
HKI193-1 × F_1-B	BC_1F_1	129	60	55	-	0.217	0.641^{ns}	-	67	62	0.704	0.401^{ns}
	BC_2F_1	229	229	-	-	-	-	-	113	116	0.039	0.842^{ns}
	BC_2F_2	166	166	-	-	-	-	38	87	41	0.493	0.781^{ns}
HKI163 × F_1-A	BC_1F_1	110	51	59	-	0.581	0.445^{ns}	-	52	58	0.327	0.567^{ns}
	BC_1F_2	235	235	-	-	-	-	61	115	59	0.140	0.932^{ns}

F_1-A (F_1 from cross CML161 × QCL3024) F_1-B (F_1 from cross CML191 × QCL3024);

$P < 0.05$, ns- non-significant

Table 4.9Number of screened SSR markers and the percentage polymorphism observed in the four crosses

Chr.	**No. of markers screened**	**HKI161 × QCL3024**		**HKI193-1 × QCL3024**		**HKI193-2 × QCL3024**		**HKI163 × QCL3024**	
		NP	**Pol. (%)**	**NP**	**Pol. (%)**	**NP**	**Pol. (%)**	**NP**	**Pol. (%)**
1	40	18	45.00	13	32.50	14	35.00	23	57.50
2	35	16	45.71	10	28.57	14	40.00	19	54.29
3	35	13	37.14	14	40.00	17	48.57	17	48.57
4	35	15	42.86	16	45.71	14	40.00	19	57.14
5	35	17	48.57	17	48.57	18	51.43	17	48.57
6	35	13	37.14	18	51.43	12	34.29	13	37.14
7	35	15	42.86	13	37.14	12	34.29	11	31.43
8	35	13	37.14	14	40.00	9	25.71	10	28.57
9	35	13	37.14	13	37.14	14	40.00	10	28.57
10	30	14	46.67	11	36.67	14	46.67	13	43.33
Total	**350**	147	42.02	139	39.77	138	39.60	152	43.51

Chr. - Chromosome; NP - No. of observed polymorphic markers; Pol. (%) - Polymorphism percentage

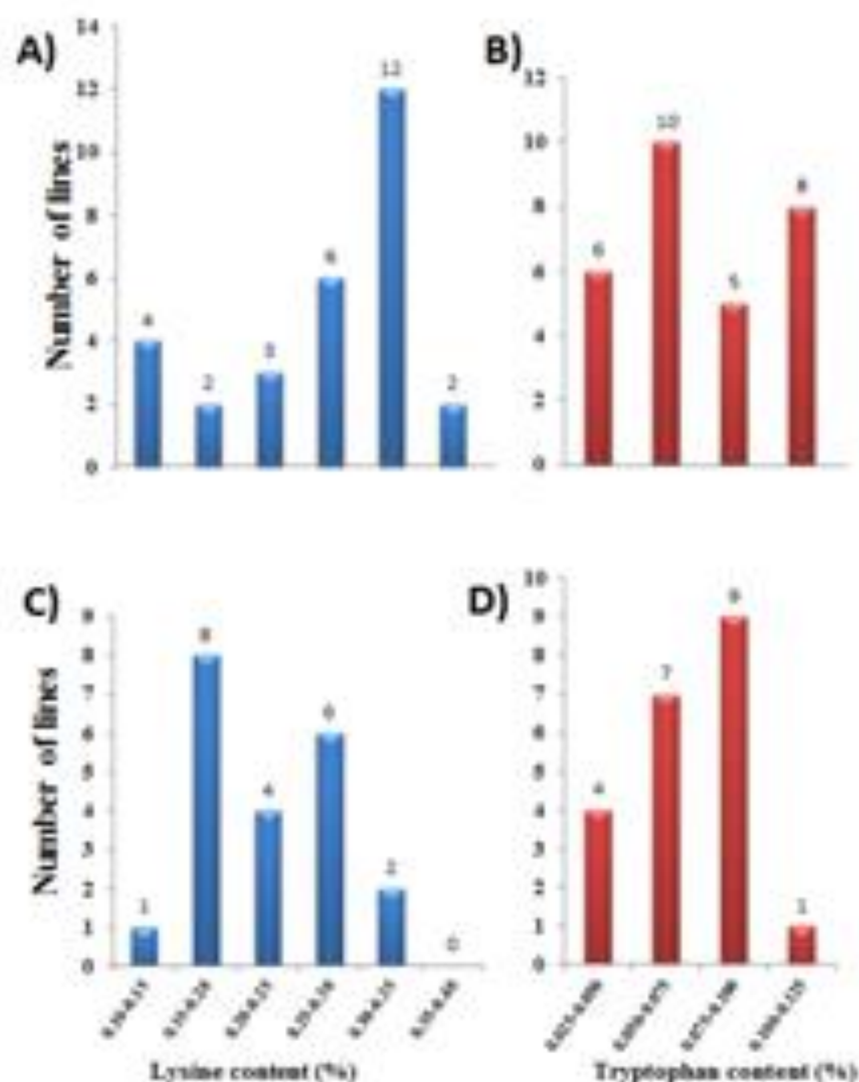


Figure 4.9 Frequency distribution of lysine and tryptophan of CML533 × QCL-3024 (A and B, respectively) and CML537 × QCL-3024 (C and D, respectively) F_3 families showing multiple peaks

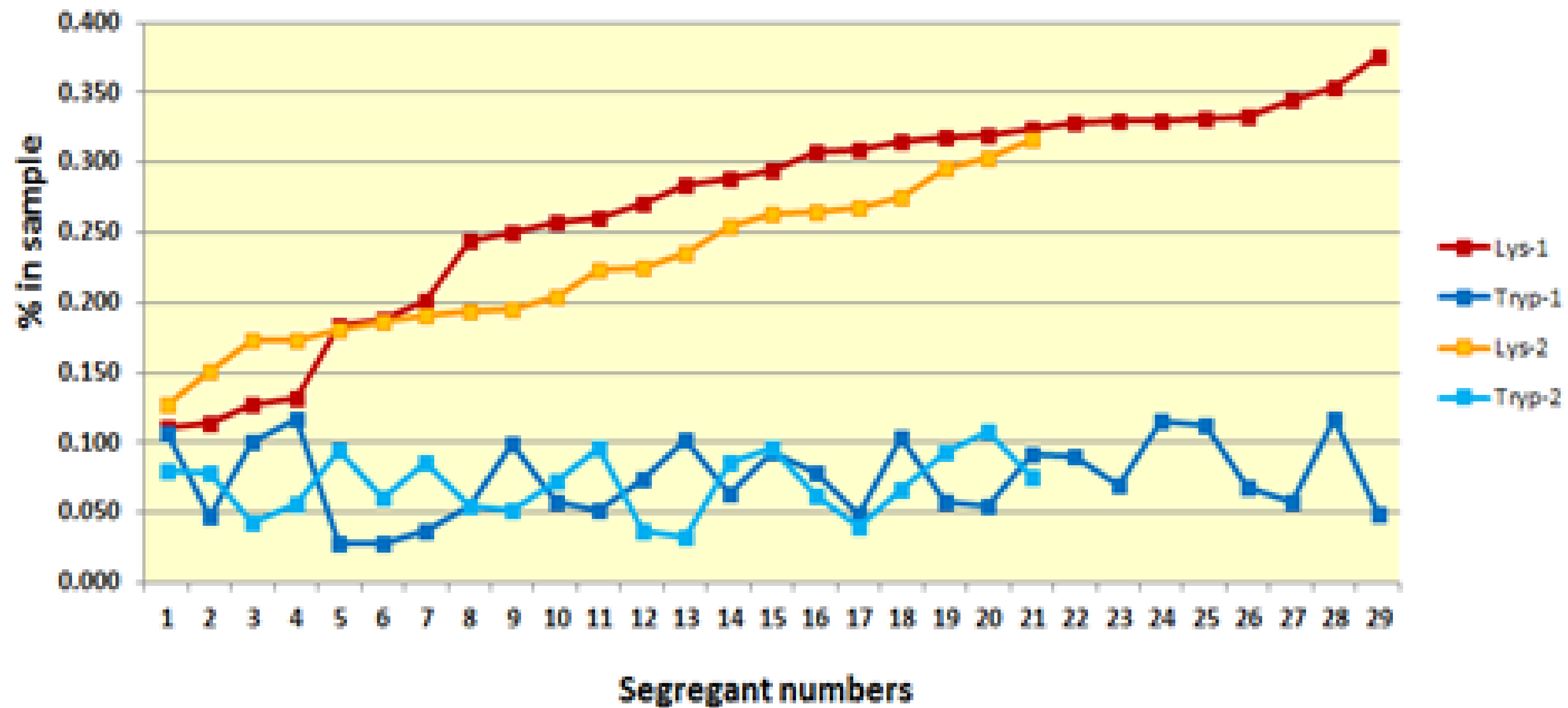


Figure 4.10 Variation in lysine and tryptophan among *o16o16* segregants of two F_2 populations (1: CML533 × QCL3024; 2: CML537 × QCL3024)

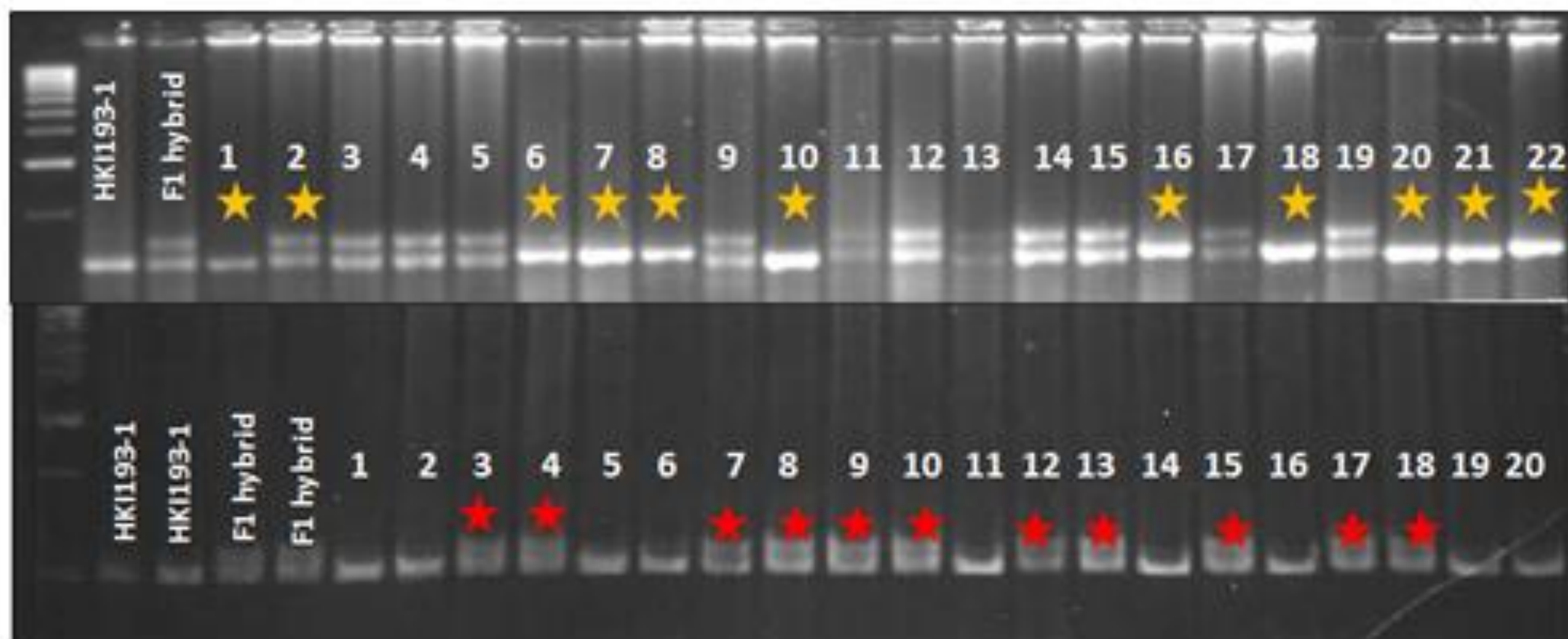


Figure 4.11 Segregation in HKI193-1 BC_1F_1 population of A) *o2-based* marker, *umc1066* B) *o16* linked marker, *umc1141*

