DETECTION OF PROMISING QPM DONORS WITH SSR MARKERS FOR CONVERTING NON QPM INBREDS OF ANGRAU

M. SURENDER

B.Sc. (Ag.)

MASTER OF SCIENCE IN AGRICULTURE

(AGRICULTURAL BIOTECHNOLOGY)

2011

DETECTION OF PROMISING QPM DONORS WITH SSR MARKERS FOR CONVERTING NON QPM INBREDS OF ANGRAU

BY

M. SURENDER

B.Sc. (Ag.)

THESIS SUBMITTED TO THE

ACHARYA N.G. RANGA AGRICULTURAL UNIVERSITY

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

AWARD OF THE DEGREE OF

MASTER OF SCIENCE IN AGRICULTURE

(AGRICULTURAL BIOTECHNOLOGY)

CHAIRPERSON: Dr. S. SOKKA REDDY

DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

RAJENDRANAGAR HYDERABAD - 500 030

ACHARYA N. G. RANGA AGRICULTURAL UNIVERSITY

2011

CERTIFICATE

Mr. M. SURENDER has satisfactorily prosecuted the course of research and that the thesis entitled "**DETECTION OF PROMISING QPM DONORS WITH SSR MARKERS FOR CONVERTING NON QPM INBREDS OF ANGRAU"** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part there of has not been previously submitted by him for a degree of any university.

Date:

Place: Hyderabad

(S. SOKKA REDDY)

Chair Person

CERTIFICATE

This is to certify that the thesis entitled "**DETECTION OF PROMISING QPM DONORS WITH SSR MARKERS FOR CONVERTING NON QPM INBREDS OF ANGRAU"** submitted in partial fulfilment of the requirements for the degree of **Master of Science in Agriculture** of the **Acharya N.G. Ranga Agricultural University, Hyderabad**, is a record of the bonafide original research work carried out by **Mr.M. SURENDER** under our guidance and supervision.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part and all assistance received during the course of the investigation have been duly acknowledged by the author of the thesis.

Thesis approved by the Student Advisory Committee

Chairperson **(S. SOKKA REDDY)**
Professor and Univ. Head
Department of Agricultural Biotechnology
College of Agriculture, ANGRAU Signature
Rajendranagar, Hyderabad – 500 030

Member **(M.R. SUDARSHAN)**
Principal Scientist , Plant Breeding
Maize Research Center, ARI
Rajendranagar, Hyderabad – 500 030 Signature

Member **(Ch.V. DURGARANI)**
Associate Professor
Department of Agricultural Biotechnology Signature
College of Agriculture, ANGRAU
Rajendranagar, Hyderabad – 500 030

Date of final viva-voce:

DECLARATION

I, **M. SURENDER** hereby declare that the thesis entitled **"DETECTION OF PROMISING QPM DONORS WITH SSR MARKERS FOR CONVERTING NON QPM INBREDS OF ANGRAU"** submitted to the **Acharya N.G. Ranga Agricultural University** for the degree of **Master of Science in Agriculture** is the result of original research work done by me. I also declare that any material contained in the thesis has not been published earlier in any manner.

Date: **(M. SURENDER)**

Place: Hyderabad **I. D. No.ABT/09-04**

LIST OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF SYMBOLS AND ABBREVIATIONS

β-ME	Beta-mercaptoethanol
CIMMYT	International Maize and Wheat Improvement Centre
CTAB	Cetyl trimethyl ammonium bromide
^{o}C	Degrees Celsius
DNA	Deoxy ribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribo Nucleotide Tri-Phosphate
DW	Distilled water
EDTA	Ethylene diamine tetra acetate
EtBr	Ethidium bromide
Fig.	Figure
g	Gram
H	Hour
IAA	Isoamyl alcohol
Kbp	Kilo base pairs
M	Mole
mA	Milli ampere
Min	Minute
µl	Microlitre
ml	Milliliter
mm	Milli mole
MW	Molecular weight
ng	Nanogram
nm	Nanometer
No	Number
OD	Optical density
%	Percentage
P mole	Pico mole
PCR	Polymerase chain reaction
PAGE	Poly acryl amide gel electrophoresis

P^H	Power of Hydrogen
QPM	Quality Protein Maize
PVP	Polyvinyl pyrrolidone
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
SDS	Sodium dodecyl sulphate
SSR	Simple Sequence Repeats
TAE	Tris Acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
UV	Ultraviolet
Viz.,	Namely
V	Volts

ACKNOWLEDGEMENTS

I am pleased to place my profound gratitude to Dr. S. SOKKA REDDY, Professor, Department of Agricultural Biotechnology, College of Agriculture, Rajendranagar and esteemed chairperson of my advisory committee for his learned counsel, unstinted attention, arduous and meticulous guidance on the work in all stages. His keen interest, patient hearing and constructive criticism have installed in me the spirit of confidence to successfully complete the task.

I deem it my privilege in expressing my fidelity to Dr. M.R. SUDARSHAN, Principal scientist, Maize Research Center Rajendranagar, Hyderabad and member of my advisory committee for his valuable suggestions during the course of my study and for providing necessary facilities to carryout the research work and also for supplying germplasm and taking special care during crop season.

I wish to take this valuable opportunity to express my deep sense of gratitude, sincere and profound thanks to Dr. Ch.V. DURGARANI, Associate Professor , Dept of Agricultural Biotechnology, College of Agriculture, Rajendranagar, Hyderabad and member of my Advisory committee for her constant encouragement, constructive suggestions and indomitable quest for science which have always been a source of inspiration to me.

I express deep sense of gratitude to Dr. K. Manorama, Professor, Dept of Agricultural Biotechnology, college of agriculture, Rajendranagar, Hyderabad for her counselling and encouragement during my post graduation as well as research period.

I express my heartfelt gratitude and thanks to Mr. A. Ravicharan for his valuable guidance in practicals.

My special thanks to Dr. J. C. Sekhar, Principal Scientist and Dr. Saikumar, Director of Maize Research, New Delhi for supplying the QPM germplasm lines for the current study.

I thank the scientists and non teaching staff of Maize Research Centre, ARI, Rajendranagar for their assistance in raising the maize crop.

I express my heartfelt gratitude and thanks to my Divisional Senior and Ph.D scholar from Department of Agricultural Biotechnology, M.S.R. Krishna, in particular, for his valuable guidance, help and encouragement.

I would like to acknowledge the help and cooperation of all my friends especially Ganga Prasad, Shiva Kumar, Kishore kumar, Sachin, Deborah, Sreevani, Roja , Lakshmi Devi , Saibaba and Pavitra for the great support they gave me.

Finally, I wish my humble thanks to one and all who have directly or indirectly contributed to the course of study.

M.SURENDER

Author	:	**M. SURENDER**
Title of the thesis	:	**DETECTION OF PROMISING QPM DONORS WITH SSR MARKERS FOR CONVERTING NON QPM INBREDS OF ANGRAU**
Degree	:	**MASTER OF SCIENCE IN AGRICULTURE**
Faculty	:	**AGRICULTURE**
Department	:	**AGRICULTURAL BIOTECHNOLOGY**
Major Advisor	:	**Dr. S. SOKKA REDDY**
University	:	**ACHARYA N. G. RANGA AGRICULTURAL UNIVERSITY**
Year of submission	:	**2011**

ABSTRACT

Development of QPM (Quality Protein Maize) with high lysine and tryptophan is foremost important task in maize breeding programme. Marker assisted selection is the easiest way of developing QPM hybrids in short time. Our investigation deals with identification of suitable donors for QPM hybrid development. 60 germ plasm lines have been screened with gene specific SSR marker: *umc1066. SSR* markers are known to distinctly identify the differences among *opaque-2* alleles. The *opaque-2* mutant allele with larger distance from wild type allele on the agarose gels is preferred as a donor in conversion of non QPM lines. However, the highest quantity of lysine and tryptophan should be kept in mind in such conversion programmes. Seven lines have shown distinct allelic differences to wild type allele in the non QPM lines. In order to identify the ease with which the seven potential donors could be used in conversion programs, they have been crossed with BML 2, BML 6, BML 7 and BML 15. The molecular weight of all the seven *opaque- 2* mutant alleles in the donors appears to be same (~156 bp) on agarose gels with metaphor: merc agarose(1:2). There appears to be a difference of less than ten bases between wild type and mutant alleles. In order to assess the visibility and feasibility of using these donors, CML181 and CML186 have been crossed with a few important non QPM inbred lines like BML 2, BML 6, BML 7 and BML 15. The gel patterns revealed a satisfactory resolution between the alleles. Since SSR markers are co-dominant, we

preferred to use them in our studies as they are found to be the best under the existing laboratory conditions next to SNPs which are costly.

Chapter I

INTRODUCTION

Maize is an important cereal food crop in the world after wheat and rice. It is a staple food for millions of people in poor countries around the world. In the world, maize is cultivated on the area of 160 million ha with a total production of 814 million metric tones giving an average yield of 3.41 metric tones/ha (Anonymous, 2011). During 2010-11, the crop was grown on 8.11 million ha in India with total production of 16.8 million metric tones. Andhra Pradesh occupies first place in the production (21%) and productivity (3248 kg/ha) in the country.

A typical mature maize kernel contains a small embryo and much larger endosperm, which are 90 per cent starch and 10 per cent protein. Approximately 70 per cent of this protein is composed of several types of prolamines known as zeins that are alcohol soluble. Four types of zeins, *viz.,* α, β, γ and δ are found in maize and they are distributed in a distinctive pattern. But it is deficient in two essential amino acids, lysine and tryptophan. Babies and adults consuming normal maize suffer from nutritious disorders such as marasmus and kwashiorkor.

However, using the maize mutant *opaque2* (o2) discovered in the early 1960s (Mertz *et al*. 1964) scientists developed high lysine and tryptophan maize with normal kernels of vitreous appearance (Ortega and Bates, 1983). More specifically CIMMYT breeders developed high lysine corn from *opaque2* genotypes by selecting for the *opaque2* phenotype with normal endosperm texture and increased levels of lysine and tryptophan. These modified *opaque2* maize lines were designated as "Quality Protein Maize" or QPM lines (Vasal *et al*. 1980).

The conventional breeding procedures were successful in releasing several QPM hybrids both in Africa and Latin America. India has imported the Mexican QPM lines and developed several QPM hybrids in the recent past. However, it is very much essential to develop the QPM hybrids which can yield on par with normal hybrids. For this purpose

molecular tools can be used to identify the high lysine and tryptophan lines after screening the potential germplasm that can help the breeder in constructing hybrids using Marker Assisted Selection.

Marker assisted selection in combination with conventional breeding can greatly accelerate the introgression of QPM genotype into normal maize. The *opaque2* trait is expressed in the recessive state whereby the mutant kernels have a typically starchy endosperm texture and low density. The *opaque2* mutation increases the lysine content in maize endosperm by decreasing the content of zeins, while *opaque2* modifiers alter the soft texture of *opaque2* kernels into hard endosperm. Breeders have systematically transferred the modifier genes into *opaque2* germplasm to develop normal looking maize with high lysine. Molecular tools have facilitated the breeders to transfer the *opaque2* along with modifiers into elite maize inbreds for construction of the hybrids. Although several QPM hybrids have been developed in India using conventional breeding procedures, VPKAS, Almora, has succeeded in developing the first QPM hybrid, named, Vivek QPM-9 (Gupta et al. 2009) using MAS.

The QPM breeding essentially needs two components, *viz*, molecular markers and QPM donors. SSR (Simple Sequence Repeats) have been considered as the best option currently to develop QPM hybrids in the absence of facilities and budget for SNPs. *Umc 1066, phi 057 and phi 112* are important opaque-2 specific markers for developing QPM hybrids. The QPM donors imported from CIMMYT have been extensively used for the purpose of conversion programmes. However the donor selection for converting the local specific lines the polymorphic studies have to be undertaken apart from tryptophan estimation. The suitable donor marker combination is yet another important step in the process of conversion of elite non QPM inbred lines.

The current study deals with identification of suitable QPM donors containing more than 4.0 per cent lysine and 0.95 per cent tryptophan with *opaque-2* specific SSR markers. F1 polymorphism was also carried out to establish the suitability of the QPM donors for conversion of elite non QPM lines used in ANGRAU breeding programmes. The current investigations were started with the following objectives:

1. To produce PCR profiles of QPM and non QPM inbreds with gene specific SSR markers.
2. To identify QPM donors for conversion of specific non QPM inbreds of ANGRAU.