★Favourable homozygous genotypes

★Favourable heterozygous *umc1141* genotypes

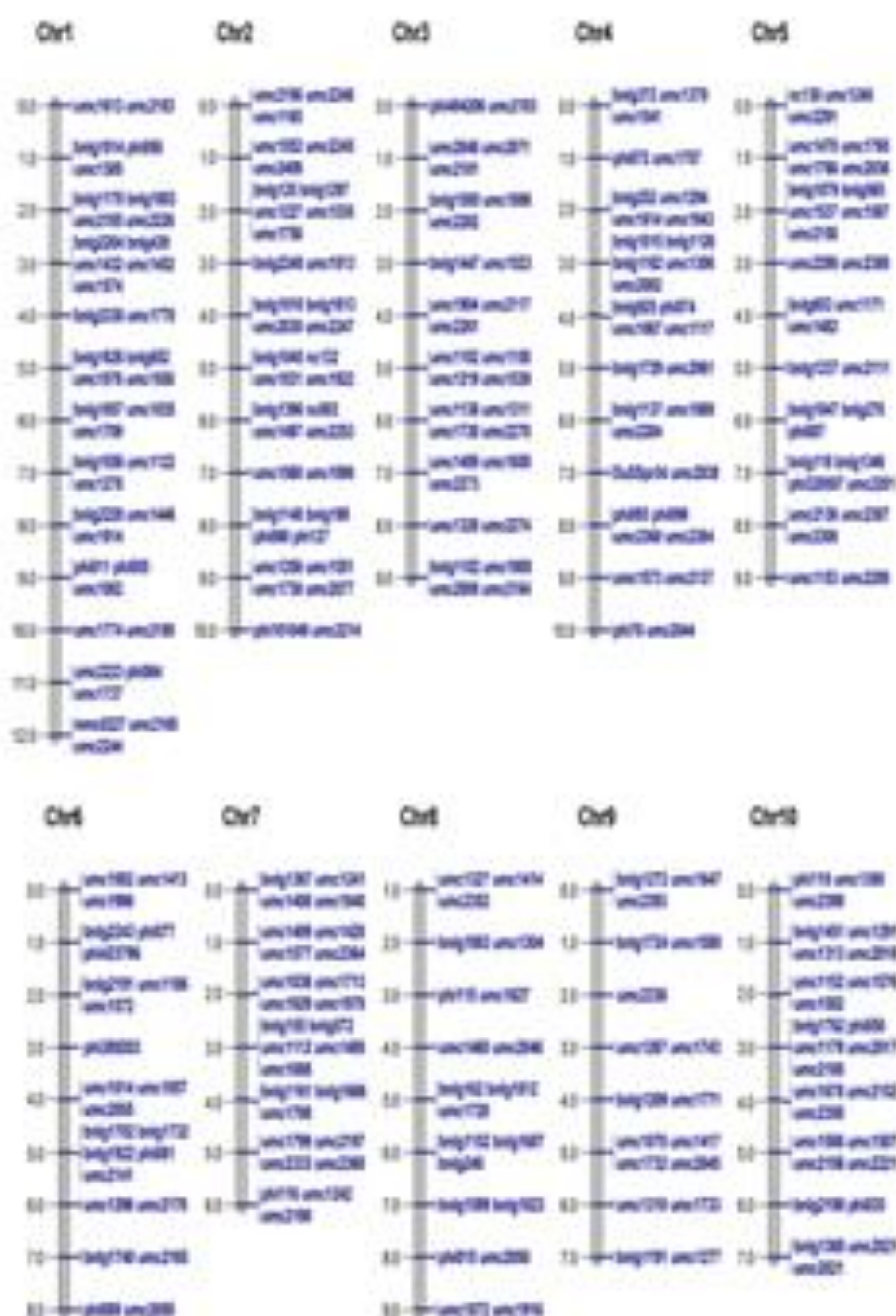


Fig 4.12 Genome-wide SSR markers used for polymorphism survey between the recurrent and donor parents

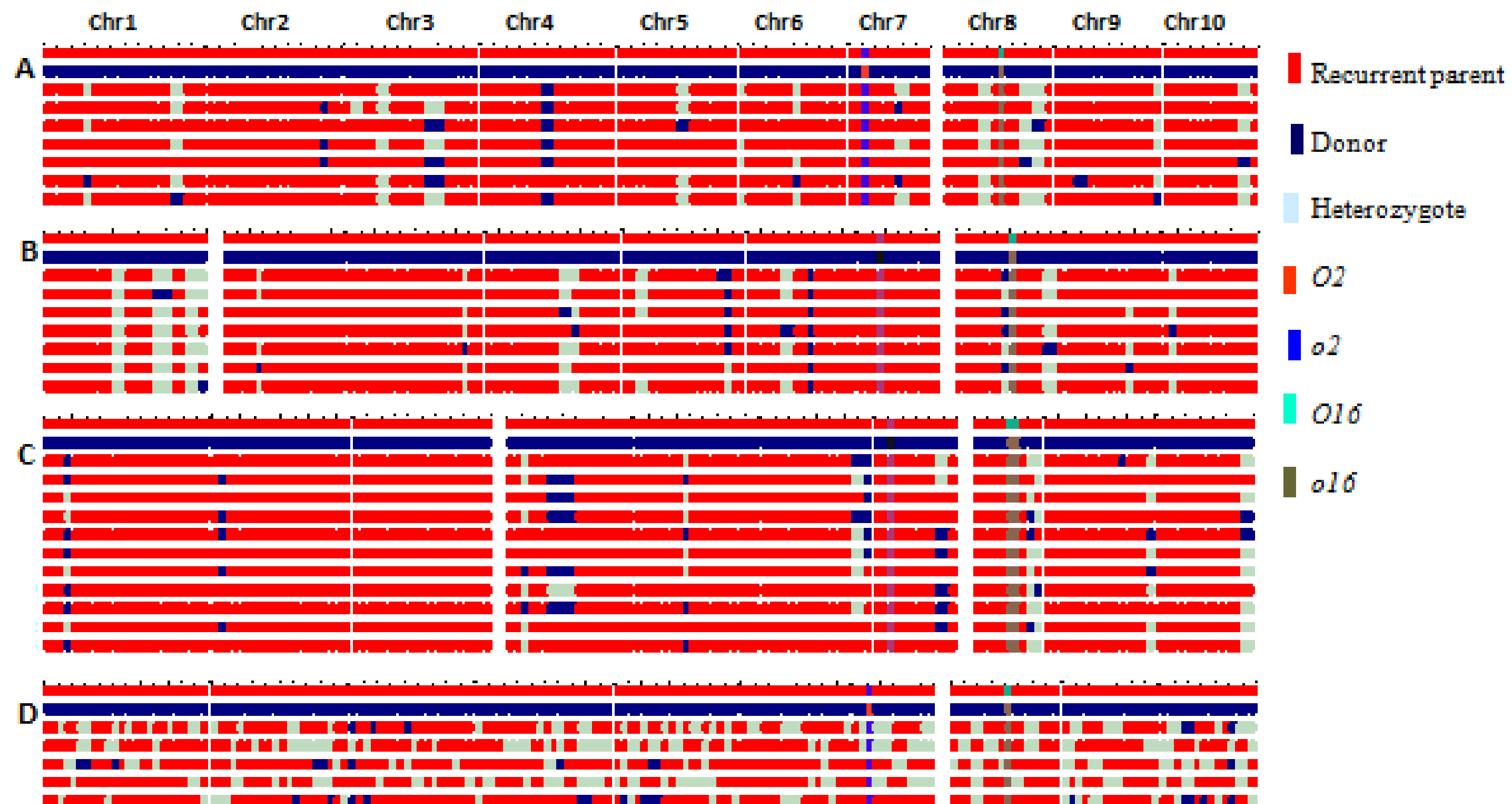


Figure 4.13 Graphical genotype in BC_2F_2 generation of the crosses whose recipient parents (A) HKI161 (B) HKI193-1 (C) HKI193-2 and BC_1F_2 generation of cross whose recipient parent (D) HKI163, indicating the recovery of the recurrent parent genome in the introgressed lines

4.8.3 *Conversion of HKI161*

A total of 115 individual plants from HKI161 based BC_1F_1 population were genotyped using *o2*-gene specific SSR, *umc1066*, and *o16*-linked SSR, *umc1149*. The progenies were segregating in the ratio of 60 (*O2o2*): 55 (*o2o2*), and 51 (*O16o16*): 64 (*O16O16*) for both the loci. Chi-square analysis revealed no significant deviation from the expected Mendelian segregation pattern of 1:1 (Table 4.8). The progenies with genotype *o2o2* and *O16o16* were selected and further subjected for background selection using 147 polymorphic markers distributed throughout the genome (Table 4.9). Subsequently, two individuals with higher RPG recovery of 82% and 75% were selected, and forwarded for advancement of next backcrossed generation.

After backcrossing of BC_1F_1 with the recurrent parent HKI161, we derived a BC_2F_1 population of size 223. There was no segregation of *o2,* since *o2* was already fixed in the BC_1F_1 generation itself; so all the individual progenies were homozygotes for *o2* locus. In the case of *o16* locus, the progenies were segregating in the ratio of 1:1 with 114 heterozygotes and 109 dominant homozygotes. Based on the morphological similarity of plant phenotypes, 15 plants of genotypes *o2o2*/*O16o16* were subjected for further background analysis with the polymorphic markers which were found to be heterozygote in the previous generation of BC_1F_1. Two plants with similar cob- and seed- characteristics with the recurrent parent, and with highest RPG recovery of 91.6% and 90.6% were selected, and used to generate BC_2F_2 population.

A total of 168 plants were raised in the BC_2F_2 population and foreground selection was carried out for *o16* loci. Of these 168 plants, 39 plants were observed to be homozygotes for *o16*. The segregation pattern with 39 (*o16o16*): 84 (*O16o16*): 45 (*O16O16*) did not show any deviation from the expected Mendelian ratio of 1:2:1. Out of 39 *o2o2*/*o16o16* plants, 16 individual segregants with similar morphological phenotype of HKI161 were subjected for background selection. The RPG recovery ranged from 93.9 to 95.3% (Table 4.10). The harvested BC_2F_3 seeds were subjected to UPLC analysis for lysine and tryptophan content and screening for plant phenotypes, ear characteristics and yield parameters. Three best families, HKI161-14-56-22-77, HKI161-14-56-22-129, and HKI161-14-56-22-130 were forwarded for generating converted lines with RPG recovery of 93.9%, 94.3% and 94.1% respectively (Table 4.10).

4.8.4 *Conversion of HKI193-1*

Population size of 126 in HKI193-1-based BC_1F_1 generation was screened using *umc1066* and *umc1141* for the targeted loci *o2* and *o16*, respectively. 58 plant homozygote for *o2* and 51 heterozygotes for *o16* based on the foreground selection were identified. Chi-square analysis revealed no deviation of segregation pattern from the expected 1:1 ratio (Table 4.8). Based on the morphological phenotypic similarity with HKI193-1, 9 plants were subjected for background selection with 139 polymorphic markers distributed across the 10 chromosomes. Based on the similarity in the characteristics of -cob and -seeds with recurrent parent along with higher RPG recovery, two plants having recovery of 89% and 87% RPG (Table 4.10) were backcrossed to generate BC_2F_1 populations. Ten plants that were homozygous for *o2* and heterozygous for *o16* in BC_2F_1 generation were subjected for background selection and 2 plants with RPG of 89% and 87% and with similar phenotypic characteristic of -cob and -seeds were further selfed to generate BC_2F_2. There was no deviation in the segregation pattern of any loci in any of these generations (Table 4.8).

Based on the similarity in the plant morphology, 17 segregants positive for both *o2* and *o16* in BC_2F_2 were further subjected for background selection. Biochemical analysis for lysine and tryptophan were performed in 10 harvested BC_2F_3 families. Phenotypic selection of -seeds and -cobs were performed. Two BC_2F_3 families (HKI193-1-14-14-15-17 and HKI193-1-14-14-15-22) with higher lysine and tryptophan with 93% and 94% RPG with similar phenotypic characteristics were forwarded for advancement.

4.8.5 *Conversion of HKI193-2*

A size of 129 individual plants in HKI193-2-based BC_1F_1 population was genotyped for *o2* and *o16* with gene-based SSR, *umc1066* and linked SSR *umc1141*, respectively. There was no deviation in the segregation pattern from 1:1 for both the loci (Table 4.8). A segregation of 60 (*o2o2*): 55 (*O2o2*), and 67 (*O16o16*): 62

Table 4.10 Recurrent parent genome (RPG) recovery % of the improved lines

Cross	Generation	Genotype	% RPG recovery	Range of RPG %
HKI161 × QCL3024	BC_1F_1	HKI161-14-56	81.9	70.5 - 81.9
	BC_2F_1	HKI161-14-56-22	91.6	81.2 - 91.6
	BC_2F_2	HKI161-14-56-22-77	93.9	93.9 - 95.3
		HKI161-14-56-22-129	94.3	
		HKI161-14-56-22-130	94.1	
HKI193-1 × QCL3024	BC_1F_1	HKI193-1-14-14	78.1	68.7 - 78.1
	BC_2F_1	HKI193-1-14-14-15	88.8	82.4 - 88.8
	BC_2F_2	HKI193-1-14-14-15-17	93.2	92.4 - 94.2
		HKI193-1-14-14-15-22	94.1	
HKI193-2 × QCL3024	BC_1F_1	HKI193-2-14-29	81.2	68.5 - 81.2
	BC_2F_1	HKI193-2-14-29-51	88.4	88.4 - 91.6
	BC_2F_2	HKI193-2-14-29-51-21	93.2	92.8 - 95.7
		HKI193-2-14-29-51-36	95.1	
		HKI193-2-14-29-51-25	94.2	
HKI163 × QCL3024	BC_1F_1	HKI163-14-54	73.5	73.4 – 77.2
		HKI163-14-19	76.2	
	BC_1F_2	HKI163-14-54-34-4	81.5	81.3 - 85.8
		HKI163-14-19-12-1	85.3	
		HKI163-14-54-56-2	84.3	

(*O16O16*) were observed. Base on the similarity of plant morphology, 14 plants homozygote for *o2* and heterozygote for *o16* were subjected for background selection with 138 polymorphic markers distributing uniformly all across the 10 chromosomes (Table 4.9). Phenotypic selection on the seeds and cob was carried out. Two lines with highest recovery of 81% each with phenotypic similarity with recurrent parent, HKI193-2 were forwarded and backcrossed to obtained BC_2F_1 generation. Eleven plants that were homozygous for *o2* and heterozygous for *o16* in BC_2F_1 generation were subjected for background selection after phenotypic screening of plants, and 2 plants with RPG of 92% and 88% with similar phenotypic characteristic of -cob and -seeds were further selfed to generate BC_2F_2. Any deviation in the segregation pattern from the expected ratio was not observed in any loci in these generations.

Based on the phenotypic similarity with recurrent parent, 21 segregants positive for both *o2* and *o16* in BC_2F_2 were further subjected for background selection. Three BC_2F_3 families, HKI193-2-14-29-51-21, HKI193-2-14-29-51-36, and HKI193-2-14-29-51-25, with high lysine and tryptophan with RPG recovery of 93.2%, 95.1% and 94.2%, respectively having similar phenotypic characteristics were advanced for further generation of advanced inbreds (Table 4.10).

4.8.6 *Conversion of HKI163*

A total of 110 individual plants in BC_1F_1 population of HKI163 were genotyped using *o2*-gene specific SSR, *umc1066* and *o16*-linked marker, *umc1149.* Chi-square analysis revealed no significant deviation from the expected Mendelian segregation pattern of 1:1 with a segregation of 59 (*O2o2*): 51 (*o2o2*), and 52 (*O16o16*): 58 (*O16O16*) for both the targeted *o2* and *o16* loci, respectively (Table 4.8). The progenies with genotype *o2o2* and *O16o16* were selected and further subjected for background selection with the identified 147 polymorphic markers (Table 4.9). Subsequently, two individuals with higher RPG recovery of 76.2% and 73.5% were selected and forwarded for advancement of next backcrossed generation.

We could not derive the BC_2F_1 population due to non-synchronization of flowering time between silk and tassel of HKI163 and BC_1F_1 selected progenies. Instead the selected individuals were selfed to obtain BC_1F_2 generation. Since the *o2* was fixed already in the BC_1F_1 generation, all the segregants were *o2o2* homozygotes. The locus was segregating with a ratio of 1:2:1 in the case of *o16* locus. The

segregating pattern did not deviate from the expected mendelian ratio. Based on the morphological similarity of plant phenotypes, 13 plants of genotypes *o2o2/o16o16* from BC_1F_2 generation were subjected for further background analysis with the polymorphic markers which were found to be heterozygote in the previous generation of BC_1F_1. The recovery of RPG ranged from 81.3 to 85.8% (Table 4.10). Eleven harvested BC_1F_3 families seeds were subjected for lysine and tryptophan analysis. Three BC_1F_3 families with similar cob and seed characteristics with the recurrent parent were selected and forwarded for next generation of selfing. HKI163-14-19-12-1 showed highest RPG recovery among the selected progenies with 85.3%.

4.8.7 *Nutritive value of o2o2/o16o16 converted lines*

The lysine and tryptophan analysis among the MABB-derived progenies of HKI161, HKI193-1, HKI193-2 and HKI163 showed a significant increase in both lysine and tryptophan over their respective recurrent parents (Fig 4.14, 4.15 & Table 4.11). The lysine in the BC_2F_3 seeds in HKI161-based MABB progenies ranged from 0.17 to 0.49% (in flour), while tryptophan varied from 0.05 to 0.13% (in flour). Among the selected progenies of HKI161 having desirable morphological characteristics and high RPG, the increase was as high as 57.37% in lysine and 91.17% in tryptophan over the recurrent parent HKI161 in the introgressed line HKI161-14-56-22-130. In HKI193-1, the lysine and tryptophan ranged from 0.16 to 0.53% and 0.05 to 0.11%, respectively. Among the forwarded progenies of HKI193-1, the increase was as high as 75.85% in lysine (HKI193-1-14-14-15-22) and 32.37% in tryptophan (HKI193-1-14-14-15-17) over the recurrent parent. Biochemical analysis for lysine and tryptophan among the BC_2F_3 families of HKI193-2 revealed that lysine ranged from 0.18 to 0.56% and tryptophan from 0.06 to 0.12%. Three BC_2F_3 families with lysine content 0.52%, 0.56% and 0.45% with tryptophan content of 0.12%, 0.10% and 0.11%, respectively, were forwarded for advancing the inbred lines. Among the three lines, HKI193-2-14-29-51-36 showed highest increased in lysine over the parent HKI193-2 and HKI193-2-14-29-51-21 showed highest increase in tryptophan. In the BC_1F_3 lines of HKI163, the increase of lysine was as high as 58.82% in introgressed line HKI163-14-19-12-1, while the same was 37.52% for tryptophan over the *o2o2* recurrent parent, HKI163.