Fig. 1.1 Quality Protein Maize

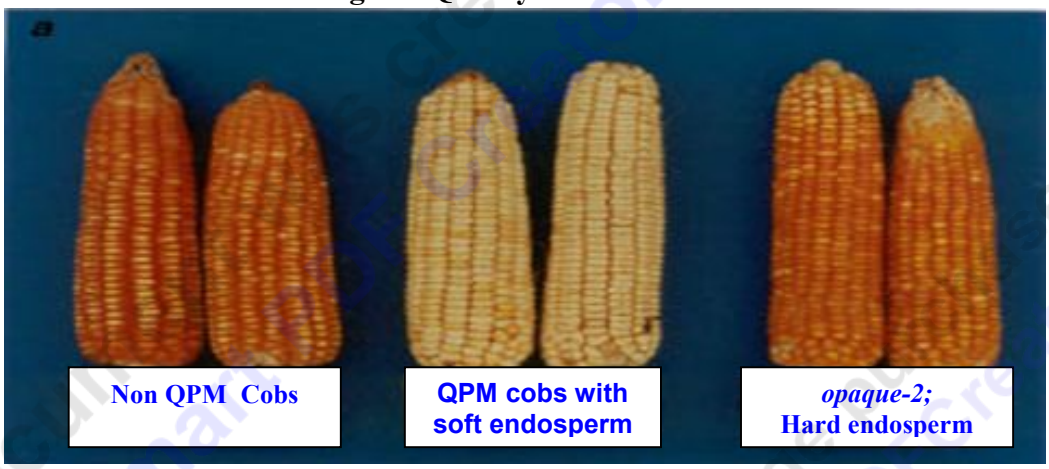

Fig. 1.2 Non QPM and QPM Cobs

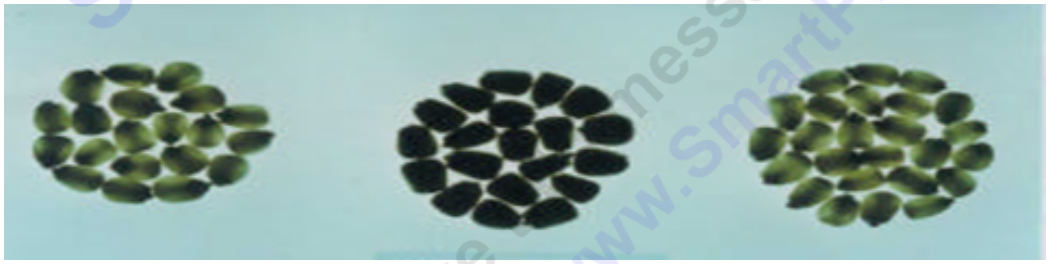

Fig.1. 3 Non QPM and QPM Kernels

Courtesy: Prasanna

Fig. 1.4 DHM 117 Hybrid with its parental lines

BML 6 INBRED

X

BML 7 INBRED

DHM 117 HYBRID

Fig. 1.5 DHM 117 Hybrid with its parental inbred cobs

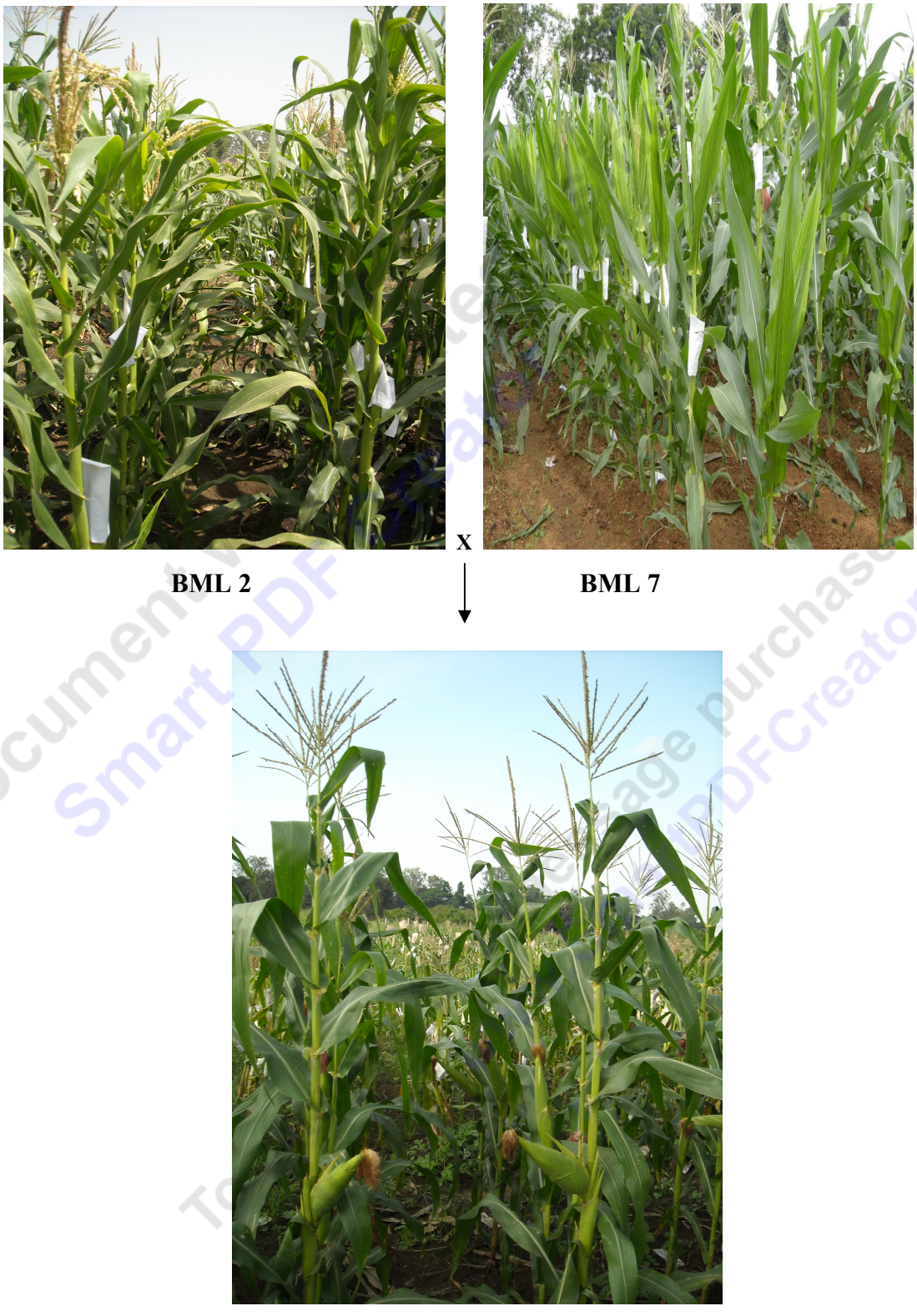

Fig. 1.6 DHM 113 Hybrid with its parents

BML 2 **X** **BML 7**

DHM 113 HYBRID

Fig. 1.7 DHM 113 Hybrid with its parental inbred cobs

Fig. 1.8 DHM 111 Hybrid with its parents

BML 6 X BML 15

DHM 111 HYBRID

Fig. 1.9 DHM 111 Hybrid with its parental inbred cobs

Chapter II

REVIEW OF LITERATURE

Maize is a cereal crop for human consumption and livestock feed as well as raw material for several industrial uses. However, conventional maize contains low protein quality due to its deficiency in two essential amino acids lysine and tryptophan (FAO, 1992). It is a primary source of energy supplement and can contribute up to 30 per cent protein, 60 per cent energy and 90 per cent starch in an animal's diet (Dado, 1999). Improving the protein quality of cereal grains has been a major concern of scientists in the last two decades. Mutant germplasm with high levels of lysine and tryptophan has been identified in maize (Mertz *et al*., 1964). The maize breeders in the International Maize and Wheat Improvement Centre (CIMMYT) have combined the high-lysine and tryptophan potential of the *opaque-2* gene with a sufficient number of modifier genes to change the original soft *opaque-2* endosperm into a hard vitreous type (Vasal *et al*., 1980). QPM populations that have superior lysine and tryptophan content with high yield and agronomic characteristics similar to those of normal corn are now available (Ortega *et al*., 1986).

2.1 INTRODUCTION AND HISTORY OF QPM DEVELOPMENT

Agricultural scientists have long had an interest in improving the protein quality of plants. Maize, one of the most important staple foods worldwide, is relatively low in protein content (generally about 10%). Also, roughly half of that protein contains almost no lysine or tryptophan, two amino acids essential for building proteins in humans and monogastric animals. Several natural maize mutants conferring higher lysine and tryptophan were identified in the 1960s and 1970s, i.e., *opaque-2* (*o2*), *floury-2* (*fl2*), *opaque-7* (*o7*), *opaque-6* (*o6*) and *floury-3* (*fl3*). The *o2* mutation was found to be the most suitable for genetic manipulation in breeding programs aimed at developing maize high in lysine and tryptophan. Maize homozygous for the *o2* (recessive) mutation was shown to have substantially higher lysine and tryptophan content than maize that was either homozygous dominant (*O2O2*) or heterozygous (*O2o2*) for the *opaque-2* locus (Crow and Kermicle., 2002). Bressani (1992b) showed that increased concentrations of these two amino acids in the grain endosperm can double the biological value of maize protein. However, the amount

of protein in such maize remains at about 10% or, in other words, the amount of common maize that needs to be consumed to achieve amino acid equilibrium is more than twice as much as the amount of *opaque-2* maize (FAO, 1992). Maize homozygous for the *o2* also has protein quality value equivalent to 90% that of milk. The discovery of *opaque-2* maize stimulated considerable research interest and activity, with high hopes of substantially improving the nutritional status of maize consumers, especially in developing countries. But, this highly desirable trait turned out to be closely associated with several undesirable ones. The *opaque-2* maize kernels were dull and chalky, had 15-20% less grain weight and were more susceptible to several diseases and insects. These obstacles made most research programs to stop their work on *opaque-2*.

Only a small number of crop research institutes continued this work, most notably the International Maize and Wheat Improvement Center (CIMMYT) in Mexico. Using conventional breeding methodologies, the CIMMYT interdisciplinary research team slowly overcame the original *opaque-2* defects while maintaining superior nutritional quality. They were able to convert the floury soft endosperm kernels into harder types, increase grain yield potential to the level of the best normal maize types, endow the *opaque-2* maize with disease and insect resistance and with utilization and storage qualities similar to those of superior normal maize materials. The new, normal-looking, normal-tasting *opaque-2* types were renamed "Quality Protein Maize" or QPM (Vivek *et al*., 2008).

2.2 UTILITY OF QPM

Maize with high lysine and tryptophan, has been used in feeding studies involving monogastric animals and humans. In one experiment it was shown that pigs raised on high lysine and tryptophan maize gained weight at roughly twice the rate of animals fed solely on normal maize with no additional protein supplements. An equal quantity of high lysine maize substituted for normal maize in pig feeds can maintain the amino acid balance and decrease the use of synthetic lysine (Burgoon *et al*., 1992). Several human nutrition studies were conducted by Akuamoa-Boateng., 2002 in Ghana, where maize is the main staple. It was shown that children fed with high lysine and tryptophan maize were healthier, had reduced stunting and better growth enhancing capabilities, compared with children fed normal maize porridge. The conclusion was that high lysine and tryptophan maize holds the promise of improving the nutritional status of vulnerable groups whose main staple is maize and who cannot afford protein-rich foods to supplement the diet. The biological value of

protein is estimated on the average proportion of absorbed protein that is successfully retained by the body for maintenance and growth. Biological value is closely related to protein quality, which in the case of maize is limited mostly due to low concentrations of essential amino acids. Several studies on children and adults have found that subjects eating QPM had significantly higher nitrogen retention than those who ate normal maize (Bressani, 1991a), indicating that QPM protein is more "bioavailable". The biological value of normal maize is about 45% and of QPM about 80%, which is similar to the biological value of milk - 90% (FAO, 1992). Besides biological value, QPM has additional improved nutritional advantages over normal maize. As the consequence of increased concentration of tryptophan, QPM has higher concentration of niacin (B3). Also, because of reduced leucine concentration absorption of potassium (Graham *et al*., 1980) and carotene (De Bosque *et al*., 1988) are improved.

Although QPM was developed primarily for utilization in the regions of poverty, maize is the main staple food (e.g., Africa, Asia), it has many advantages for production and consumption in other parts of the world, too. QPM can be used for production of conventional and new animal feed, as well as for human nurture. Utilization of QPM could discourage import of protein additives which are used in animal feed composites. As the rate of animal weight gain is doubled with QPM and protein availability is better, a part of normal maize production could be available for other purposes, such as, ethanol production.

2.3 GENETIC BIOCHEMICAL AND MOLECULAR BASIS OF QPM

The endosperm of maize (*Zea Mays* L.) kernel is a triploid tissue originating when a male gamete fertilizes diploid central cell in a process parallel to the fertilization of the egg cell that gives origin to diploid zygote. The maize grain largely consists of endosperm that is rich in starch (71%). Protein constitutes < 10% of the kernel. In normal maize, 50-70% of the endosperm proteins are of prolamin type (zeins). Zeins are a class of alcoholic soluble proteins that are specific to endosperm of maize. (Prasanna *et al*., 2001)

These zeins consist of albumins, globulins, glutelins and prolamins. The prolamins of maize grain are called zeins and are often classified by differential solubility and mobility through gels as α zein, β zein, δ zein and γ zein (Coleman *et al*., 1997). The zein fraction α is in cysteine while β and γ fractions are rich in methionine. These 4 types constitute about 50-70% of maize endosperm and are essentially rich in glutamine, leucine

and proline and are poor in lysine and tryptophan. The non zein fraction is balanced and is rich in lysine and tryptophan (Vasal, 2000).

Zeins, particularly α zeins are the most abundant proteins in the grain endosperm but are also characteristically poor in amino acids, lysine and tryptophan (Gibbson *et al.*, 2005). The high proportion of zeins in endosperm which results in a lack of lysine and tryptophan is primary reason for poor protein quality of maize. Interest in improving the protein quality of cereals led to the identification of maize mutant that resulted in increased lysine and tryptophan (Vasal, 2000).

A natural spontaneous mutation of maize with soft, opaque grain was discovered and delivered to Connecticut Experimental Station (Vietmeyer., 2000). This maize mutant was eventually named *Opaque-2* (*o2*) by a Connecticut Researcher (Singleton, 1939).

In its dominant form *o2* regulates the expression of 22 kDa α zeins (Damerval and Devienne., 1993) and other genes including lysine ketoglutarate reductase. The mutant allele *o2* typically causes a significant increase in non zein fraction associated with decrease in lysine poor α zein protein. Moreover, the defective lysine ketoglutarate reductase enzyme causes increased levels of free lysine.

The well studied *opaque-2* mutant in maize was originally reported by Emerson *et al*., (1935). Mertz *et al.,* 1964 reported that maize genotypes homozygous for the mutant allele *o2* had a considerably higher content of lysine and tryptophan in the grain when compared with wild type genotypes. The recessive mutant allele of *opaque-2* genes is the central component of QPM breeding. Characterization of this gene has identified it as encoding a transcription factor (a gene regulator) of zein synthesis (Schmidt *et al.*, 1990).

2.4 PROBLEMS ASSOCIATED WITH *OPAQUE-2* MUTANT ALLELES

The high lysine mutants aroused tremendous interest and enthusiasm for their possible use in developing maize with superior protein quality, but rapidly the negative pleiotropic effects of such mutations began to be recognized. These undesirable features were limiting factors to its wide spread use and adoption (Lauderdale, 2002).