Table 4.11 Percent increase in lysine and tryptophan in the selected*o16o16*introgressedQPM inbred lines over their respective *o2o2* recurrent parents

Pedigree	**Lysine (%)**	**% lysine increased over recurrent parent**	**Tryptophan (%)**	**% tryptophan increased over recurrent parent**
HKI161	0.31	-	0.07	-
Range	0.17-0.49	-	0.05-0.13	-
HKI161-14-56-22-77	0.43	38.23	0.14	91.17
HKI161-14-56-22-129	0.43	40.22	0.13	67.33
HKI161-14-56-22-130	0.49	57.37	0.10	39.66
HKI193-1	0.30	-	0.08	-
Range	0.16-0.53	-	0.05-0.11	-
HKI193-1-14-14-15-17	0.53	75.24	0.11	32.37
HKI193-1-14-14-15-22	0.53	75.85	0.09	9.27
HKI193-2	0.35	-	0.06	-
Range	0.18-0.56	-	0.06-0.12	-
HKI193-2-14-29-51-21	0.52	48.81	0.12	87.39
HKI193-2-14-29-51-36	0.56	60.26	0.10	57.11
HKI193-2-14-29-51-25	0.45	29.18	0.11	70.25
HKI163	0.34	-	0.08	-
Range	0.15-0.40	-	0.06-0.14	-
HKI163-14-54-34-4	0.41	20.59	0.12	50.23
HKI163-14-19-12-1	0.54	58.82	0.11	37.52
HKI163-14-54-56-2	0.49	44.12	0.11	37.73

Table 4.12 Plant phenotypes and other parameter attributing to yield in the *o16o16*introgressed QPM inbreed lines with their respective *o2o2* recurrent parents

Genotypes	**Plant height (cm)**	**Ear height (cm)**	**Days to 50% tassel**	**Days to 50% silk**	**Cob length (cm)**	**Cob width (cm)**	**No. of rows**	**No. of kernels/row**	**100SW (g)**
HKI161-14-56-22-77	159.0	60.7	73	74	9.3	4.3	12.7	18.7	26.5
HKI161-14-56-22-129	161.0	65.0	71	74	10.1	3.7	13.3	19.0	23.1
HKI161-14-56-22-130	159.0	64.3	74	75	10.3	3.7	13.3	18.3	26.0
HKI161	154.7	65.0	72	74	10.0	3.8	14.0	19.0	25.3
HKI193-1-14-14-15-17	152.0	54.3	76	78	9.8	3.0	12.0	18.7	17.3
HKI193-1-14-14-15-22	157.3	58.3	74	76	11.1	3.2	12.0	20.0	18.7
HKI193-1	152.7	53.0	75	76	10.4	3.0	12.0	19.0	18.5
HKI193-2-14-29-51-21	142.0	68.7	74	76	12.7	3.8	12.0	23.7	14.9
HKI193-2-14-29-51-36	142.3	68.0	75	78	11.3	3.3	12.7	17.3	17.4
HKI193-2-14-29-51-25	139.0	77.3	75	77	11.8	3.2	12.7	21.7	16.6
HKI193-2	143.3	68.7	75	77	10.9	3.1	12.0	21.0	18.0
HKI163-14-54-34-4	146.3	71.7	76	78	11.9	3.2	12.0	18.7	18.2
HKI163-14-19-12-1	150.7	74.0	78	78	11.3	3.4	12.7	22.0	20.0
HKI163-14-54-56-2	143.7	68.3	77	79	12.3	3.1	12.0	17.3	17.9
HKI163	149.3	70.3	77	78	10.7	3.2	12.0	19.7	24.6
CD (5%)	3.96	3.82	1.05	0.94	0.54	0.21	0.35	0.99	2.25

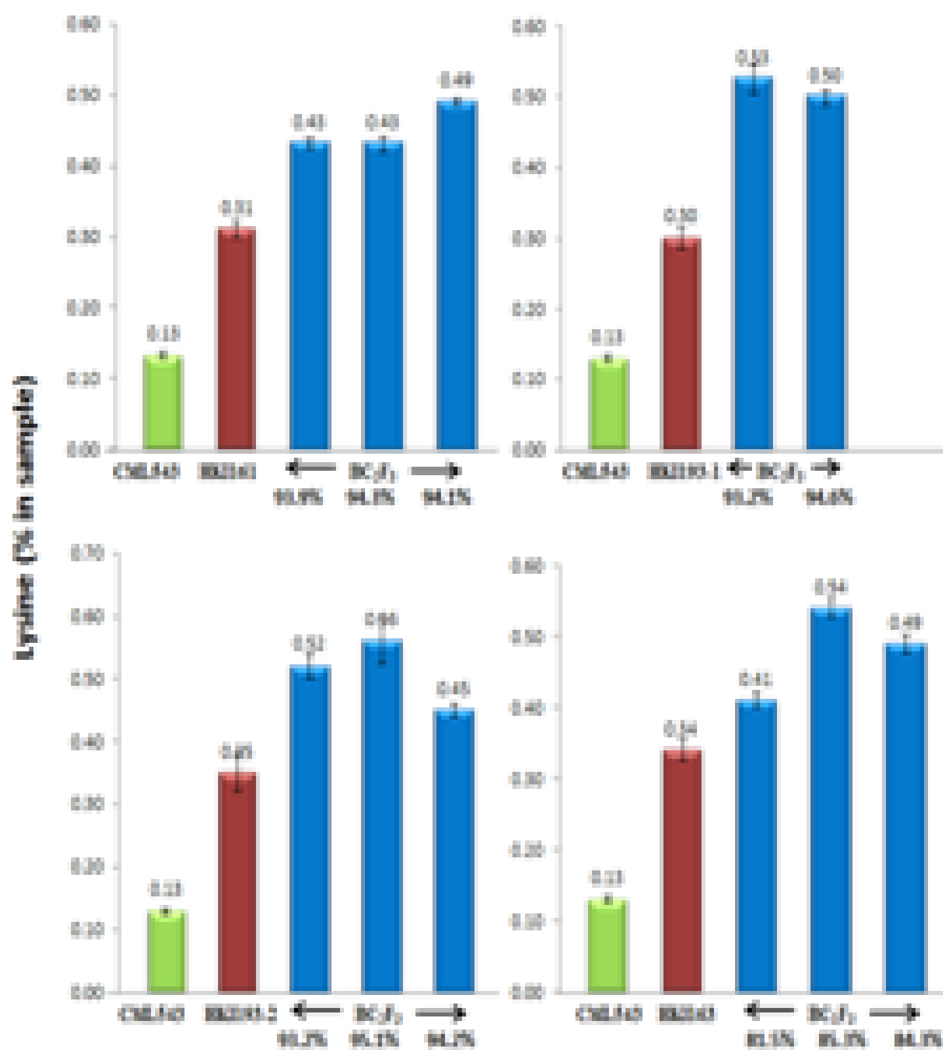


Figure 4.14 Lysine content of the selected *o2o2/o16o16* introgressed families for generation of advanced inbred lines

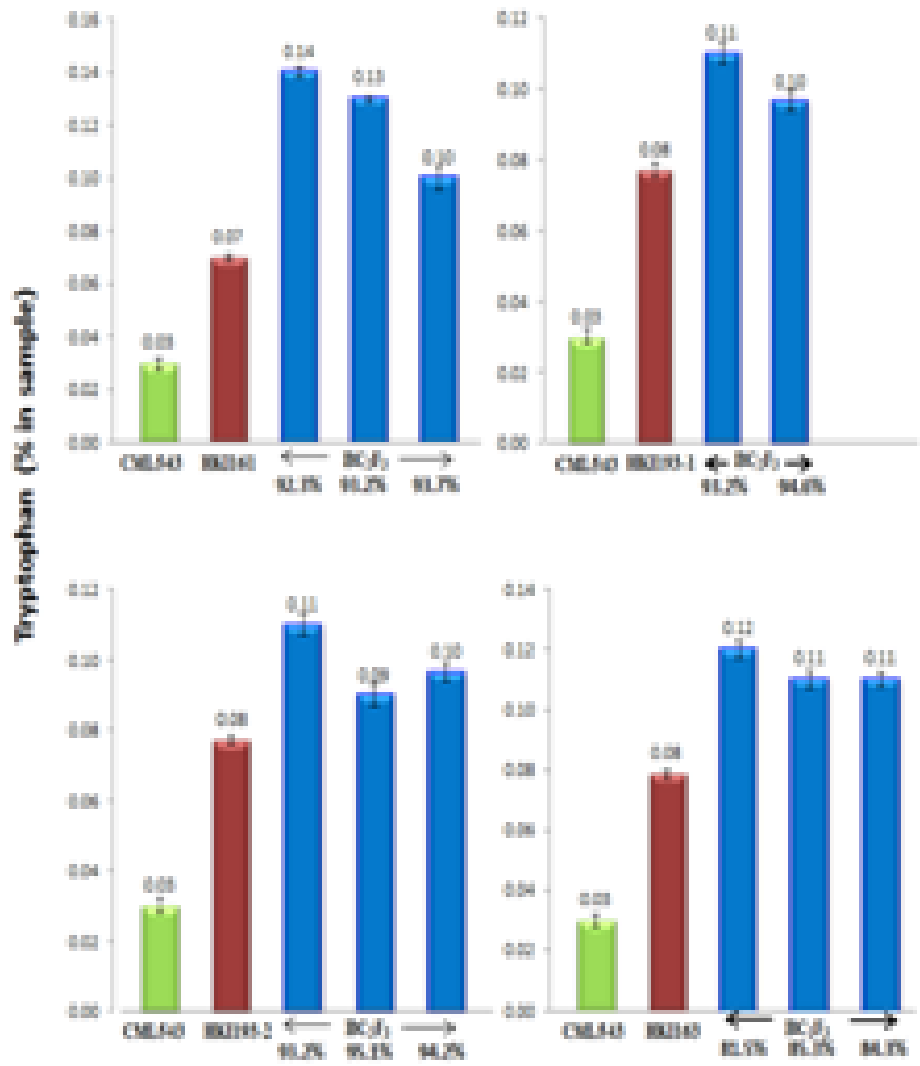


Figure 4.15 Tryptophan content of the selected *o2o2/o16o16* introgressed families for generation of advanced inbred lines

4.8.8 *Morphological phenotypes of derived o2o2/o16o16 lines*

For every backcrossed and selfed generation in all the crosses, we carried out stringent screening of plant phenotypes, seeds and cobs characteristics and selected the lines which had got the maximum similarity with the recurrent parents. The plant phenotypic parameters of forwarded introgressed lines and the other parameters attributing to yield are presented in the Table 4.12. The forwarded lines with favourable allele of *o2* and *o16* in all the derived introgressed lines were well within the critical difference. Among the three lines of HKI161 based introgressed lines, HKI161-14-56-22-129 showed the maximum similarity with the recurrent parent in terms of plant characteristics (Fig. 4.16). The opaqueness of the three forwarded introgressed lines was about 30 to 40 % which was similar with the recurrent parent HKI161 seeds whose opaqueness was about 25 to 40% (Fig 4.17). In the case of HKI193-1 based introgressed lines, both the lines showed similarity but HKI193-1-14-14-15-17 was showing maximum similarity in the plant and cobs characteristics and did not show significant deviation from their respective recurrent parent. The endosperm modification of the recurrent parent was 40 to 50% and the opacity of the derived lines was 35 to 40% (Fig. 4.18). The ratio of the soft and hard region of the grain was comparable between the derived lines and the recurrent parent. In the HKI193-2 based introgressed lines, HKI193-2-14-29-51-21 showed highest degree of resemblance with the recurrent parent HKI193-2 with respect to plant and cob characteristics. Among the targeted four parental inbreds, HKI193-2 showed highest modification with almost 95% of opacity. This opacity could also be observed in the derived *o16* introgressed lines with 90 to 100% (Fig. 4.19). In HKI163 based derived lines, even though the lines were backcrossed only once, the genome could be recovered as high as 85% and so the phenotypic characteristics of plants, and cob parameters could be observed to be similar with that the recurrent parent HKI163. HKI163 showed the least opacity among the four parental inbreds with 20 to 35%. The derived *o16* lines showed an opacity of 30 to 35% which was similar to the recurrent parent HKI163 (Fig. 4.20). So we successfully could derive *o16* introgressed lines with improved protein quality lines with similar morphological, and cob and seed characteristics with their respective recurrent parents.

Figure 4.16 Resemblance for plant characteristics of the HKI161-based introgressed lines with the recurrent parent HKI161

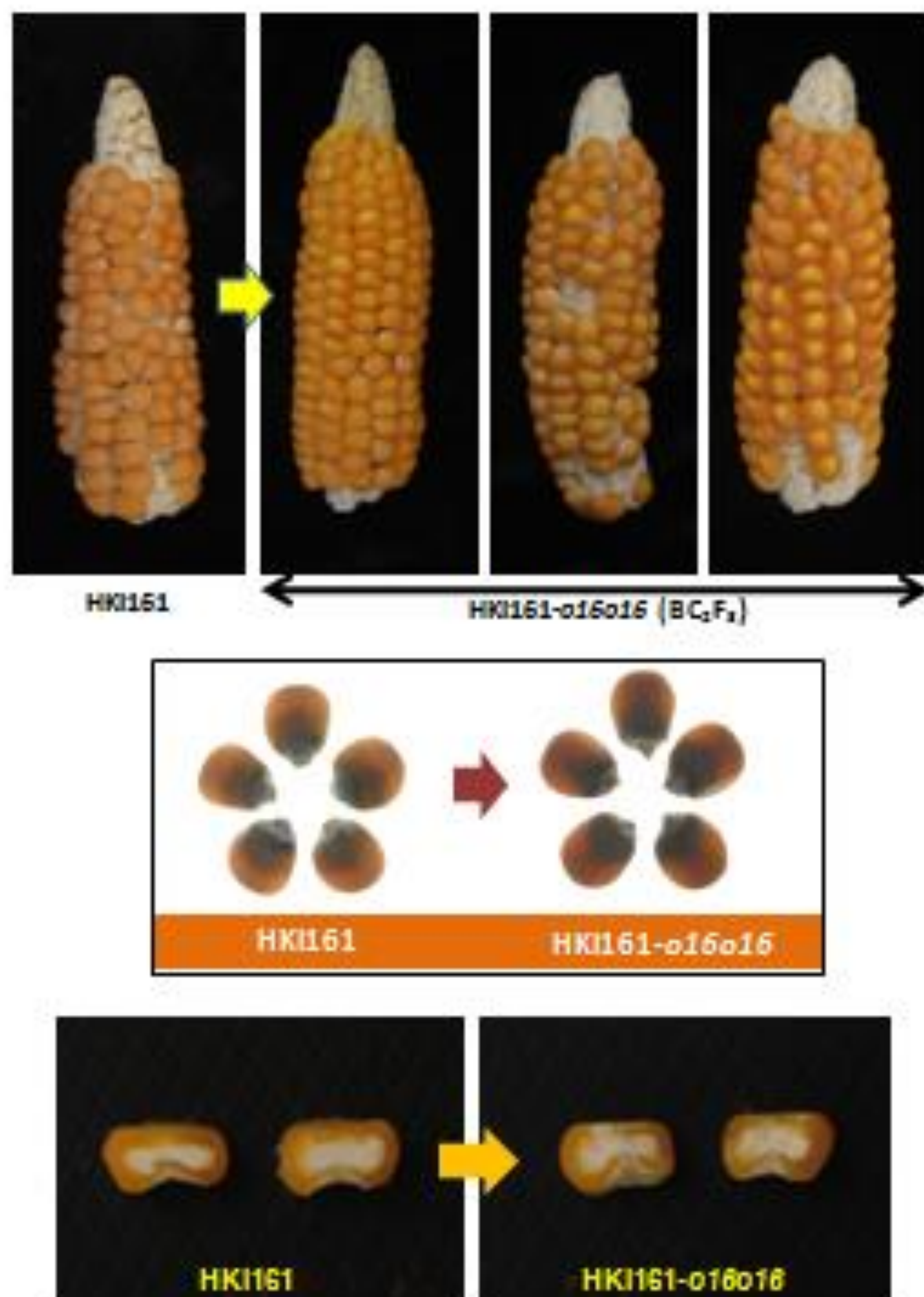


Figure 4.13 Comparison of ear and grain characteristics of recurrent parent HKI161 with *o2o2*/*o16o16* introgressed lines

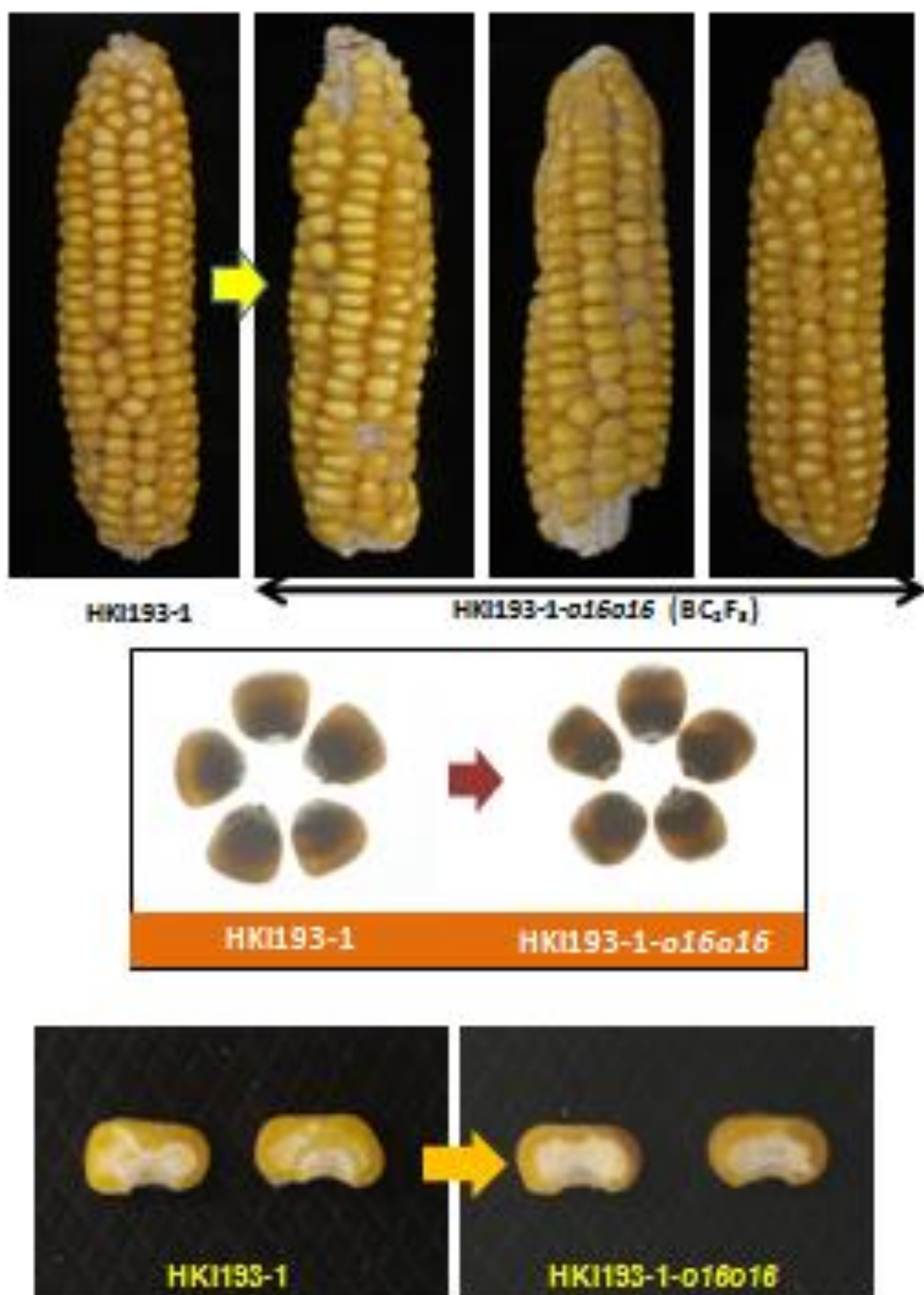


Figure 4.18 Comparison of ear and grain characteristics of recurrent parent HKI193-1 with *o2o2*/*o16o16* introgressed lines

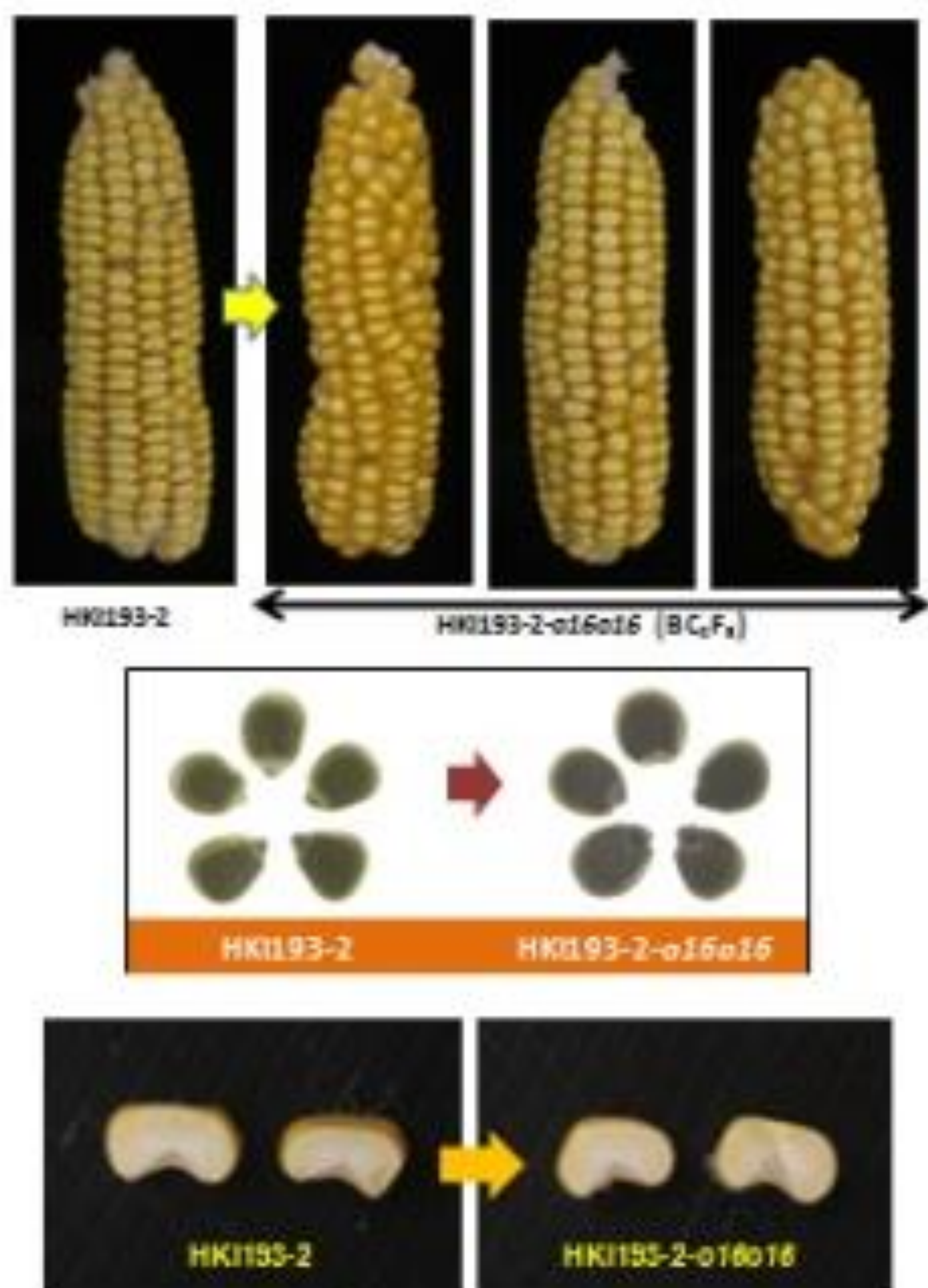


Figure 4.19 Comparison of ear and grain characteristics of recurrent parent HKI193-2 with *o2o2/o16o16* introgressed lines