In particular, *o2* genotypes had soft kernels that were prone to mechanical damage, yield reductions of 8 – 15 % relative to *O2* plants and greater susceptibility to fungi and insect damage (Lambert *et al.*, 1969).

The soft endosperm texture of *o2* germplasm is associated with the reduction in the proportion of α zein protein (Huang *et al*., 2004). It has been postulated that the absence of specific zeins in *o2* genotypes causes the formation of smaller protein bodies and therefore alters the packing of the starch fraction during seed desiccation, resulting in abnormally soft endosperm (Schmidt *et al.,* 1990). The undesirable characteristics also include reduced yield than normal maize, low grain consistence and farinaceous endosperm that retains water (Toro *et al.*, 2003). These features result in soft, chalky endosperm that dried slowly making it prone to damage, a thick pericarp, more susceptibility to diseases and pests, higher storage losses and also effects harvest ability since the kernel weight is reduced due to less density per unit volume as starch is loosely packed with lot of air spaces. There is corresponding decline in the yield (Singh and Venkatesh., 2006) which can be almost to the tune of 10% or above.

2.5 DEVELOPING QPM LINES THROUGH CONVENTIONAL BREEDING METHODS

Soon after the discovery of the nutritional benefits of the o2 mutation, it began to be incorporated into many breeding programmes world wide, with a major emphasis on conversion of normal endosperm populations and inbred lines to o2 versions through a backcross approach (Prasanna *et al.*, 2001).

Backcross breeding in maize (*Zea mays* L.) has been extensively used to transfer favourable alleles for monogenic traits from donor genotype to elite inbred lines (Openshaw *et al.*, 1994) but high heritability polygenic traits have also been transferred through this method (Rinke and Sentz., 1961; Shaver., 1976).

The conventional backcross strategy for conversion of normal maize to QPM suffers from two major problems. One that *o2* being a recessive trait, selection has to be carried out at each backcross in order to fix the recessive *o2* allele, prior to selection for endosperm modification, there by extending the time period for line conversion . Moreover, the quality traits such as grain protein content cannot be selected prior to seed formation making screening very difficult. Besides, low cost and reliable methods of screening are not available.

2.6 DEVELOPING QPM THROUGH MARKER ASSISTED BREEDING

Marker assisted selection is an appropriate technology for traits such as high lysine in maize and can be a cost effective procedure for selecting *o2* locus in breeding populations.

According to Tanksley *et al*., (1989), the main advantage of using DNA markers as opposed to conventional selection is to accelerate the fixation of recipient alleles in non target regions and to identify the genotypes containing crossovers close to target genes (Ribaut and Hoisington, 2002). According to Frisch *et al*., (1999a), molecular markers are used in backcross breeding for two purposes:

A, as a diagnostic tool to trace the presence of a target allele when direct selection is difficult or impossible such as recessive alleles are expressed late in plant development or quantitative trait loci.

B, to identify individuals with a low proportion of undesirable genome from the donor parent, this approach being called 'background selection' and was first proposed by Tanksley *et al*., (1989), and then by Hillel *et al*.,(1990) and was further investigated by Hospital *et al*., (1992) and later reviewed by Viescher *et al*.,(1996).

The use of markers as a diagnostic tool was first proposed by Tanksley (1983) and reviewed by Melchinger *et al*., (1990). Both foreground MAS and background MAS can be effectively employed for selecting o2 phenotype besides ensuring maximum recovery of recurrent parent. Babu *et al*., (2005) used MAS for development of QPM parental lines of Vivek Hybrid-9 and developed QPM hybrid in less than half the time required through conventional breeding. Danson *et al*., (2006) used various markers to introgress o2 gene in to herbicide tolerant elite maize inbred lines.

Introgression of *o2* genotype into normal elite maize lines through conventional breeding has been on going. To accelerate the time taken to obtain a true QPM, marker assisted breeding is gaining recognition as it can reduce time, costs and also ensure quality of QPM maize (Frisch *et al.*, 1999a, Frisch *et al.*, 1999b; and Hospital and Charcosset,1997).

Hamada *et al,* (1982) described a novel repeated element termed simple sequence repeat (SSR) or micro satellite, which is a short tandem repeat DNA sequence. These sequences spread widely through out the nuclear genome of eukaryotes. The variation in

number of repeats for a defined locus among different genotypes is easily detected with PCR. Micro satellite markers have been used for, individual identification , diversity analysis (Powell *et al*., 1996 ; Yang *et al*., 1996), evolution studies , population structure of related species and for mapping genomes of cereal species (Davierwala *et al.,* 2000).

Microsatellite loci have been proved to be highly polymorphic and useful as genetic markers in many plant species including *Arabidopsis* (Depeiges *et al*., 1995), bur oak (Dow *et al*., 1995), maize (Senior and Heun, 1993), seashore paspalum (Liu *et al*., 1995), rapeseed (Kresovich *et al*., 1995; Charters *et al*.,1996), soybean (Akkaya *et al*., 1992; Rongwen *et al*., 1995), sugar beet (Morchen *et al*., 1996), sweet potato (Jarret and Bowen, 1994) and wheat (Plaschke *et al*., 1995; Roder *et al*., 1995).

SSR markers are widely preferred for genotype characterisation, genome analysis and gene mapping in various crop species, including maize as these are PCR – based, codominant, locus specific, highly reproducible, hyper variable, informative and reasonable easy to use (Powell *et al*., 1996). They can also provide greater power of discrimination than RFLPs, and can reveal genetic associations that are reflective of the pedigree of the inbreds (Smith *et al*., 1997; Senior *et al*., 1998).

A large no of marker systems are now available that are associated with *o2* and endosperm modification phenotype (Lopez *et al*., 2004; Bantle and Prasanna, 2003). Recent investigations on the abundance and allele variation for SSRs in maize (Senior and Heun, 1993, Chin *et al*., 1996; Taramino and Tingey, 1996) led to the identification of hundreds of mapped SSR loci dispersed over entire maize genome. The utility of SSR markers in characterisation of maize germplasm has been well demonstrated in recent years (Senior and Heun, 1993; Senior *et al*., 1996; Smith *et al*., 1997).

The effectiveness of SSR markers in QPM genotype discrimination and analysis of genetic relationship and their potential contribution towards effective utilisation of the elite QPM germplasm in Indian Maize breeding programmes is demonstrated by Bantle and Prasanna (2003).

Three micro satellite markers were discovered that lie within the *opaque–2* gene following successful cloning of this gene and their primer sequences released in 2001 to the website www. Argon. missari. edu and has since facilitated study and application of the o2 gene (Mbogori *et al*., 2006).

Within the *opaque–2* gene sequence, three different internal repetitive elements (SSR markers) have been identified – *phi057*, *phi112* and *umc1066*. The internal position of these markers enables direct selection of the *opaque-2* gene without false positive and false negative results in breeding programmes, i.e., enables marker assisted selection – MAS (Babu *et al.*, 2005; Danson *et al.*, 2006)

The *phi112* SSR is located between the G – box and 3 upstream open reading frames (u ORFs) (Schmidt *et al.*, 1990) in the leader sequence of the gene and its mutation can affect transcription of the *o2* gene. The *umc1066* and *phi057* SSRs are located in exon1 and exon 6, respectively. These are the largest exons among six exons with in the *o2* gene (www. ncbi. nlm. nih.gov, Gene Bank accession numbers X 15544 and X16618). Mutations at these two exons will increase or decrease the number of prolines in the *o2* protein and affect the activity of the position of *o2* protein as a transcriptional activator because the position of the proline residue influences the direction and degree of turns in the three dimensional structure of the *o2* protein (Shen and Wang , 1998)

On the other hand, Lazzari *et al.,* (2002) revealed a very high degree of homology among the different alleles of the *o2* gene, except for two hyper variable regions in the exon 1. There fore, using *umc1066*, *phi057*and *phi112* markers, the allelic SSR variations in the leader region and the 5' region and 3' region of the *o2* gene can represent the variation with the *o2* gene.

Phi112, which exhibits dominant polymorphisms, cannot be used for discriminating homozygous and heterozygous backcross progeny. This marker could be of use in checking the seed purity during routine field maintenance of *opaque-2* or QPM inbred lines. (Ignjatovic - Micic *et al.,* 2009)

But *phi057* and *umc1066*, could be recommended for discriminating between homozygous and heterozygous progeny during MAS process (Babu *et al.,* 2005 and Ignjatovic – Micic *et al.,* 2009). *Phi057*, would be applied as marker assisted selection for improvement of efficiency of QPM breeding and obtain QPM hybrid variety in a short period of time (Jompuk *et al*., 2006).

To detect heterozygous maize for backcross breeding program, *phi057* was considered more feasible than *phi112* as marker assisted selection (MAS) for *opaque-2* and to identify QPM line in the short period of time (Jompuk *et al.*, 2006).

According to Yang *et al*, all the three markers, *umc1066*, *phi057* and *phi112* not just one of them, shall be used to screen out the mutant lines with larger variations at the three SSR sites in comparison with the wild type when applying the three markers to MAS for high lysine maize materials.

The new QPM has desirable features such as, uniform ear placement, resistance to ear rot and root lodging and better grain protein profile (Cordovo, 2001).

Breeding strategies aimed at improving the protein profile of maize will go a long way in reducing prevalence and persistence of malnutrition in developing world (Sofi *et al.,* 2009).

In addition to the molecular work, a lot of biochemical research is also being done in *opaque-2* mutants to know the amino acid content. Wallace *et al*., (1990) used SDS-PAGE method, Western blotting and ELISA techniques to extract and analyse the zein content of several quality protein maize varieties developed by CIMMYT.

2.7 LYSINE AND TRYPTOPHAN ESTIMATION:

These methods have been used over the tears for lysine and tryptophan estimation: colorimetric method, Near Infrared Transmittance and High Performance Liquid Chromatography. CIMMYT employed HPLC method to estimate lysine and tryptophan before characterizing the QPM CML lines and use in Corn Improvement Programme. However, the other methods were used for initial screening of large populations. The QPM CML lines became the only source for India to implement QPM programmes.

2.8 RESEARCH AND DEVELOPMENT EFFORTS ON QPM IN INDIA

India received QPM germplasm accessions from International Centre for Maize and Wheat improvement (CIMMYT), Mexico, during 1990 tested them at different centres of All India Coordinated Research Project on Maize, (AICRPM). Through acclimatization and repeated selection, hard endosperm modified *opaque2* maize inbred lines and their crosses have been identified for better protein quality and higher yield potential. The Directorate of Maize Research (DMR), New Delhi, developed first QPM composite variety, Shakthi-1 with 0.63 per cent tryptophan in the year, 1997, which was released and recommended for general cultivation by Indian farmers in 1998. The QPM research gained further momentum by launch of National Agricultural Technology Project (NATP) on QPM in 1998 by the

Indian Council of Agricultural Research (ICAR). In this project a multidisciplinary team of multiple institutes involving DMR, New Delhi, Punjab Agricultural University (PAU), Ludhiana, Choudhary Charan Singh Haryana Agricultural University (CCSHAU), Karnal, Acharya N. G Ranga Agricultural University(ANGRAU), Hyderabad, Rajendra Agricultural University (RAU), Pusa, wherein the QPM germplasm received from CIMMYT was acclimatized to suit the local agroclimatic conditions in India. The lines were evaluated for their productivity and deployed in combination breeding which led to release of first QPM three way cross hybrid, Shakthiman-1 by RAU, Pusa during 2001 followed by release of first QPM single cross hybrid, Shakthiman-2 during 2004 for their cultivation in Bihar state (Jat *et al.* 2009).

Bantle and Prasanna (2003) reported the effectiveness of SSR markers in QPM genotype discrimination and analysis of genetic relationships and potentially contribute towards effective utilization of the elite QPM germplasm in Indian maize breeding programmes. Babu *et al.* (2005) reported two generation marker based backcross breeding program for incorporation of the *opaque-2* gene along with phenotypic selection for kernel modification in the background of an early maturing normal maize inbred line, V 25. In 2005, CCSHAU, Karnal released another QPM single cross hybrid HQPM-1, which is the first yellow grain QPM single cross hybrid released for its cultivation across the country. Later in series of QPM, Shakthiman – 3 and Shakthiman – 4 were released by RAU, Pusa during 2006 for their cultivation in Bihar.

In 2007, another QPM single cross hybrid, HQPM-5 from CCSHAU, Karnal and Vivek QPM-9 from Vivekananda Parvatiya Krishi Anusandhan Sansthan (VPKAS), Almora have been identified during 2008. Vivek QPM-9 has a unique distinction of the first molecular marker assisted (MAS) product of normal hybrid, Vivek-9, (Jat *et al.* 2009).

Department of Biotechnology has recently funded State Agricultural Universities and ICAR institutes for development of QPM single cross hybrids using marker assisted selection (MAS).

Chapter III

MATERIAL AND METHODS

The present investigation entitled "Detection of promising QPM donors with SSR markers for converting non QPM inbreds of ANGRAU'' was carried out during *Kharif* and *Rabi,* 2010 at Maize Research Centre, Agricultural Research Institute, Rajendranagar, Hyderabad.