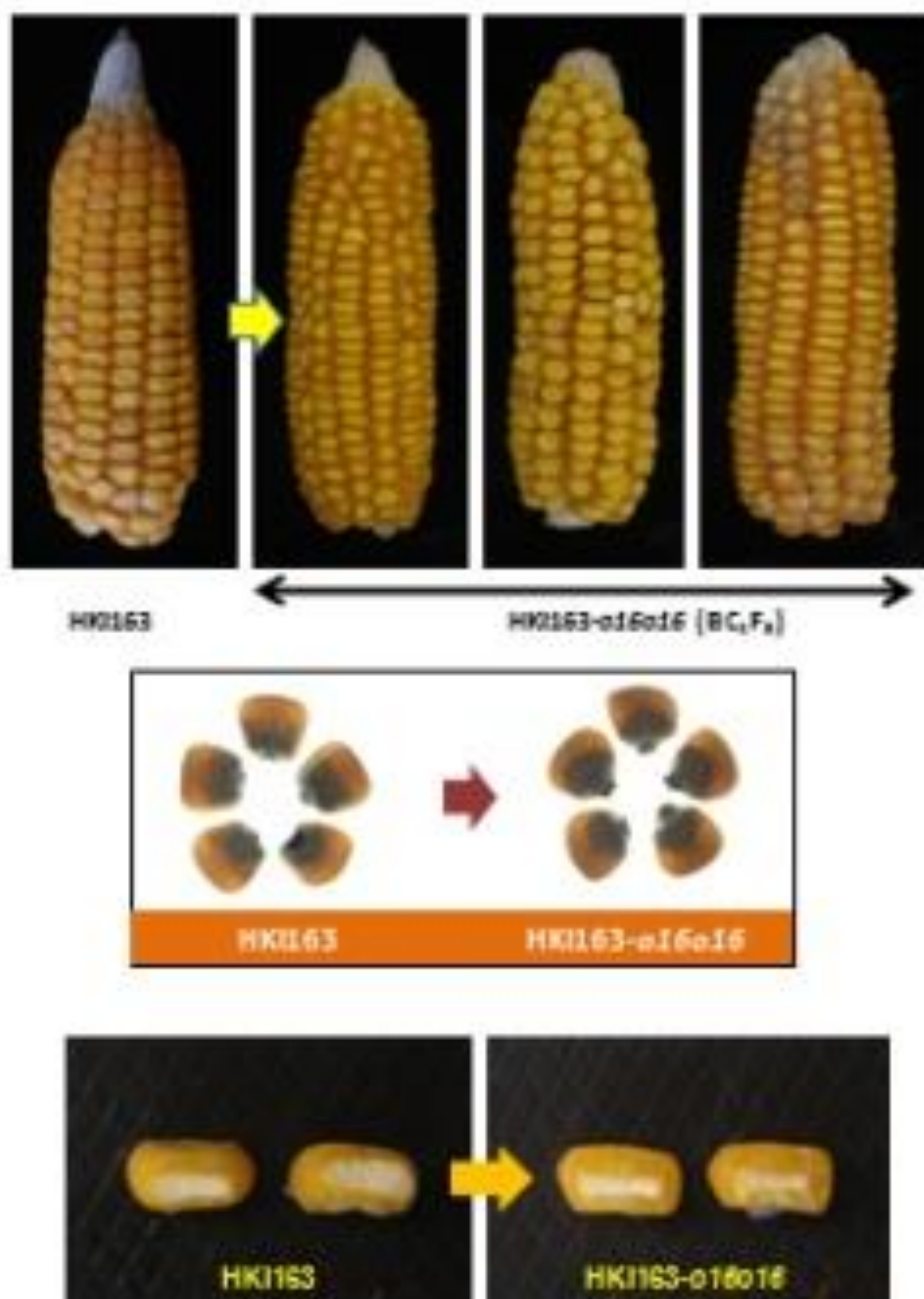


Figure 4.20 Comparison of ear and grain characteristics of recurrent parent HKI163 with *o2o2/o16o16* introgressed lines

Discussion

5 Discussion

Maize endosperm protein is of poor nutritional value due to reduced concentration of essential amino acids lysine and tryptophan (Gupta et al. 2013). Monogastric animals such as poultry birds, pigs including humans cannot synthesize lysine and tryptophan in their body, thus are required to be provided through diet (Prasanna et al. 2001). The discovery of nutritional benefits of recessive *o2* located on chromosome 7 was a significant breakthrough, as it makes the grain proteins nearly twice as nutritious as in normal maize endosperm (Mertz et al. 1964). The *o2* coupled with 'endosperm modifiers' led to the birth of 'Quality Protein Maize' (QPM) (Vasal et al. 1980). An array of QPM cultivars with diverse adaptation to various agro-climatic conditions has been developed and commercialized worldwide (Yadav et al., 2015). Though various mutants viz., *o1*, *o5*, *o9-11*, *o13*, *o17*, *fl1*, *fl2*, *fl3*, *Mc* and *Def-B30* have been discovered and experimentally tried singly or in combinations, but could not be successfully deployed due to negative pleiotropic effects (Salamini et al. 1983; Huangs et al. 2004; Gibbon and Larkin 2005). Recently, Yang et al. (2005) reported that a novel recessive *opaque16* (*o16*) mutant located on chromosome 8, increased lysine by 30% when present with *o2* compared to *o2o2* or *o16o16* alone (Zhang et al. 2010; 2013). Dr. Wenpeng Yang at Guizhou Institute of Upland Food Crops, Guizhou Academy of Agricultural Sciences, China has reported the utilization of *o16* in the breeding programme. So far, *o16* has not been utilized elsewhere including the Indian maize breeding programme. In the present study an effort was directed to understand the influence of *o16* on endosperm texture and nutritional attributes in germplasm adapted to Indian conditions, and improve elite QPM inbreds for nutritional quality.

5.1 *Analyzing the effects of o16 through F_2 population*

5.1.1 *Segregation of o16 in various populations*

Among the three reported *o2* gene-based SSR markers viz., *phi112*, *phi057* and *umc1066* (Lin et al. 1997), *umc1066*, and *o16*-linked SSRs viz., *umc1141* and *umc1149* segregated co-dominantly in all four F_2 populations without any deviation from 1:2:1 Mendelian segregation (Yang et al. 2005; Muthusamy et al. 2014). Gupta et al (2013) also reported *umc1066* to be inherited co-dominantly and used it for selecting individual plants positive for *o2* allele in the breeding programme. Yang et al. (2013) and Zhang et al. (2013) used *umc1141* for selecting the individuals possessing *o16* allele. Thus,

umc1066, *umc1141,* and *umc1149* were successfully implemented in genotyping the F_2 populations, and classifying the individual plants into different genotypic classes across crosses.

5.1.2 Influence of o16 on endosperm modification

Phenotypic screening of the individual seed endosperm opacity under light box is the most convenient and efficient strategy for studying the endosperm modification (Prasanna et al. 2001; Hossain et al. 2008a, b; Gupta et al. 2013). In the present investigation, selected F_2 seeds of CML161 × QCL3024 and CML193 × QCL3024, segregating for *o2* and *o16* showed significant degree of endosperm opaqueness. While F_2 populations (CML533 × QCL3024 and CML537 × QCL3024) where *o16* segregating alone did not exhibit significant opaqueness. This suggests that *o16* did not influence endosperm modification as opposed to *o2* which played major role in effecting various degree of endosperm opaqueness (Morton et al. 2015). The opacity in F_2 population of CML161 × QCL3024 and CML191 × QCL3024 were ~25% (Table 2), and it is expected if *o2* alone is affecting the modification and segregating in the ratio of 3:1 (translucent/normal : opaque). The varied degree of opaqueness observed in *o2o2* genetic background is due to the accumulation of different endosperm modifier loci present in the genetic background of individual plants (Lopes and Larkins 1995). The *o2*-based endosperm modifiers are polygenic in heritance with complex phenotypic effects (Ufaz and Galili 2008) in the endosperm with reportedly governed by several QTLs (Holding et al. 2011; Pandey et al. 2015). This observation was further confirmed through F_3 seed analyses. The extent of opaqueness in F_2-derived F_3 seeds of *o2o2/o16o16* was comparable to *o2o2/O16O16*, while *O2O2/o16o16* resembling *O2O2/O16O16.* It is therefore, established from the current investigation that *o16* alone does not increase opaqueness in *o2o2/o16o16* but solely due to influence by the presence of *o2* allele in the genetic background.

Grain hardness corresponds to resistance towards infestation of the storage pests and fungal infection, and it also determines the kernel density required for post-harvest processing (Hossain et al. 2007a; Siwale et al. 2009; Garcia-Lara & Bergvinson 2014; Zunjare et al. 2014). In the investigation, *O2O2/o16o16* possessed hard endosperm similar to *O2O2/O16O16* (corresponding wild type of the populations as well as normal check, CML543) genotypes in all the four crosses. The results thus clearly demonstrated that *o16* alone does not induce softness in the endosperm. However, the

degree of softness in *o2* genetic background is determined by the presence of modifier loci. In case of *o2o2/o16o16* and *o2o2/O16O16*, the grains were almost entirely soft, since not much favourable modifiers were present in the genetic background. But grains of QPM having *o2* allele were much harder due to the presence of favourable modifier loci (Vasal et al. 1980; Hossain et al. 2008 a, b; Pandey et al. 2015). The *o16* therefore, did not have any negative impact on the endosperm hardness unlike *o2* which generally inflicts softness in the kernel. This was also evident from the proportion of hard- (orange or yellow translucent portion) and soft- (white portion) endosperm in the grains of *o2o2*-soft, QPM, normal (*O2O2*) and *o16o16* genotypes.

Microscopic organization of the starch granules and protein bodies showed starch granules in *o16o16* are angular polygonal shape with proteinaceous matrix surrounding them which was characterized by tightly packed structure with no air space and showed similarity with wild line, CML543. During desiccation of seeds, rough endosplasmic reticulum (ER) membranes break down exposing the zein protein mixing with the other content of the cytoplasm. It acts as cementing glue thereby providing an airtight interaction with starch granules in normal vitreous seed endosperm (Wu et al. 2010 a, b). Thus this also explained the hardness of *o16o16* grains being similar to the wild check and corresponding wild type of the same population. But significant reduction in the proteinaceous matrix adhering to the starch granules induced loose packaging with relatively large intergranular space in *o2o2* which led to the opacity of endosperm. The opacity is due to the diffraction of light caused by the air spaces left due to loose packaging of protein and starch granules in the endosperm (Wu et al. 2010a). In HKI193-1, though the starch granules were spherical and smooth, a relatively more proteinaceous matrix adhering to the starch granules was observed which revealed a tighter interaction with less air space. QPM seeds were thus more vitreous and hard as compare to *o2* line due to accumulation of *o2* modifiers in the genetic background (Wu et al. 2010b). The structure of *o2o2/o16o16* was intermediate between *o2o2* and *o16o16,* having semi-polygonal starch granules and proteinaceous matrix being present but less packed compared to *o1616*. The compact protein bodies and its interaction with starch granules through amorphous, non-crystalline amylopectin molecules at the surface links starch granules together, and makes the packaging more compact and grain appearance as vitreous (Wu et al. 2010b; Gibbon et al. 2003). The compact packaging of starch and protein bodies in *o16o16* thus conferred vitreous

kernels, while the air space left due to weak interaction made the kernels of *o2o2* and *o2o2/o16o16* as soft and opaque.

SDS-PAGE was used to compare qualitatively and to some extent quantitatively as well for prolamin fraction in the lines (Hunter et al. 2002). Since the *o2* mutant reduces the synthesis of 22-kDa α-zein by coding a less active transcriptional factor, thus *o2o2* in absence of favourable endosperm modifier loci (viz., MGUQ-102) produces less 22-kDa and in turn develops soft and opaque endosperm (Lending and Larkins 1989). We could also observe nearly two-fold increase in the expression of 16-, 27- and 50-kDa γ-zein in modified-*o2o2* (QPM: HKI193-1) compared to fully opaque *o2o2* line. Enhanced synthesis of γ-zein has been identified as the major factor in endosperm modification (Wu et al. 2010 b). Several studies demonstrated a positive relationship between the content of 27-kDa γ- zein and endosperm vitreousness (Geetha et al. 1991; Lopes and Larkins 1995). Segal et al. (2003) induced a full opaque kernel phenotype by silencing the 22-kDa α- zeins by RNAi, while the overproduction of 27-kDa γ-zein enhanced protein body number resulting with more vitreous phenotype in QPM (Moro et al. 1995). The disulfide bonds of cystein residues in γ-zein helps in extensive cross-linking and covalent linkage between protein bodies could provide a mechanism for cementing protein bodies around starch grains (Lopes and Larkins 1991).

The findings here thus established beyond doubt that the mechanism of higher synthesis of lysine and tryptophan in *o16* mutant is entirely different from the *o2*. *O2* located on chromosome 7 codes for a DNA binding protein belonging to basic leucine zipper class of transcriptional factors, and acts as transcriptional activator of 19- and 22-kDa α-zein genes (Hartings et al 1989; Schmidt et al. 1992). The mutant *o2*-based protein induces an overall reduction of 50-70% in zein protein which increases non-zein proteins proportionally, resulting in twice the lysine content than in normal maize (Mertz et al. 1964). Since, zein profiles *o16o16* differs considerably from *o2o2*, the mechanism of enhanced nutritional value needs further investigation. It is worth to mention here that among various high lysine mutants discovered; only *o2*, *fl2* and *Def-B30* affect different aspects of storage protein synthesis and alter zein content and compositions (Morton et al. 2016). The other mutants such as *o5, o15*, *fl1*, *Mc* do not induce significant changes in zein content and composition. The *o15* mutation exerts its effect primarily on the 27-kDa γ-zeins (Dannenhoffer et al. 1995). The *fl1* mutation is

rather resulted due to abnormal placement of α-zeins within the protein bodies. *Fl1* encodes a transmembrane protein that is located in the protein body ER membrane (Holding et al. 2007). Similarly, *o5* mutant phenotype is caused by a reduction in the galactolipid content of the maize endosperm, with no change in zein proteins (Myers et al. 2011). The increase in lysine and tryptophan in *o16* thus warrants further investigation.

5.1.3 Influence of o16 on accumulation of lysine and tryptophan

The recessive *o16o16* possessed almost two-fold increase in both lysine and tryptophan as against the corresponding wild type line of the respective population. Yang et al. (2005) reported similar degree of increase only for lysine with 0.206% in *O16O16* and 0.447% in *o16o16* genotypes. Further, Yang et al. (2013) introgressed the *o16* allele into two waxy inbreds and reported a lower degree of increase (16-28%) in lysine. Thus, the earlier reports of enhancement of lysine by *o16* in Chinese genetic background are also validated in these two new populations. In addition, it is also established here that *o16* also plays important role in higher accumulation of tryptophan as well in maize endosperm. The lysine and tryptophan content showed a wide variation among *o16o16*. It was thus amply clear that though recessive *o16* plays major role in enhancement of lysine and tryptophan, the amino acid modifier genes in the background could be an important factor in deciding the final nutritive value. Yang et al (2005) reported transgressive segregation of lysine in F_2; and suggested that accumulation of lysine is also governed by modifier loci. It is worth mentioning here, that though *o2* causes significant increase of lysine and tryptophan, it showed wide variation in different genetic background (Vivek et al. 2008), suggesting the importance of favourable amino acid modifier in *o2o2* genetic background for realizing maximum gain (Pandey et al. 2015). Among the two populations, *o16o16* segregants of CML533 × QCL3024 in general possessed higher lysine and tryptophan than the same from CML537 × QCL3024. This is possibly because CML533 contributed more favourable modifier loci (than CML537) that alone or in interaction with modifiers from QCL3024 caused higher accumulation of lysine and tryptophan.

The increase in the lysine accumulation in some of the families was comparable with the *o2o2* genotypes but we did not observe a significant correlation between these two amino acids. In contrast, tryptophan and lysine possess strong correlation (r = 0.99) in *o2* genetic background, with concentration of lysine being four times that of

tryptophan (Vivek et al. 2008). Though *o16o16* possesses higher average lysine and tryptophan than wild type, the simultaneous enhancement of similar degree may not be always achieved. Since lysine estimation is cumbersome, while tryptophan can be easily estimated through colorimetric method, selection of QPM genotypes (possessing *o2o2*) in early segregating generations is undertaken based on tryptophan only. However, for *o16o16*, it warrants estimation of both lysine and tryptophan in order to select individuals high in both the amino acids. It is worth mentioning here, that the average levels of lysine and tryptophan in *o16o16* was less than the *o2o2*, but were much higher than the wild type (*O16O16*). Thus *o16* is a major locus that can enhance lysine and tryptophan in normal maize by almost two fold, and can be as good as *o2* once favourable modifier loci interact with *o16* in the genetic background. These segregants with high lysine and tryptophan could serve as novel genetic resource in the breeding programme, as these newly-derived lines would further diversify QPM genetic base which has traditionally used *o2* for the nutritional enhancement.

5.2 *Marker-assisted improvement of QPM inbreds for nutritional quality*

5.2.1 Marker-assisted foreground and background selection

In the present investigation, MABB was undertaken to enhance the lysine and tryptophan content of maize endosperm in four elite QPM inbred parental lines (HKI161, HKI193-1, HKI193-2 and HKI163) which are the parents for commercial four QPM hybrids viz. HQPM-1, HQPM-4, HQPM-5 and HQPM-7 with wide adaptability all over the country. The *o2* gene-based marker viz., *umc1066* and *o16* linked markers, viz., *umc1141* and *umc1149* could be successfully used for pyramiding both the mutants (Gupta et al. 2013; Yang et al. 2013; Zhang et al. 2013). Marker-assisted background selection could be successfully used for recovery of recurrent parent genome (RPG) which ranged from 81 to 85% for HKI163-based lines to 93.2 to 95.1% across the remaining populations (HKI161-, HKI193- and HKI193-2 based). The lesser recovery in HKI163 based lines (~83%) is due to one backcross as compared to two backcrosses attempted in other three crosses (Muthusamy et al 2014).

5.2.2 Effects of introgressed inbreds for accumulation of lysine and tryptophan

The lysine and tryptophan content on an average across the introgressed lines of the four QPM inbreds was 0.48% and 0.11%, respectively. The enhancement of concentration of both the lysine and tryptophan was as high as 0.56% and 0.14%,

respectively. Yang et al. (2005) and Zhang et al. (2013) reported synergistic effects of both *o2* and *o16* on accumulation of more lysine in *o2o2/o16o16* than *o2o2* alone. Thus, the present investigation led to the initial understanding of how the *o16* is affecting the endosperm. The novel high lysine and tryptophan mutant, *o16* possessed no adverse effect on the endosperm modification when present alone. The recessive *o16* alone improves the nutritional quality of maize, can be utilized as effectively as *o2* (Sarika et al. 2016). The novel *o16* mutant thus holds significant promise in quality breeding programme. MABB has led to the accelerated development of elite inbreds with enhanced lysine and tryptophan concentration. These newly developed inbreds hold significant promise to provide a balanced amino acid food source to the poor section of society and improved feed as well.

5.2.3 Effects of introgressed inbreds on phenotypic characteristics

The backcross derived progenies with high recovery of RPG showed high degree of resemblance with their corresponding recurrent parent with respect to plant architecture and ear characteristics (Gupta et al. 2013; Choudhary et al. 2014). This high degree of phenotypic similarity among these introgressed inbreds (with their respective recurrent parents) is attributed to the high recovery of RPG achieved through genome-based SSR markers used in the background selection (Gupta et al. 2013; Muthsamy et al. 2014; Choudhary et al. 2014). The present investigation thus, demonstrated the successful conversion of lines by MABB programme. Further, the degree of modification among the selected introgressed inbreds was *at par* with their respective recurrent parents. This was possible as *o16* alone does not influence the degree of opaqueness (Sarika et al. 2016), and favourable modifier loci could be accumulated due to stringent background selection followed by selection of kernels through light box test (Hossain et al 2008 a, b; Pandey et al. 2015).

Conventional breeding for enhancement of lysine and tryptophan is expensive, laborious and time consuming due to large scale phenotyping of the segregating population and accumulation of the modifier loci which is of very complex inheritance. MABB has been successfully utilised for the improvement of parental lines of normal maize hybrids into quality protein maize (QPM) through the introgression of *o2* (Babu et al. 2005; Danson et al. 2006; Gupta et al. 2013) and *o16* (Yang et al. 2005, 2013; Zhang et al. 2010, 2013).