3.1 MATERIAL

The germplasm lines for the present study were procured from Directorate of Maize Research, New Delhi and Maize Research Centre, Rajendranagar. The list of the germ plasm lines is shown in the Table.3. 1

Table3. 1 List of germplasm lines used in present investigation

S. No	Name of line	S. No	Name of line	S. No	Name of line
1	BML 6	21	CML 142	41	S 771
2	BML 7	22	CML 150	42	S 5180
3	BML 10	23	CML 153	43	S 5291
4	BML 13	24	CML 154	44	S 5258
5	CM 104	25	CML 157	45	S 5170
6	CM 105	26	CML 158	46	S 5175
7	CM 114	27	CML 163	47	S 5163
8	CM 115	28	CML 164	48	S 5347
9	CM 118	29	CML 165	49	S 5555
10	CM 119	30	CML 167	50	S 5367
11	CM 120	31	CML 171	51	S 5204
12	CM 121	32	CML 172	52	S 5342
13	CM 131	33	CML 175	53	S 5340
14	CM 132	34	CML 176	54	S 5207
15	CM 133	35	CML 177	55	S 5330
16	CM 201	36	CML 181	56	S 5238
17	CM 202	37	CML 182	57	S 5121
18	CM 211	38	CML 186	58	S 76
19	BML15	39	CML 191	59	SHKI 163
20	BML 2	40	CML 192	60	SHKI 193

SSR Primers: Three SSR markers are used in the present study with the details in the following Table.3.2

Table3. 2 Primer sequences of three SSR markers employed in the present study

S.No	Marker	Forward primer Sequence	bps	Reverse primer Sequence	bps
1.	*Umc1066*	ATGGAGCACGTCATCTCAATGG	22	AGCAGCAGCAACGTCTATGACACT	24
2.	*Phi057*	CTCATCAGTGCCGTCGTCCAT	21	CAGTCGCAAGAAACCGTTGCC	21
3.	*Phi112*	TGCCCTGCAGGTTCACATTGAGT	24	AGGAGTACGCTTGGATGCTCTTC	23

3.2 METHODS

The methods used for the accomplishment of this study are as follows:

1. Genomic DNA Isolation
2. DNA Purification & Quantification of DNA
3. Normalization of DNA concentration
4. Polymerase Chain Reaction with SSR markers
5. SSR Product visualization on Agarose Gel Electrophoresis
6. Detection of polymorphic donors
7. Confirmation of parental polymorphism

3.2.1 Genomic DNA Isolation:

Plant sample collection:

1. Fresh young leaves were harvested from two week old seedlings from the field (2-4g) and collected in the ice. Low temperature prevents nucleases from degrading the DNA.
2. The leaves were folded into 10-15cm sections and placed in an Aluminum foil and labeled.
3. The samples were placed in deep freezer (-80^{0}C).

DNA Isolation Procedure:

1. Pre-chilled plant leaves (3 g) at -80ºC were crushed in liquid nitrogen with a mortar and pestle.
2. This homogenate is poured into a 50 ml microfuge tube.

3. A pre-heated (65ºC) extraction buffer (35 ml) containing 100 mM Tris-HCI (pH8); 1.4 mM NaCl; 20 mM EDTA; 0.1% 2-mercaptoethanol GR (Merck) and 2% CTAB powder is added to crushed leaf material.
4. The mixture was incubated at 65ºC for 45 min in water bath (Lab Tech) followed by addition of equal volume of Chloroform: isoamyl alcohol (24:1) (Thermo Fisher Scientific India Pvt. Ltd) mixture and the homogenate is incubated for 15 min at room temperature.
5. Centrifugation was done for 10 min at a speed of 10,000 rpm in a centrifuge machine (Eppendorf) at room temperature.
6. The upper aqueous phase was carefully removed into a new tube (avoiding transfer of any material from the interface) with a pasture pipette and an equal volume of Chloroform: isoamyl alcohol (24:1) (Thermo Fisher Scientific India Pvt. Ltd) mixture was added and again the homogenate was centrifuged for 10 min at 10,000 rpm.
7. The upper aqueous phase was again removed carefully into a new tube and an equal volume of ice-cold isopropanol was added to this to precipitate the DNA.
8. The tube was kept on ice for 10 minutes.
9. This mixture was centrifuged at maximum speed for 10 min at room temperature to collect the DNA pellet at the bottom of the tube.
10. The DNA pellet was washed by adding 200 ml 70% ethanol and centrifuged at maximum speed of 10,000 rpm for 2 min.
11. The pellet was dried in a vacuum centrifuge (Speed Vac) and re-suspended in 100 µl sdH_2O (sterile distilled water).
12. Any contaminating RNA was removed by addition of 10 µl of a 10 µg/ml stock solution of Rnase H and incubated at 37ºC for 45 min.
13. DNA was removed by the addition of 1/10 volume of 3M sodium acetate (pH 6.8) and 2 volumes of 96% ethanol to the DNA containing solution.
14. This was incubated on ice for 10 min.
15. Centrifugation was done at maximum speed of 10,000 rpm for 5 min at room temperature to pellet the DNA.
16. DNA pellet was washed and dried as above and finally dissolved in 50 µl sdH2O.

3.2.2a Purification of DNA samples

1. 20 µl of Rnase H (final concentration of 100 µg/ml) was added and incubated overnight at 4^0C.
2. Next day it was incubated at 37^0C for 30 min. to bring the tubes to room temperature.
3. An equal volume of Phenol: Chloroform solution was added to the tubes.
4. The contents of the tubes were mixed thoroughly and centrifuged at 10,000 rpm for 10 min.
5. The aqueous layer was transferred to a new 15 ml Polypropylene tube.
6. 1/10 volume of Sodium acetate (3M, pH-5.2) was added to this.
7. The precipitated DNA was spooled out with a glass hook into a 1.5 ml eppendorf tube.
8. The DNA was washed with 70% Ethanol for 2-3 times.
9. After washing, it was air dried and then $T_{10}E_1$ (50-400µl) was added to the tubes depending on the quantity of DNA.

Phenol was added as a mixture of Phenol: Chloroform: Isoamyl alcohol in a 25:24:1 ratio to remove proteins. It was added to the solution during chloroform step instead of adding only Chloroform. It was required to remove all traces of Phenol after the above step. Hence, further washes with chloroform were required.

Agarose Gel Electrophoresis

Procedure for preparing a 0.8% Agarose gel

1. 0.8 g of agarose was weighed and taken into a 250 ml conical flask. Then 100 ml of 1X TAE was added to the former. Swirling was done to mix properly.
2. The mixture was boiled in a microwave oven until a clear solution resulted and the agarose was dissolved completely.
3. It was left to cool for 5-7 min down to about 60^0C (just too hot to hold with bare hands).
4. 1µl of EtBr was added to this solution (10 mg/ml) and swirled to mix.
5. The molten agarose was poured into the gel casting tray slowly. The benefit of pouring slowly was that most bubbles stay up in the flask.

6. Bubbles were pushed away to the side using a disposable tip. The comb was inserted and double checked so that it was correctly positioned.
7. The gel was left to set for at least 30-50 min.
8. 1X TAE buffer was poured into the gel tank to submerge the gel to 2-5 mm depth.
9. The gel was placed along with the tray in the electrophoresis tank and the comb was removed slowly so as not to damage the wells.
10. DNA sample of 2-8 µl was loaded along with 2 µl of loading dye into the well using a micropipette.
11. The gel tank was closed and the power supply was switched ON and the gel was run at 60 V.
12. The gel was stopped after it was run for $3/4^{th}$ the length. Then the gel was carried to the gel documentation system to observe the DNA bands and a photograph of the gel was taken

3.2.2b Quantification of DNA by Spectrophotometry

1. The Spectrophotometer in the photometric mode was adjusted with wavelengths set at 260 nm and 280 nm.
2. The spectrophotometer was adjusted with TE buffer as blank.
3. 5 µl of DNA sample was taken with a cut tip and added to 995 µl of TE in a 1ml cuvette. This was mixed well by gentle inversion and the OD readings were noted.
4. The same procedure was repeated with another lot of the same sample.
5. The $OD_{260:280\ ratios}$ were calculated which represents the purity of DNA.
6. The amount of DNA in the sample was calculated using the formula:

   1-OD = 50 µg DNA taking the dilution factor into consideration.

   DNA (µg/ml) = OD_{260}× Dilution factor × 50
7. Purity of the DNA was given by the ratio $OD_{260/280}$. This should be approximately 1.8-2.0 for pure DNA and will fluctuate in case of presence of contaminants like RNA or proteins.

3.2.3 Normalization of DNA concentration

Normalization of DNA samples was done to equalize the concentrations of all the DNA samples that were to be run in PCR. The purpose of normalization was to avoid

erroneous analyses due to differences in the brightness of the bands obtained after electrophoresing the PCR products.

Normalization was done by diluting the DNA samples with DW to their required dilution factor, which in turn depends upon the initial concentration of DNA sample (found from spectrophotometric readings) and the type of analysis done (markers used). After normalization of the samples the concentration of DNA was 20-50 ng/µl (in case of SSRs).

3.2.4 Polymerase chain reaction (PCR)

The polymerase chain reaction was a three-step process, which is repeated in several cycles (Fig. 3.1). The three steps are denaturation, annealing and extension. In the first of the chain reaction, denaturation, the two DNA strands are separated by heating the DNA to 94ºC. Heat treatment breaks the relatively weak bonds between the DNA bases yielding two long single strands. In the second step, annealing, two primers attach themselves to the single strands. These primers are small, synthetic stretches of single-stranded DNA each about 20 base pairs long. They are selected so that they are complementary to the two single strands. The primers track down and bind themselves to the region being sought by framing the target sequence within the long DNA strand. The annealing temperature is usually in the range of 50-65ºC depending on the length and composition of the primer. Once the primers have attached themselves, two short stretches of double-stranded DNA are generated. In the third step, extension, these short stretches serve as starting blocks for the enzyme, Taq polymerase. Starting of the 3' –end of the primers, the enzyme adds the nucleotides complementary to the template at about 72ºC, linking them together. It extends the primers in the direction of the target sequence, thereby making a double strand out of the two single strands. From the original double strand, at the end of this first cycle, two new DNA double strands identical with the first one are made. The three-step cycle can now be repeated as often as necessary where only the sequence flanked by the two primers is amplified. The new DNA sequences provide the template in the next cycle for the creation of new strands, resulting in four strands in the next cycle and then in 8, 16, 32 copies and so on.

The enzyme used in this experiment was always put in ice and was removed from the freezer only before starting the required experiment.

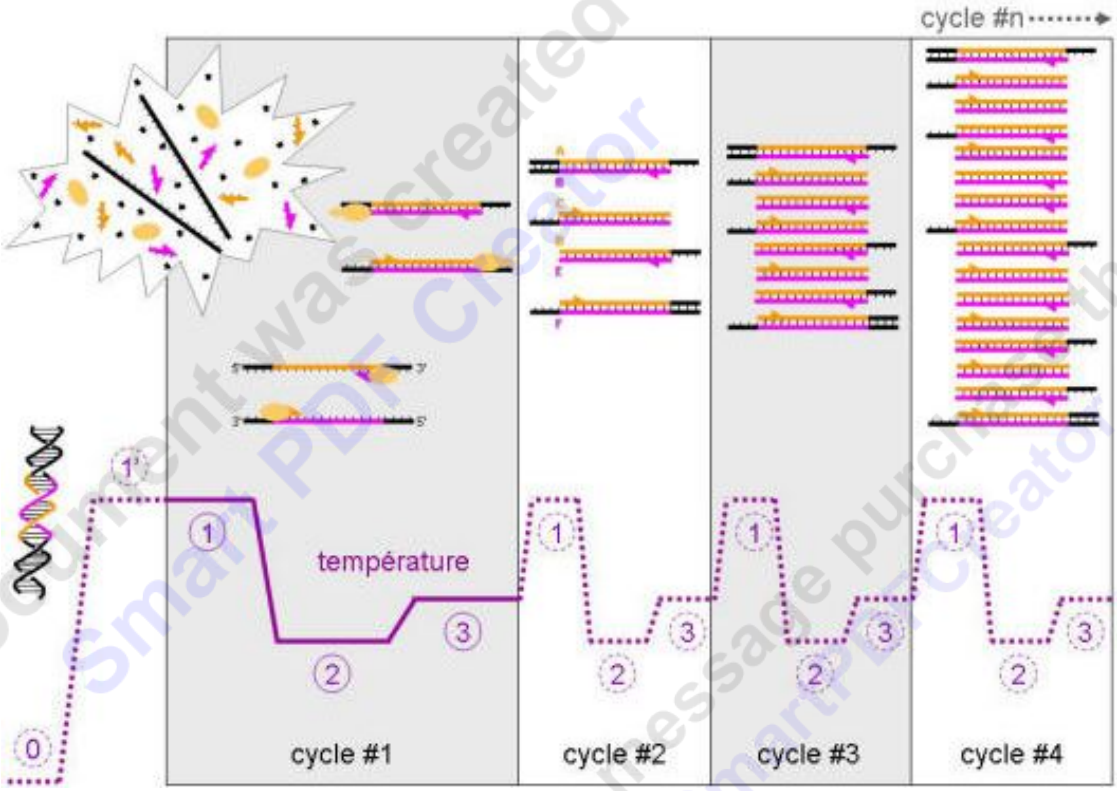

Fig. 3.1 Polymerase Chain Reaction

PCR reaction was carried out in small PCR tubes with genomic DNA as template and the primer(s) that will amplify DNA sequence were used. The PCR reaction was carried out in 10 µl volumes, which contains 2 µl maize genomic DNA, 0.3 µl of forward and reverse primers each, 1 µl dNTPs, 1 µl Taq buffer and 2 units of Taq polymerase.

The PCR components were mixed to give a final reaction volume of 10 µl per sample (Table. 3.3). For every 5 samples, one extra master mix was made in order to avoid the loss due to pipetting error.

Table3.3: List of components used in polymerase chain reaction:

Components	**Stock concentration**	**Quantity required for 1 reaction (µl)**
Sterile distilled water	-	5
PCR buffer	10 x	1.0
dNTP's	2.5 mM	1.0
Primer F	2.5 pmole	0.4
Primer R	2.5 pmole	0.4
Taq DNA Polymerase	5U / µl	0.2
		8.0 µl

Template DNA – 2.0 µl (20ng/µl)

Thermo cycling was carried out on a 96- well PCR System (eppendorf) which holds 0.2 ml thin walled PCR tubes. All tubes are placed into the thermocycler and the following DNA amplification programme was used with different annealing temperatures for three different primers used. The annealing temperatures (Table. 3.4) were calculated based on the primer sequences of the three markers used in our study (Table.3. 2)

Table.3. 4: PCR programming for three SSR markers used in the present study:

Steps follwed in Thermal cycler	Temperature in degree celcius for one cycle			Time for one cycle
	Umc1066	*Phi057*	*Phi112*	
Step 1	94 ºC	94 ºC	94 ºC	5 minutes
Step 2	94 ºC	94 ºC	94 ºC	1 minute
Step 3	56 ºC	56 ºC	56 ºC	45sec
Step 4	72 ºC	72 ºC	72 ºC	1minute
All the above four steps are repeated for 35 cycles				
Step 5	72ºC	72 ºC	72 ºC	10 minutes
Step 6	hold at 4ºC until ready to load onto gel			

The amplification will last about 4 hours.