Summary and Conclusion

6 Summary and Conclusion

Deficient in the limiting essential amino acids like lysine and tryptophan is one the most important nutritional deficiencies prevailing in maize. Development of high lysine and tryptophan maize therefore, holds great significance for nutritional security. So far, the recessive *o2* has been traditionally used for the enrichment of lysine and tryptophan in QPM genotypes. Though several mutations have been discovered during the last few decades, none could be successfully utilized, primarily due to negative pleotropic effects. A recently discovered recessive *o16* mutant holds a significant promise in further enhancement of nutritional quality of maize. Thus investigating and understanding the effect of this novel mutant on the grain quality and endosperm attributes holds an important place in successfully deploying the newly discovered mutant, *o16.* In the present study, the effect of *o16* was analyzed in various segregating generations, and the salient findings of the investigation are as follows:

- Enhancement of both lysine and tryptophan was recorded in *o16o16* segregants, with almost double the concentration as compared to *O16O16* segregants. Lysine in CML533 × QCL3024 increased from 0.134% (*O16O16*) to 0.270% (*o16o16*), while tryptophan was enhanced from 0.044% (*O16O16*) to 0.075% (*o16o16*). In CML537 × QCL3024, the enhancement for the same was from 0.117 to 0.224% and 0.026 to 0.070% for lysine and tryptophan, respectively.
- Some of the *o16o16* segregants (0.376% lysine and 0.117% tryptophan) were *at par* with *o2o2*-based inbreds, viz. MGUQ-102 (0.378% lysine and 0.083% tryptophan) and HKI163 (0.342% lysine and 0.076% tryptophan).
- Among *o16o16* segregants, reasonably wider range for lysine (0.111 to 0.376%) and tryptophan (0.027 to 0.117%) was observed across CML533 × QCL3024 and CML537 × QCL3024. This is possibly due to amino acid modifiers present in the genetic background. Similar observation in *o2*-genetic background is well established.
- The newly developed *o16o16*-based progenies developed here would serve as a valuable genetic resource in the QPM breeding programme. This is the first report on effect of *o16* on accumulation of tryptophan in maize endosperm.
- The analysis also revealed that *o16* possessed no adverse effect on the endosperm modification when present alone. Since the *o16o16* genotypes possessed vitreous

endosperm with similar grain hardness to normal line, the mutant provides tremendous advantage to the breeders as accumulation of endosperm modifiers in the genetic background need not to be looked into while breeding for high lysine and tryptophan, which otherwise is mandatory for *o2*-based breeding.

- The recessive *o16* did not alter the microscopic organisation of starch granules and protein bodies or matrix as depicted by SEM, and thus it formed a compact tight packaging explaining the hard vitreous endosperm of *o16o16* lines.
- The mechanism of *o16* on nutritional improvement seemed to be completely different from the *o2*. Synthesis of zein fractions among *o16o16* was not affected when investigated through protein profiling in SDS-PAGE.
- The recessive *o16* has been successfully pyramided with *o2* through MABB approach. The effect of *o2* and *o16* has been synergistic in accumulating lysine and tryptophan. The maximum increase among introgressed lines was to the tune of 57.37% (lysine) and 91.17% (tryptophan) over HKI161. In the case of HKI193-1 based introgressed line, 75.24% (lysine) and 32.35% (tryptophan) increase over the recurrent parent could be obtained. Similar trend was also observed among introgressed lines of HKI193-2 (60.26% and 87.39% increase for lysine and tryptophan over recurrent parent, respectively) and HKI163 (58.82% and 50.23% increase for lysine and tryptophan over recurrent parent, respectively).
- The degree of opaqueness in *o2o2/o16o16* depended on the *o2*-based endosperm modifiers only, as *o16o16* alone did not cause opaqueness. Several QTLs for these modifiers have been identified, and with the availability of SSRs associated with *o2*, *o16* and QTLs linked to modifier loci, marker-assisted selection would provide great opportunity to develop high lysine and tryptophan maize with hard endosperm.
- Thus, *o16* mutant alone or in combination with *o2* thus provides ample opportunity to enhance the nutritional quality of maize grains. This is the first ever study on effects of *o16* in relation to tryptophan concentration and endosperm modification. The information generated and the improved elite inbreds developed here, hold great significance in maize biofortification programme.

Abstract

Abstract

The improvement of protein quality in maize so far has been based on recessive *opaque2* (*o2*) mutant that along with endosperm-modifiers led to development of quality protein maize (QPM) having higher lysine and tryptophan. Many other mutants having potential to increase lysine and tryptophan in endosperm though discovered, could not be successfully deployed due to various negative pleiotropic effects. The recent discovery of nutritional benefits of recessive *opaque16* (*o16*) mutant was of immense significance for the improvement of protein quality in maize. In the current study, the influence of *o16* was investigated using four F_2 populations segregating for *o2* and/or *o16* alleles. The recessive *o16* enhanced the lysine and tryptophan by almost two-fold (0.246% lysine and 0.072% tryptophan) as against the corresponding *O16* (0.126% lysine and 0.035% tryptophan) with some segregants *at par* with QPM check (*o2o2*). The *o16o16* alone did not cause opaque phenotype in endosperm, and its grain hardness was comparable with *O16O16* segregants, normal maize as well as QPM. The pattern of microscopic arrangement of starch granules and protein matrix in *o16o16* was similar to hard endosperm maize, but distinct from the *o2o2*-soft genotype. The zein profiling of the storage protein and γ-zein in particular among *o16o16* also resembled the normal and QPM genotypes, but completely different from *o2o2*-soft genotype. The pattern of packaging of starch granules with protein matrix among *o2o2/o16o16* was unique, and zein fractions were different from both *o2o2* and *o16o16*. We demonstrated here, that *o16* did not possess any influence on grain endosperm modification, and had a completely different mechanism of increasing lysine and tryptophan from *o2*. Marker-assisted introgression of *o16* into *o2o*2-based parental inbreds (HKI161, HKI193-1, HKI193-2 and HKI163) of four commercial Indian QPM hybrids (HQPM-1, HQPM-4, HQPM-5 and HQPM-7) led to the enhancement of lysine and tryptophan to a level of as high as 75% and 91%, respectively over the respective recurrent parents. The selected introgressed inbreds (*o2o2/o16o16*) possessed 81 to 96% RPG, and with considerable phenotypic resemblance to their respective recurrent parents. The degree of opaqueness among the introgressed lines was similar to the respective parents. The study signified the role of *o16* as alternative as well as supplementary to *o2* for nutritional quality enhancement in maize. This is the first ever study on effects of *o16* in relation to tryptophan concentration and endosperm modification. The information generated and

the improved elite inbreds developed here, hold great significance in maize biofortification programme.

सारांश

अभी तक मक्का की प्रोटीन–गुणवत्ता में सुधार अप्रभावी *ओपेक2* (*o2*) उत्परिवर्ती पर आधारित रहा है जो भ्रूणपोष–रूपांतरकों के साथ, उच्चतर लायसीन एवं ट्रिप्टोफेन युक्त, गुणवत्ता प्रोटीन मक्का (क्यू पी एम) विकसित कर सका है। यद्यपि भ्रूणपोष में लायसीन एवं ट्रिप्टोफेन की बढ़ोतरी करने में सक्षम अनेक अन्य उत्परिवर्तितों की खोज की जा चुकी है किन्तु कई प्रतिकूल बहुप्रभाविताओं के कारण उनका फसल प्रत्यारोपण नहीं हो सका। अप्रभावी *ओपेक16* (*o16*) उत्परिवर्ती के पोषण संबंधी लाभों की हाल में खोज, मक्का में प्रोटीन गुणवत्ता में सुधार हेतु अत्यंत महत्वपूर्ण है। वर्तमान अध्ययन में, *o2* एवं/अथवा *o16* युग्मविकल्पियों हेतु विसंयोजी हो रही चार एफ$_2$ आबादियों का उपयोग कर, *o16* के प्रभाव का अध्ययन किया गया। तद्नुसार *o16* (0.12% लायसीन एवं 0.035% ट्रिप्टोफेन) के विपरीत $_o$16 वे लायसीन एवं ट्रिप्टोफेन की मात्रा में लगभग दोगुना की बढ़ोतरी की (0.246% लायसीन एवं 0.072% टिप्टोफेन), कुछ विसंयोजी क्यू पी एम चैक (*o2o2*) के समकक्ष थे। अकेले *o16o16* ने भ्रूणपोष में पारभाषी लक्षणप्ररूप उत्पन्न नहीं किया तथा इसकी दाना कठोरता, *o16o16* संपृथक्कृतों, सामान्य मक्का तथा साथ ही क्यू पी एम के साथ तुलनीय थी। *o16o16* में स्टार्च कणों एवं प्रोटीन मैट्रिक्स की सूक्ष्मदर्शीय व्यवस्था का ढ़ंग, कठोर भ्रूणपोष वाली मक्का के समान था किन्तु *o2o2*–मुलायम जीनप्ररूप से भिन्न था। *o16o16* में भण्डारण प्रोटीन की जाइन प्रोफायलिंग तथा विशेष रूप से γ–जाइन ने भी सामान्य एवं क्यू पी एम जीनप्ररूपों के साथ समानता दर्शायी किन्तु ये *o2o2*–मुलायम जीनप्ररूप से पूर्णयता भिन्न थी। *o2o2/o16o16* के मध्य, प्रोटीन मैट्रिक्स के साथ स्टार्च कणों की पैकेजिंग का ढ़ंग, अनूठा था तथा जाइन प्रभाज *o2o2* एवं *o16o16*, दोनों से भिन्न थे। हमने यहाँ दर्शाया है कि *o16* दाना रूपांतरण पर कोई प्रभाव नहीं रखती थी तथा उसकी लायसीन एवं ट्रिप्टोफेन में बढ़ोतरी की क्रियाविधि, *o2* से पूर्णतया भिन्न थी। चार वाणिज्यिक भारतीय क्यू पी एम संकर (एच क्यू पी एम–1, एच क्यू पी एम–4, एच क्यू पी एम–5 एवं एच क्यू पी एम–7, के *o2o2*–आधारित जनक अंतःप्रजातों (एच के आई 161, एच के आई 193–1, एच के आई 193–2 एवं एच के आई 163) के भीतर चिह्नकों की सहायता से *o16* के अतंर्वेशन के फलस्वरूप क्रमानुसार आवर्ती जनकों की तुलना में लायसीन एवं ट्रिप्टोफेन में क्रमशः 75% एवं 91% की उच्चस्तरीय बढ़ोतरी हुई। चयन किए गए अंतर्वेशित अंतःप्रजात (*o2o2/o16o16*), 81 से 96% आर पी जी रखते थे और इनकी अपने–अपने आवर्ती जनकों के साथा उल्लेखनीय लक्षणप्ररूपी समानता थी अंतर्वेशित क्रमों के मध्य पारभासकता का स्तर उनके अपने जनकों के समान था। यह अध्ययन, मक्का में पोषण संबंधी गुणवत्ता में बढ़ोतरी हेतु *o2* के विकल्प तथा साथ ही पूरक के रूप में *o16* के महत्व को दर्शाता है। ट्रिप्टोफेन सांद्रता एवं भ्रूणपोष–रूपांतरण के संदर्भ में *o16* के प्रभावों पर यह सर्वप्रथम किया गया अध्ययन है। इस अध्ययन से उत्पन्न जानकारी एवं विकसित किए गए उन्नत, श्रेष्ठ अंतःप्रजात, मक्का जैवप्रबलीकरण कार्यक्रम में अत्यंत महत्वपूर्ण सिद्ध होंगे।

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