For *umc1066*, the following PCR programme was used: a pre-denaturation step at 94^0C for 5min, followed by 35 cycles of a denaturation at 94^0C for 1min, annealing at 56^0C for 45sec, and extension at 72^0C for 1min. A final extension step at 72^0C for 10min was included, followed by termination of the cycle at 4^0C.

For *phi057*, the following PCR programme was used: a pre-denaturation step at 94^0C for 5min, followed by 35 cycles of a denaturation at 94^0C for 1min, annealing at 64^0C for 45sec, and extension at 72^0C for 1min. A final extension step at 72^0C for 10min was included, followed by termination of the cycle at 4^0C.

For *phi112*, the PCR profile consisted of a pre-denaturation step at 94^0C for 5min, followed by 35 cycles of a denaturation at 94^0C for 1min, annealing at 59^0C for 45sec, and

extension at 72^0C for 1min. A final extension step at 72^0C for 10min was included, followed by termination of the cycle at 4^0C.

3.2.5 SSR product visualization on Agarose gel electrophoresis

PCR products for *umc1066* were separated in 3.0% Metaphor agarose gels (2:1 ratios of agarose and metaphor) and 1X TAE buffer. Samples were run in gels at 50 V for 3-4 hrs, and visualized under Gel documentation system and the gel documented.

The PCR amplified products were mixed with 3 µl of 6X loading dye and electrophoresed. The gel was run at a constant voltage of 90 V for 3-4 hrs. The gel was observed under a gel documentation system and documented.

3.2.6 Detection of polymorphic donors

The distance between the polymorphic bands was taken in to consideration to select the potential donors. 50bp marker was used to visualize the distance between the alleles

3.2.7 Confirmation of parental polymorphism

The two QPM donors with distinct polymorphism (CML181 and CML 186) were crossed with four non QPM inbreds (BML 2, BML 6, BML 7 and BML 15) in different combinations (Table. 3.5) for confirmation of F1 polymorphism.

.

Table3. 5: List of crosses made for F1 polymorphism study

S.No	Cross	S.No	Cross
1	BML 6 X CML 181	**4**	BML 15 X CML 186
2	BML 7 X CML 181	**5**	BML 6 X CML 186
3	BML 2 X CML 186	**6**	BML 7 X CML 186

Chapter IV

RESULTS AND DISCUSSION

Maize is an important cereal crop for human and animal consumption. Of late the attention of the breeders is diverted to develop crop varieties with high quality and yield using biotechnological tools especially Marker Assisted Selection. Thus there is a tremendous hope for future to provide food and nutritional security. Increase in Beta carotene, iron, zinc, lysine and tryptophan along with low phytate is an immediate goal of corn breeders for Biofortification.

The present study deals with attempts to identify the donors and markers required for conversion of non QPM inbreds used in ANGRAU maize breeding programmes. *Opaque-2* is an important gene to provide increased lysine and tryptophan in homozygous recessive condition. However, all the known non QPM inbreds have homozygous dominant condition as far as *opaque-2* is concerned. The goal is to search for potential QPM lines in gemplasm with *opaque-2* in homozygous recessive state with high lysine and tryptophan. To achieve the objective, we have collected the germplasm from Directorate of Maize Research, New Delhi and Maize research Station, Rajendranagar, Hyderabad. The lines have been sown at MRS, Rajendranagar in *kharif* and *rabi*-2010 and screening work was carried out at the Department of Agricultural Biotechnology, College of Agriculture, Rajendranagar. The leaf samples have been collected from 15 day old seedlings and DNA was isolated and purified. Subsequently this genomic DNA was used for screening with gene specific SSR markers: *umc1066, phi057 and phi112*. These markers provided the polymorphism sufficient enough to detect the donors for QPM and ultimately the *umc1066* was used for all the subsequent studies because it provided the distinct polymorphism. The following paragraphs narrate the work carried out briefly.

DHM111, DHM 113 and DHM 117 are the ruling hybrids of ANGRAU at present. In addition, several hybrids are in pipe line. Some new inbred lines have been developed by the breeders which are involved in hybrid development. All these inbreds have been included in our study for working out the possibility of converting with the identified QPM donors. Seven potential donors have been identified with umc1066 that

can convert the elite inbreds available with ANGRAU and currently involved in hybrid production.

Umc1066

The marker *umc1066* has amplified all the 60 genomic DNA samples that have been subjected for PCR analysis and out of them 53 lines have shown monomorphism with a molecular weight of around 145bps on 3.0% gels with 2:1 ratio of Merc agarose and metaphor agarose. Figures 4.1-4.2 indicate the seven polymorphic lines identified in the study. These lines have shown polymorphism with molecular weight of ~155bps. These lines could be considered as QPM donors. However, data on lysine and tryptophan levels shall strengthen the donor status. It is imperative that homozygous *o2* lines with distinctly scorable polymorphic bands may be considered for using in QPM conversion programmes as it is currently followed in some universities and ICAR institutes in the event of unavailability of reliable quality control laboratories.

The polymorphic status of CML 181 and CML 186 could be seen in Fig. 4.1 They look like excellent QPM lines for conversion purposes. The tryptophan content of CML 181 is 0.86% and that of CML186 is 1.02% as revealed from CIMMYT and IARI records. These could be used to convert our four promising non QPM lines *viz*., BML 6, BML 7, BML 13 and BML 15. These four non QPM lines were used in construction of different single cross hybrids of high popularity in Andhra Pradesh (DHM 111, DHM 113, and DHM 117 (Fig. 1.4 to Fig1.9).

Seven polymorphic lines are visible in Fig. 4.2a (CML 142, CM 201, CM 202, CM 119, CM 121, CML 186 and CML 181). The molecular weight of these lines is same as those of polymorphic lines shown in Fig. 4.1 .The polymorphic nature of the lines is not distinct in gels with phi057 marker (Fig. 4.2b). Moreover the non specific bands were also observed indicating the requirement for better standardization. The *phi112* profiles could not give satisfactory results. Perhaps further standardization is needed.

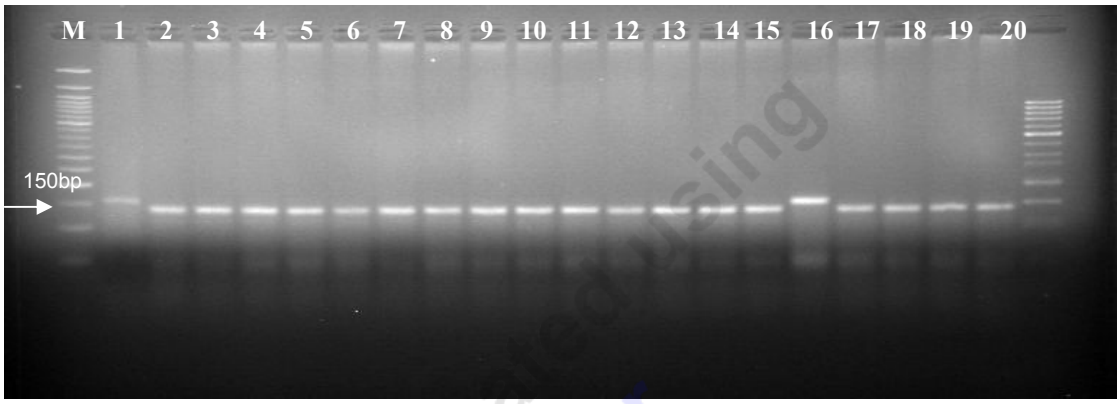

Fig. 4.1 SSR profile of maize germplasm generated by *umc1066*

M:50 bp Ladder, 1:CML 186; 2:CML 150; 3:CML 157; 4:CML 158; 5:CML 154; 6:CML 153; 7:CML 176; 8:CML 177; 9:CML 172; 10:CML 163; 11:CML 167; 12:CML 191; 13:CML 194; 14:CML 164; 15:CML 165; 16:CML 181; 17:CML 171; 18:CML 182; 19:CML 142; 20:CML 175

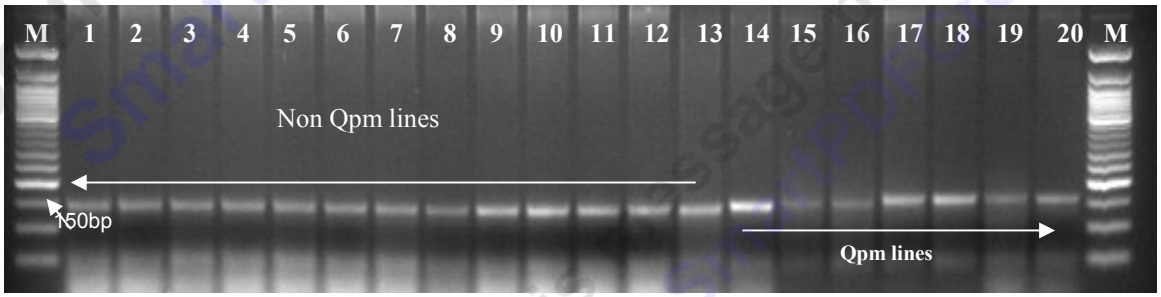

Fig. 4.2a SSR profile of maize germplasm generated by *umc1066*

M: 50bp ladder; 1:BML 2; 2:BML 6; 3:BML 7; 4:BML 10; 5:BML 13; 6: BML 15; 7: CM 104; 8: CM133; 9:S 5180; 10:S 5258; 11:S 5347; 12:S 5330; 13:S 5238; 14:CML 142; 15:CM 201; 16:CM 202; 17:CM 119; 18:CM 121; 19:CML 186; 20:CML 181.

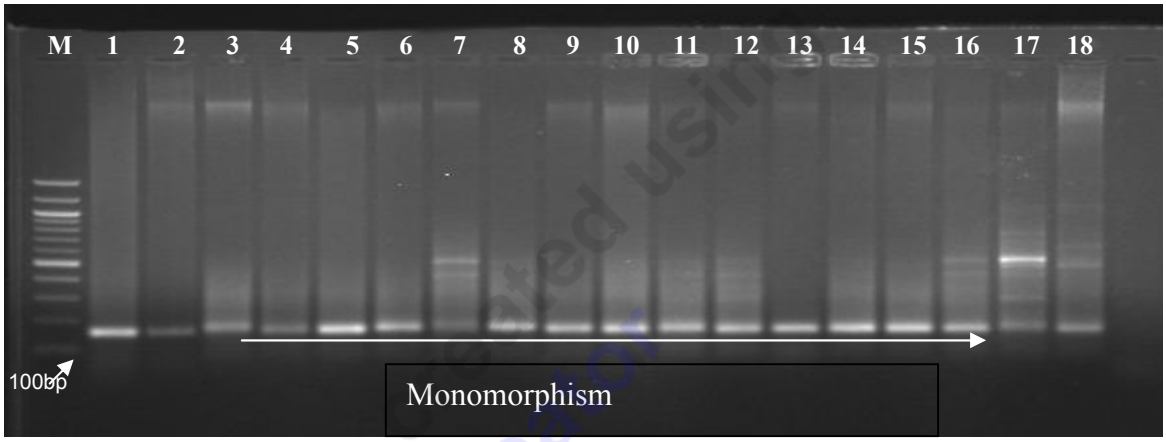

Fig. 4.2b SSR profile of maize germplasm generated by *phi057*

M:100bp ladder; 1: BML 7; 2:BML 6;3: BML 10; 4: BML 13; 5: CM 104; 6: CM 105; 7: CM 114; 8: CM 115; 9: CM 118; 10: CM 120; 11: CM 131; 12: CM 133; 13: CM 211; 14: BML15; 15: BML 2; 16: CM 132; 17: CML 181; 18: CML 186

The figure (Fig.4.3a) clearly demonstrates the difference between the molecular weight of non QPM inbreds (BML 2, BML 6, BML 7 and BML 15) with the QPM inbreds (CML 181 and CML 186) whereas the (Fig. 4.3b) depicts the differences between non QPM inbreds (BML2, BML6, BML7, BML15, CML176 CML 154, S 5204 and S 5175) and QPM inbreds (CML186, CML181, CML172, CM119, CM121, CM201, CM202, CML142). The clarity in the profiles suggests that the QPM lines could be used as potential donors in the conversion programmes.

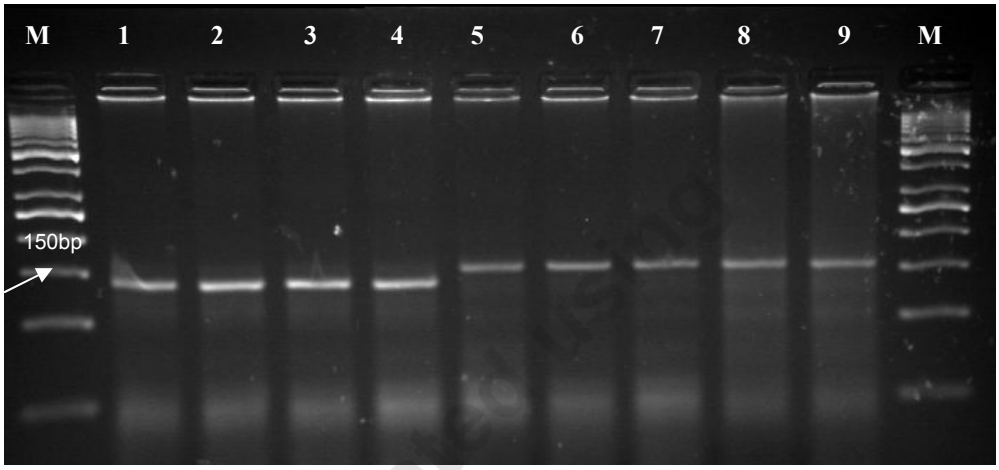

Fig.4.3a SSR profile of non Qpm and Qpm lines with marker, *umc1066*

M.50bp ladder, 1. BML 2; 2. BML 6; 3. BML 7; 4. BML 15; 5- 6. CML 181; 7-9 . CML 186.

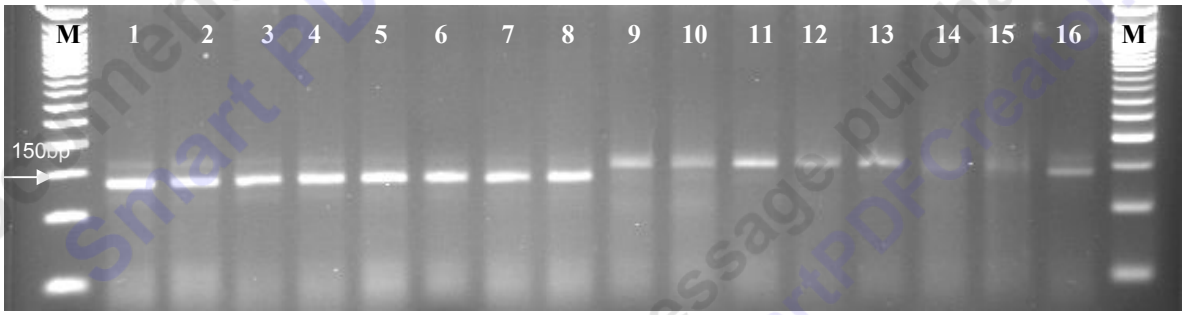

Fig. 4.3b SSR profile of non Qpm and Qpm lines with marker, *umc1066*

M: 50bp ladder; 1:BML 2; 2:BML 6; 3:BML 7; 4:BML 15; 5:CML 176; 6: CML 154; 7: S 5204; 8: S 5175 ; 9:CML186; 10:CML 181; 11:CML 181; 12:CM 119; 13:CM 121; 14:CM 201; 15:CM 202; 16:CML 142.

The donor CML 181 was crossed with BML 6 and BML 7; and the donor CML 186 with BML 2, BML 6, BML 7 and BML 15 to study the F1 polymorphism. SSR markers are co-dominant and the same is observed in all the crosses made with distinct polymorphism indicating that they can be used in conversion of the respective non QPM lines (Fig. 4.4).

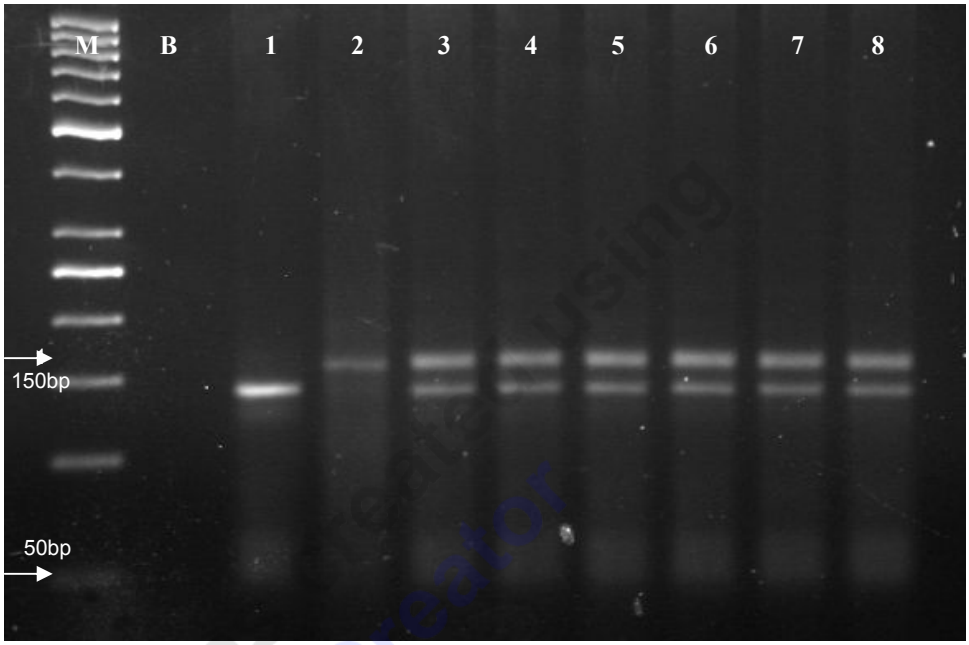

Fig.4.4 F1 polymorphism generated by umc1066 in cross between BML15 and CML186

M:50bp ladder; B: Blank; 1:BML 15; 2:CML186; 3-8 F1s (BML6XCML186)

The pictures of all the above mentioned hybrids and the corresponding parental lines have been presented in Figures 4.5-4.10**.**

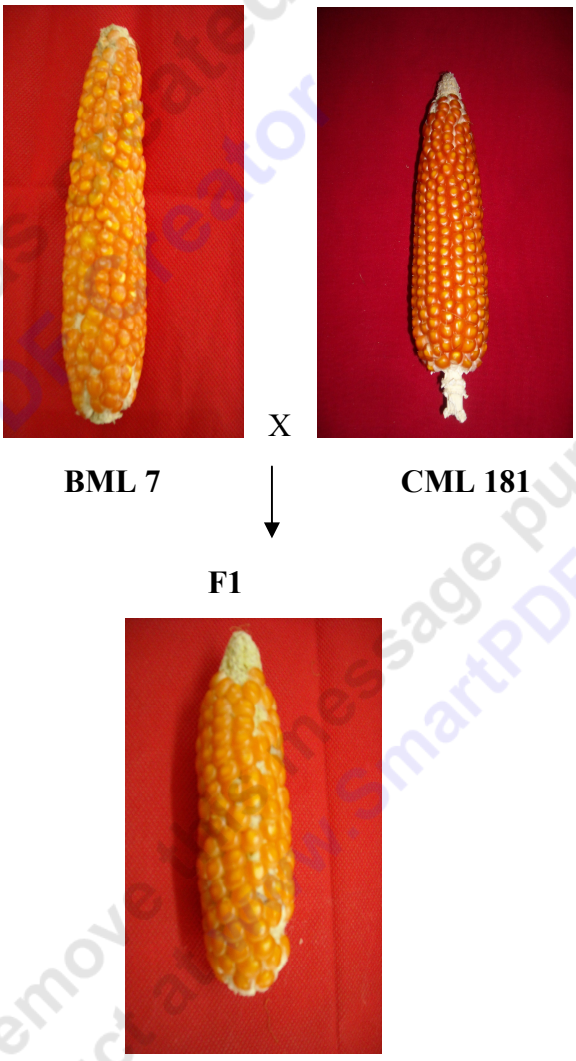

Fig.4.5 Cross between non QPM inbred BML 7 and QPM donor CML 181

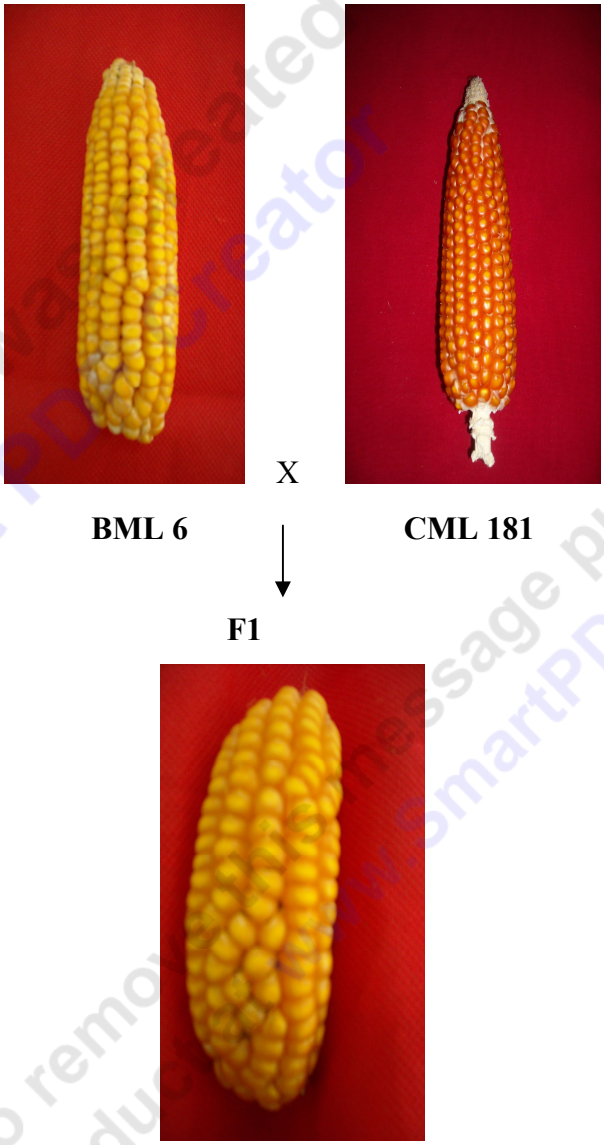

Fig.4.6 Cross between non QPM inbred BML 6 and QPM donor CML 181

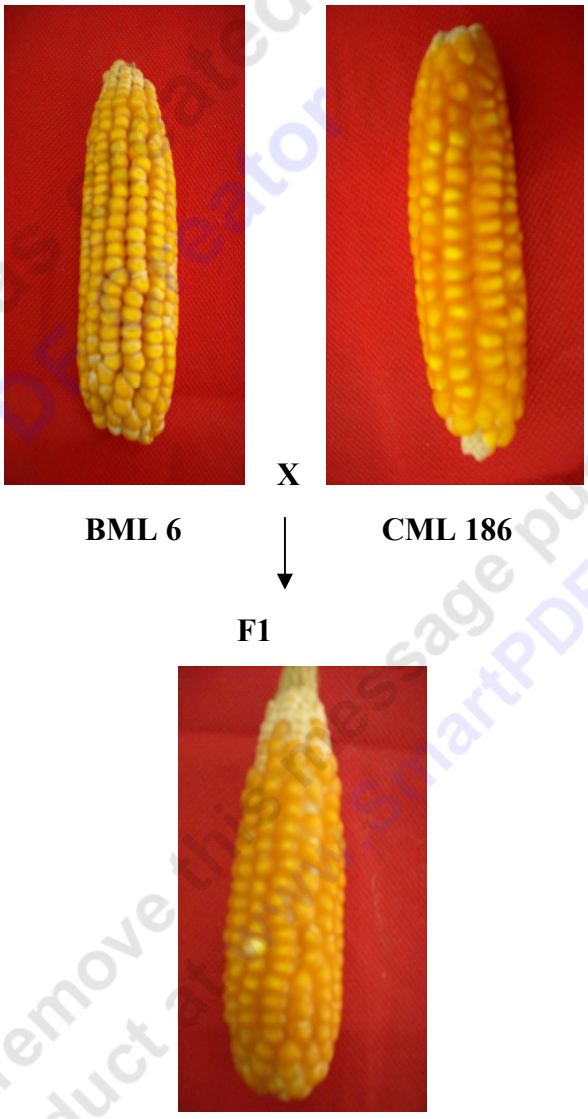

Fig.4.7 Cross between non QPM inbred BML 6 and QPM donor CML 186

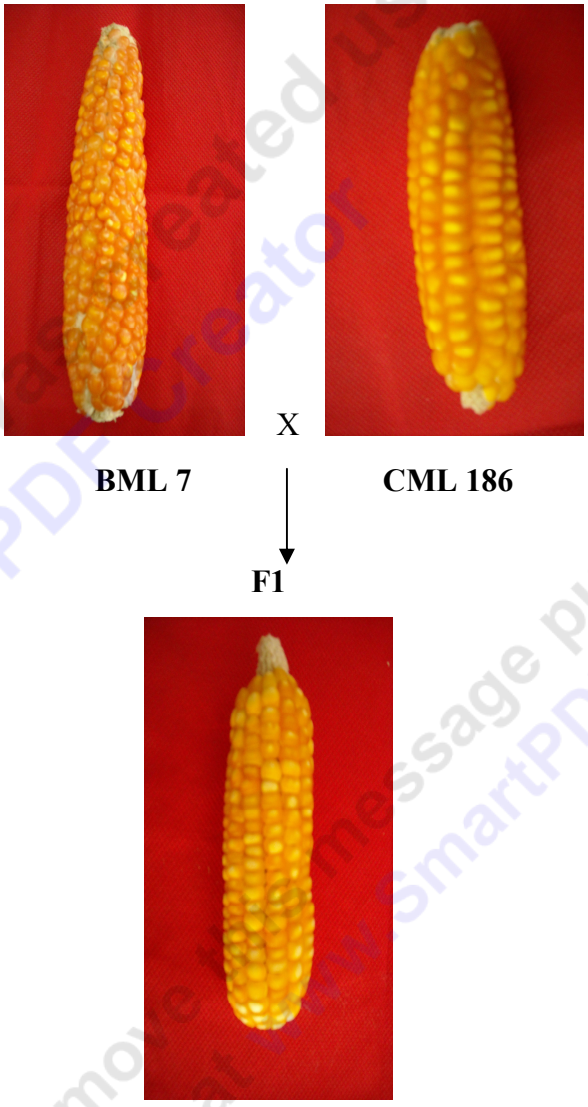

Fig.4.8 Cross between non QPM inbred BML 7 and QPM donor CML 186

BML2 X CML 186

↓

F1

Fig.4.9 Cross between non QPM inbred BML 2 and QPM donor CML 186

BML15 **X** **CML186**

F1

Fig.4.10 Cross between non QPM inbred BML15 and QPM donor CML186

The present studies have emerged with the seven potential QPM donors for conversion of non QPM lines used in ANGRAU breeding programmes (Fig. 4.11).
The F1s produced can be used to advance the conversion programmes.

The *opaque-2* specific SSR markers have been used in MAS programmes by several workers successfully (Gupta *et al, 2009* and Prasanna *et al.,* 2001*)*. Our results are in conformity with all other scientists who worked on similar lines.

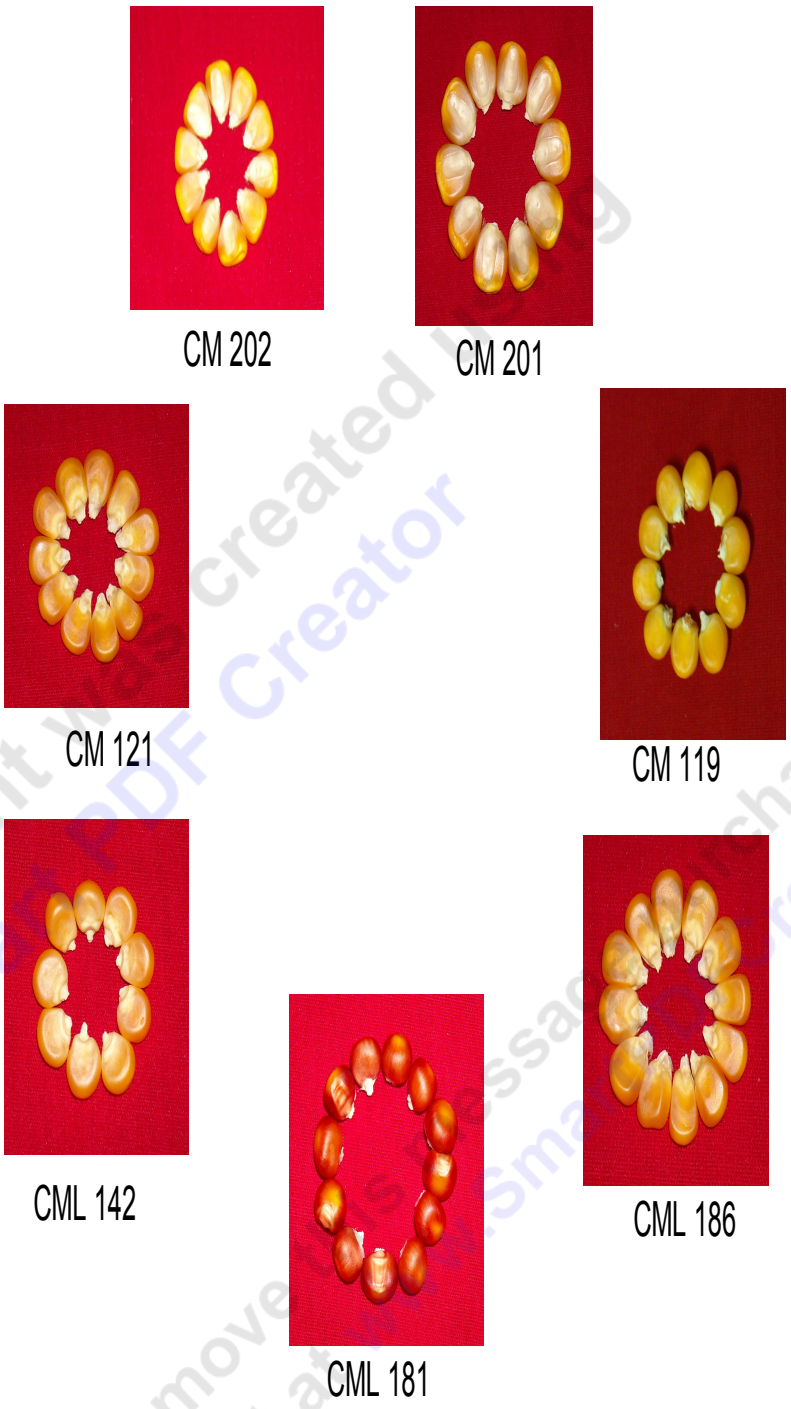

Fig.4.11: The kernels of potential QPM donors for conversion of ANGRAU elite non QPM lines

Chapter V

SUMMARY AND CONCLUSIONS

Maize is an important cereal crop for human and animal consumption. Of late the attention of the breeders is diverted to develop crop varieties with high quality and yield using biotechnological tools especially Marker Assisted Selection. Thus there is a tremendous hope for future to provide food and nutritional security. Increase in Beta carotene, iron, zinc, lysine and tryptophan along with low phytate is an immediate goal of corn breeders for Biofortification.

The present study deals with attempts to identify the donors and markers required for conversion of non QPM inbreds used in ANGRAU maize breeding programmes. Opaque-2 is an important gene to provide increased lysine and tryptophan in homozygous recessive condition. However, all the known non QPM inbreds have homozygous dominant condition as far as opaque-2 is concerned. The goal is to search for potential QPM lines in gemplasm with opaque-2 in homozygous recessive state with high lysine and tryptophan. To achieve the objective, we have collected the germplasm from Directorate of Maize Research, New Delhi and Maize research Station, Rajendranagar, Hyderabad. The lines have been sown at MRS, Rajendranagar in *kharif* and *rabi*-2010 and screening work was carried out at the Department of Agricultural Biotechnology, College of Agriculture, Rajendranagar. The leaf samples have been collected from 15 day old seedlings and DNA was isolated and purified. Subsequently this genomic DNA was used for screening with gene specific SSR markers: *umc1066, phi057 and phi112*. These markers provided the polymorphism sufficient enough to detect the donors for QPM and ultimately the *umc1066* was used for all the subsequent studies because it provided the distinct polymorphism.

DHM111, DHM 113 and DHM 117 are the ruling hybrids of ANGRAU at present. In addition, several hybrids are in pipe line. Some new inbred lines have been developed by the breeders which are involved in hybrid development. All these inbreds have been included in our study for working out the possibility of converting with the

identified QPM donors. Seven potential donors have been identified with umc1066 that can convert the elite inbreds available with ANGRAU and currently involved in hybrid production.

The potential QPM donors CML 181 and CML 186 with known lysine and tryptophan concentrations have been crossed with elite non QPM inbreds of ANGRAU such as BML 2, BML 6, BML 7 and BML 15 which have yielded popular hybrids in Andhra Pradesh.

The QPM donors identified in the study may be immediately use in QPM projects of ANGRAU and the crosses made may be advanced for conversion purpose. Lysine and tryptophan profiles may be worked out further strengthen the work. The allelic polymorphism among the tested lines may be further investigated which could lead to better QPM donors compared to the Known ones. The germ plasm could be exchanged with QPM net working projects in the country including IARI, VPKAS, Almora, Ludhiana University, Madhya Pradesh Agricultural University and other active centres. The DNA fingerprinting work of these donors may be under taken. The new alleles that may be introduced in to the country such as like opaque16 could be pyramided with these alleles. Further standardisation at the laboratory level and attempts to use SNPs may strengthen our efforts in developing QPM hybrids at ANGRAU.

LITERATURE CITED

Akkaya, M.S., Bhagwat, A.A and Cregn, P.B. 1992. Length polymorphisms of simple sequence repeat DNA in soybean. Genetics. 132: 1131-1139.

Akuamoa-Boateng, A. 2002. Quality protein maize infant feeding trials in Ghana. Ghana Health Service. Ashanti, Ghana.

Anonymous. 2010. International Grain Council. http://www.igc.int/en/.aspx.

Babu, R., Nair, S., Kumar, A., Venkatesh, S., Shekhar, J., Singh, N.N and Gupta, H. 2005. Two generation marker aided backcrossing for rapid conversion of normal maize lines to quality protein maize. Theoretical and Applied Genetics. 111: 888-897.

Bantle, K and Prasanna, B.M. 2003. Simple sequence repeat polymorphism in QPM lines. Euphytica. 129: 337-344.

Bressani, R. 1991a. Protein quality of high lysine maize for humans. Cereal Foods World. 36: 806-811.

Bressani, R. 1992b. Nutritional Value of high-lysine maize in humans. In E.T. Mertz (ed.). Quality Protein Maize. American Association of Cereal Chemists, St. Paul, MN.

Burgoon, K.G., Hansen, J.A., Knabe, D.A and Bockholt, A.J. 1992. Nutritional value of quality protein maize for starter and finisher swine. Journal of Animal Science. 70: 811-817.

Charters, Y.M., Robertson, A., Wilkinson, M.J and Ramsay, G. 1996. PCR analysis of oil rape cultivars (Brassica napus L.ssp.oleifera)using 5'-anchored simple sequence repeat (SSR) primers. Theoretical and Applied Genetics. 92: 442-447.

Chin, E.C.L., Senior, M.L., Shu, H and Smith, J.S.C. 1996. Mize simple repetitive DNA sequence: abundance and allelic variation. Genome. 39: 866-873.

Coleman, C.E., Damenhoffer, J.M and Larkins, B.A. 1997. The prolamin proteins of maize, sorghum and coix. Cellular and molecular biology of seed development. Kiuwer academic Dordrecht. 257 – 288.

Cordova, H. 2001. Quality protein maize: improved nutrition and livelihoods for the poor. Maize Research High lights 1999-2000, CIMMYT. pp- 27-31.

Crow, J.F and Kermicle, J. 2002. Oliver Nelson and quality protein maize. Genetics. 160:819.

Dado, R.G. 1999. Nutritional benefits of speciality maize grain hybrids in dairy diets. Journal of African science. 77(suppl.2) / Journal of Dairy Science. 82(suppl.2) 197-207.

Damerval, C and Devienee, D. 1993. Qualification of dominance for proteins, pliotropically affected by opaque – 2 in maize. Heredity. 70: 38 – 51.

Danson, J., Mbogori, M., Kimani, M., Lagat, M., Kuria, A and Diallo, A. 2006. Marker assisted introgression of *opaque-2* gene in to herbicide tolerant elite maize inbred lines: African Journal of Biotechnology. 5: 2417-2422.

Davierwala, A.P., Ramakrishna, W., Ranjekar, P.K and Gupta, V.S. 2000. Sequence variations at a complex microstellite locus in rice and its conservation in cereals. Theoretical and Applied Genetics. 101: 1291-1298.

De Bosque, C., Castellanos, E.J and Bressani, R. 1988. In ICAP Report Annual, INCAP, Guetamala.

Depeiges, A., Goubley, C., Lenior, A., Cocherel, S., Pichard, G., Raynal, M., Grellet, F and Delseny, M. 1995. Identification of the most represented repeated motifs in *Arabidopsis thaliana* microsatellite loci. Theoretical and Applied Genetics. 91: 160-168

Dow, B.D., Ashley, M.V and Howe, H.F. 1995. Characterization of highly variable GA/CT microsatellites in the bur oak, Quercus macrocarpa. Theoretical and Applied Genetics. 91: 137-141

Emerson, R.A., Beadle, C.W and Fraser, A.C. 1935. A summary of linkage studies in maize. Cornell University, Agricultural Experiment Station. Mem. No.180.

FAO. 1992. Maize in human nutrition. FAO Food and Nutrition Series No: 25, Rome, Italy.

Frisch, M. 1999a. Comparison of selection strategies for marker assisted back crossing of a gene. Crop Science 39:1295-1301.

Frisch, M. 1999b. Minimum sample size and optimum positioning of flanking markers in assisted back crossing for transfer of a target gene. Crop Science. 39:1903.

Gibbson, B and Larkin's, B.A. 2005. Molecular genetic approaches to developing quality protein maize. Trends in Genetics. 21: 227 – 233.

Graham, G., Placko, R.P and Maclean, W.C. 1980. Journal on Nutrition, 110:1070-1074.

Gupta, H.S., Agrawal, P.K., Mahajan, V., Bisht, G. S., Kumar, A., Verma, P., Srivastava, A., Saha, S., Babu, R., Pant M. C and Mani, V. P. 2009. Quality protein maize for nutritional security: Rapid development of short duration hybrids through molecular marker assisted breeding. Current Science. 96(2): 230-237.

Hamada, H., Petrino, M.G and Kakunaga, T. 1982. A novel repeayed element with Z-DNA forming potential is widely found in evolutionary diverse eukaryotic genomes. Proceedings of the National Academy of Science, USA. 79: 6465-6469.

Helentjaris, T., Weber, D and Wright, S. 1988. Identification of the genomic locations of duplicate nucleotide sequences in maize by analysis of restriction fragment length polymorphism. Genetics. 118: 353-363.

Hillel, J., Schaap, T., Haber feld, A., Jeffreys, J., Plotzky, Y., Cahaner, A and Lavi, U. 1990. DNA finger prints applied to gene introgression in breeding programs. Genetics. 124: 783-789.

Hospital, F and Charcosset, A. 1997. Marker assisted introgression of quantitative trait loci. Genetics. 147: 1469-1485.

Hospital, F., Chevalet, C and Mulsant, P. 1992. Using markers in gene introgression breeding programs. Genetics. 132: 1199-1210.

Huang, S.S., Adams, W.R., Zhou, Q., Malloy, K.P., Voyles, D.A., Anthony, J., Kriz, A.L and Luethy, M.H. 2004. Improving nutritional quality of maize proteins by expressing sense and antisense zein genes. Journal on Agriculture and Food Chemistry. 52: 1958-1964.

Ignjatovic, V., Mara, Kovic, K., Ristic, D., Mladenovic Drinic, S., Stankovic, S., Jancic, L and Denic, M. 2009. Variability analysis of normal and opaque 2 maize inbred lines. Genetika. 41(1) 81 – 93.

Jarret, R.L and Bowen, N. 1994. Simple Sequence repeats (SSRs) for sweet potato germplasm characterization. Plant Genetics Research Newsletter. 100: 9-11.

Jat, M. L., Dass, S., Yadav, V. K., Sekhar, J. C and Singh, D. K. 2009. Quality Protein

Maize for Food and Nutritional security in India. DMR Technical Bulletin, 4:1-5

Jompuk, P., Wongyai, W., Jampatong, C and Apisitvanichs. 2006. Detection of Quality protein maize (QPM) using Simple Sequence Repeat (SSR) Markers and Analysis of Tryptophan content in endosperm. Nature Science. 40: 768-774.

Kresovich, S., Szewc-Mc, Fadden, A.K., Blick, S.M and Mc Ferson, J.R. 1995. Abundance and characterization of simple-sequence repeats (SSRs) isolated from a size-fractionated genomic library of *Brassica napus* L.Crapeseed. Theoretical and Applied Genetics. 91: 206-211.

Lambert, R.J., Alexander, D.E and Dudley, J.W. 1969. Relative performance of normal and modified protein (opaque – 2) maize hybrids. Crop Science. 9:242-243.

Lauderdale, J 2002. Issues regarding targeting and adoption of quality protein maize CIMMYT working paper. p.31.

Lazzari, B., Cosentino, C and Viotti, A. 2002. Gene products and structure analysis of wild type and mutant alleles at the *Opaque-2* locus of *Zea mays*. Maydica. 47: 253-265.

Liu, Z.W., Jarret, R.L., Kresovich, S and Duncan, R.R. 1995. Characterization and analysis of simple sequence repeat (SSR) loci in seashore paspalum (Paspalum Vaginatum Swartz). Theoretical and Applied Genetics. 91: 47-52.

Lopez, M., Gloverson, L and Larkin's, B. 2004. Genetic mapping of *opaque -2* modifier genes. Maize Genetics Newsletter. 69: 165.

Manna, R., Okello, D.K., Imanywoha, J., Pixley, K and Edema, R. 2005. Enhancing introgression of *Opaque2* trait into elite maize lines using simple sequence repeats. African Crop Science Journal. 13(4): 215- 226.

Mbogori, M.N., Kimani, M., Kuria, A., Lagat, M and Danson, J.W. 2006. Optimization of FTA technology for large scale plant DNA isolation for use in marker assisted selection African Journal of Biotechnology. Vol. 5(9): 693-696.

Melchinger, A.E., Lee, M., Lamkey, K.R., Hallaver, A.R and Woodman, W. L. 1990. Genetic diversity for restriction fragment length polymorphisms and heterosis for two diallel sets of maize inbreds. Theoretical and Applied Genetics. 80: 488-496.

Mertz, E.F., Nelson, O.E and Bates, L.S. 1964. Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science. 145: 279 – 280.

Morchen, M., Cuguen, J., Michaelis, G and Saumitou-Laprade, P. 1996. Abundance and length polymorphism of micro satellite repeats in Beta vulgaries L. Theoretical and Applied Genetics. 92: 326-333.

Openshaw, S.J., Jarboe, S.G and Beavis, W.D. 1994. Marker assisted selection in backcross breeding Proceedings of the Symposium "Analysis of Molecular Marker Data", Joint plant Breeding Symposium Series, American Society for Horticultural Science/Crop Science of America Corvallis, Oregon. 41-43.

Ortega, E.I., Villegas, E and Vasal, S.K. 1986. A comparative study of protein changes in normal and quality protein maize during tortilla making. Cereal Chemistry. 63(5): 446-451.

Plaschke, J., Ganal, M.W and Roder, M.S. 1995. Detection of genetic diversity in closely related bread wheat using micro satellite markers. Theoretical and Applied Genetics. 91: 1001-1007.

Powell, W., Machray, G.C and Rovan, J.P. 1996. Polymorphism revealed by simple sequence repeats. Trends in plant science. 1: 215-222.

Prasanna, B.M., Vasal, S.K., Kassahun, B and Singh, N.N. 2001. Quality protein maize. Current Sciences. 81: 1308-1319.

Ribaut, J.M and Hoisington, D. 2002. Stimulation experiments on efficiencies of gene introgression by back crossing. Crop Science.42: 557-565.

Rinke, E.H and Sentz, J.C. 1961. Moving Corn-belt germplasm northward. Annual Hybrid Corn Industrial Conference.16: 53-56.

Roder, M.S., Plaschke, J., Konig, S.U., Borner, A., Sorrels, M,E., Tansksley, S.D and Ganal, M.W. 1995. Abundance Variability and chromosomal location of microsatellites in wheat. Molecular and General Genetics. 246: 327-333.

Rongwen, J., Akkaya, M.S., Bhagwat, A.A., Lavi, U and Cregen, P.B. 1995. The use of micro satellite DNA markers for soybean genotype identification. Theoretical and Applied Genetics.90: 43-48.

Schmidt, R.J, Burr, F.A., Aukerman, M.J and Burr, B. 1990. Maize regulatory gene *opaque –2* encodes a protein with a leucine – zipper motif that DNA. Proceedings of the National Academy of Sciences, USA.87: 46-50.

Senior, M.L., Chin, E.C.L., Lee, M., Smith, J.S.C and Stuber, C.W. 1996. Simple sequence report markers developed from maize sequences found in the Gene Bank data base: map construction. Crop Science.36: 1676-1683.

Senior, M.L and Heun, M. 1993. Mapping maize micro satellites and polymerase chain reaction confirmation of the target repeats using a C.T primer .Genome.36: 884-889.

Senior, M.L., Murphy, J.P., Goodman, M.M and Stuber, C.W. 1998. Utility of SSRs for determining genetic similarities and relationships in maize using an agarose gel system. Crop Science.38: 1088-1098.

Shaver, D.L. 1976. Conversion for earliness in maize inbreds. Maize Genetics Cooperative Newsletter.50: 20-23.

Shen, T and Wang, J.Y. 1998. Biochemistry, 2nd Ed, pp 163. Higher Educational Press, Beijing (Chinese).

Singh, N.N and Venkatesh, S. 2006. Development of quality protein maize inbred lines in: Heterosis in crop plants. Ed.Kaloo G, Rai M, Singh M, Kumar S. Book centre, New Delhi.102-113.

Singleton, W.R. 1939. Recent linkage studies in maize: V. Opaque endosperm -2 (o2). Connecticut experiment station, New Haven. Genetics. 24: 61.

Smith, J.S.C., Chin, E.C.L., Shu, H., Smith, O.S., Wall, S.J., Senior, M.L., Mitchell, S.E., Kresovitch, S and Ziegle, J. 1997. An evolution of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): Comparisons with data from RFLPs and pedigree. Theoretical and Applied genetics.95: 163-173.

Sofi, P.A., Shafiq, W.A., Rather, A.G and Shabir, W.H. 2009. Review article: Quality protein maize (QPM): Genetic manipulation for the nutritional fortification of maize. Journal of plant breeding and crop science Vol.1 (6):244-253.

Tanksley, S.D. 1983. Molecular markers in plant breeding. Plant Molecular Biology Reporter. 1: 3-8.

Tanksley, S.D., Young, N.D., Paterson, A.H and Bonierbale, M.W. 1989. RFLP mapping in plant breeding : New tools for an old science. Bio/Tech 7: 257-264.

Taramino, G and Tingey, S. 1996. Simple sequence repeats for germplasm analysis and mapping in maize. Genome. 39: 277-287.

Toro, A., Medici, L., Sodek, L., Lea, P and Azevedo, R. 2003. Distribution of soluble amino acids in maize endosperm mutants. Scientia Agricola. 60: 91-96.

Vasal, S.K. 2000. Quality protein maize story. Proceedings of workshop on improving human nutrition through agriculture. The role of international agricultural research, IRRI.1 – 16.

Vasal, S.K., Villegas, E., Bjarnason, M., Gelaw, B and Goertz, P. 1980. Genetis modifiers and breeding strategies in developing hard endosperm *opaque2* materials. Improvement of quality traits of maize for grains and silage use. 37-73

Viescher, P.M.S., Haley, C.S and Thompson, R. 1996. Marker assisted introgression in backcross breeding programs. Genetics.144: 1923-1932.

Vietmeyer, N.D. 2000. A drama in three long acts: the story behind the story of the development of quality protein maize. Diversity.16: 29 – 32.

Villegas, E., Vasal, S.K and Bjarnason, M. 1992. Quality protein maize – what is it and how was it developed? In: E.T.Mertz. eds. Quality Protein Maize. American Association of Cereal Chemists. Minnesota, USA. 24-48

Vivek, B.S., Krivanek, A.F., Palacios-Rojas, N., Twumasi-Afriyie, S and Diallo, A.O. 2008. Breeding Quality Protein Maize (QPM): Protocols for Developing QPM Cultivars. Mexico, D.F.:CIMMYT.

Wallace, J.C., Lopes, M.A and Paiva, E., Larkins, B.A. 1990. New Methods for Extraction and Quantification of Zeins Reveal a High Content of γ-Zeins in Modified *opaque-2* Maize. Plant Physiology. 92: 191-196.

Wendel, J.F., Stuber, C.W., Edwards, M.D and Goodman. 1986. Duplicated chromosome segments in maize (*Zea mays L.*): further evidence from hexokinase isozymes. Theoretical and Applied Genetics. 72: 178-185.

Yang, W.P., De Oliveria, A.C., Godwin, I., Schertz, K and Bennetzen, J.L. 1996. Comparison of DNA marker technologies in characterizing plant genome diversity: Variability in Chinese Sorghums. Crop Science.36: 1669-1676.

Yang, W., Zheng, Y., Ni, S and Wu, J. 2004. Recessive Allelic Variations of three Micro satellite Sites with in the O2 gene in Maize. Plant Molecular Biology Reporter. 22: 361 – 374.

APPENDIX A

LIST OF CHEMICALS

1. Agarose (sigma)
2. Bromophenol blue (sigma)
3. Chloroform (Qualigens)
4. Cetyl Trymethyl Ammonium bromide (CTAB)
5. dNTP's (Deoxy NucleosideTriphosphate)
6. Ethylene Diamine Tetra Acetic Acid (EDTA)
7. Ethidium bromide (10 mg / ml)
8. Icecold isopropanol
9. Isoamyl alcohol
10. Liquid Nitrogen
11. 2- Mercaptoethanol GR
12. NaOH pellets
13. NaCl
14. Nitric Acid (HNO_3 65%)
15. Phenol
16. Poly vinyl pyrrolidone
17. Proteinase K
18. RNase H
19. Taq polymerase
20. Sodium acetate
21. Sodium chloride
22. Tris base

23. 50 bp ladder
24. 100 bp ladder
25. 70 %Ethanol

APPENDIX B

BUFFERS AND STOCK SOLUTIONS

CTAB buffer 100ml

2.0 g CTAB (Hexadecetyl trimethyl- ammonium bromide)

10.0 ml M Tris pH 8.0

4.0 ml 0.5 M EDTA pH 8.0 (Ethylenediamine tetraacetic acid Di-sodium salt)

28.0 ml 5 M NaCl

40.0 ml H_20

1g PVP 40 (Polyvinyl pyrrolidone- vinyl pyrrolidine homopolymer,MW 40,000)

Adjust all to pH 8.0 with HCl and make up to 100 ml with H_2O

0.5 M Tris Buffer (pH 8.0)

Dissolved 60.55 g of Tris base in 400 ml of distilled water. Adjust pH to 8.0 by adding HCl. Adjust the volume to 500 ml with H_20. Sterilized using an autoclave.

1M EDTA (Ethylenediamine tetra acetic acid)

Dissolved 186.1 grams of EDTA, free acid in about 200 ml of distilled water. Adjust the pH to 8.0 with NaOH and make up the vol to 500 ml with distilled water. Sterilized by autoclaving.

Ethidium Bromide

Stock 20mg / ml can be prepared by dissolving 1gm of ethidium bromide in 50 ml of water.

Chloroform: Isoamyl alcohol (24:1)

Chloroform: isoamyl alcohol (24:1) were mixed and stored at room temperature.

Phenol: Chloroform: Isoamyl alcohol (25:24:1)

Phenol: Chloroform: isoamyl alcohol (25:24:1) were mixed and stored at room temperature.

TAE buffer (Tris / acetate / EDTA) 50X stock solution

242 g Tris base

57.1 ml Glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

Adjusted the pH to 8.3 with acetic acid and make up to the volume to 1 lit with distilled water. Sterilization is done by autoclaving.

TE buffer (pH 8.0)

10 mM Tris HCl

1 mM EDTA.

2 ml of 0.5 M Tris-Cl pH 8.0 was mixed with 0.2 ml of 0.5 EDTA, make up to the vol to 100 ml with sterile distilled water.

6X Gel loading buffer

0.25% (W/V) Bromo phenol blue

40% (W/V) sucrose in water

Dissolved 0.25g of Bromo phenol blue was mixed with 40g of sucrose, make up the vol to 100ml with distilled water.

RNase preparation

RNase buffer

A.1M Tris (pH 7.5)

B. 5M NaCl

Take 0.5ml of 1M Tris (final concentration 10mM) and 75 µl of 5M NaCl (final concentration 15mM) and make up the volume to 50 ml. Weighed 25 mg of ribonuclease H

into a tube and add RNase buffer to a final volume of 5 ml (so final concentration 5mg / ml). Kept the tube in a boiling water bath for 10 min, cool and made aliquots of 1 ml in 1.5 ml Eppendorf tubes and stored at -20^{0}C.

APPENDIX C

EQUIPMENTS USED

- Agarose gel electrophoresis system (CBS Scientific, USA)
- Autoclave
- Cuvettes
- Electronic balance
- Eppendorf tubes
- Freezer of -20^0 and -80^0C
- Gel Documentation System (Geneflash; SYNGENE, U.K)
- Glass hooks
- DNA Thermal Cycler (Eppendorf, Germany)
- Ice maker(Sanyo)
- Incubator(Sanyo)
- Incubator shaker 37^0C
- Microwave oven
- Microcentrifuge
- Microcentrifuge tubes
- Micropipettes
- Pasture pipettes
- pH meter
- Power supply unit
- UV-absorption spectrophotometer (Beckman DU 650 model)
- UV- transilluminator
- Vaccum centrifuge

- Vortex mixer
- Water bath