# THESIS APPROVAL

## GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Tropical Agriculture)
**DEGREE**

Tropical Agriculture
**FIELD**

Interdisciplinary Graduate Program
**PROGRAM**

**TITLE:** Improvement of Quality Protein Maize using Marker-Assisted Selection (MAS)

**NAME:** Mrs. Peeranuch Jompuk

**THIS THESIS HAS BEEN ACCEPTED BY**

_____ **THESIS ADVISOR**
( Associate Professor Wasana Wongyai, D.Agr )

_____ **COMMITTEE MEMBER**
( Associate Professor Somsak Apisitwanich, Dr.Agr.Sci. )

_____ **COMMITTEE MEMBER**
( Mrs. Chaba Jampatong, Ph.D. )

_____ **PROGRAM CHAIRMAN**
( Associate Professor Somnuk Wongthong, Ph.D. )

**APPROVED BY THE GRADUATE SCHOOL ON** _____

_____ **DEAN**
( Associate Professor Vinai Artkongharn, M.A. )

# THESIS

# IMPROVEMENT OF QUALITY PROTEIN MAIZE USING MARKER-ASSISTED SELECTION (MAS)

**PEERANUCH JOMPUK**

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Tropical Agriculture)
Graduate School, Kasetsart University
2007

Peeranuch Jompuk 2007: Improvement of Quality Protein Maize using Marker-Assisted Selection (MAS). Doctor of Philosophy (Tropical Agriculture), Major Field: Tropical Agriculture, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Wasana Wongyai, D.Agr. 68 pages.

Quality protein maize (QPM) controlled by *opaque-2* ($o_2o_2$) gene can help alleviate human malnutrition and reduce costs of animal feed because it contains higher tryptophan and lysine content in endosperm than normal maize.The *opaque-2* gene could be detected by molecular marker. The objectives of this study were to (i) improve inbred lines for quality protein maize (QPM) using marker assisted selection (MAS) and (ii) estimate general and specific combining ability (GCA and SCA) of QPM inbred lines. Three populations, Pop61$C_1$, Pop62$C_6$ and Pop65$C_6$ developed by the International Maize and Wheat Improvement Center (CIMMYT) were used. $S_0$-plants of preferred morphological characters were self-pollinated to produce $S_1$ to $S_3$ lines. For $S_1$-seed, the average tryptophan content in endosperm of QPM and non-QPM as detected by phi057 marker was 0.66% (for QPM), 0.38% (for non-QPM), 0.38% for Suwan 1 (a non-QPM) and 0.80% for the Opaque-2 standard variety. Moreover, those QPM and non-QPM plants detected by phi112 showed the same result of total protein and tryptophan content in endosperm. Ten $S_3$ inbred lines were examined for the *opaque-2* gene using the phi057 marker and they were crossed in a diallel method. The protein content in endosperm of these inbred lines, Opaque-2 and Suwan 1 had no significant differences among them. On the other hand, tryptophan content was significantly different. Results studied on grain yield of 45 $F_1$ hybrids showed that the superior tested hybrid gave 6.10 t $ha^{-1}$ which was not significantly different from the non-QPM hybrid. Protein content was not significant among tested hybrids, QPM population and non-QPM hybrids. Tryptophan content in endosperm of tested hybrids was higher than non-QPM. Inbred P10 had the best combination of GCA effects for grain yield. Moreover, inbred lines, P1, P7, P8 and P9 also gave positive GCA effects. Therefore, these inbred lines can be used as tester lines for the quality protein maize program or the positive GCA effect lines could be used as the lines to form new quality protein maize populations. The results showed that it would be developed QPM inbred lines from the cross P9 x P4. In addition, the best QPM hybrid could possibly be grown in Thailand without any differences in grain yield and has a greater advantage, in terms of grain nutrition, than normal maize.

_____ _____ ____ / _____/ _____

Student's signature Thesis Advisor's signature

# ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and profound gratitude to Assoc. Prof. Dr. Wasana Wongyai, my thesis advisor for guidance, support throughout this study and valuable suggestion for completely writing this thesis. I am also extends my appreciation and gratitude to Assoc. Prof. Dr. Somsak Apisitwanich and Dr. Chaba Jampatong, my graduate committees for their advice, suggestions and kindness to review this thesis as well as their encouragement. I am also thankful for the comments from Dr. Sorasak Maneekao, the graduate school representative.

I would like to gratefully thank Mr. Pichet Grudloyma, senior maize breeder from Nakhon Sawan Field Crops research Center, Thailand, for kindly providing the seeds of QPM populations used in this research. I am also thankful to Assoc. Prof. Dr. Amara Thongpan for her suggestion to write the publication in KU journal.

I also express my appreciation to Mr. Surapol Chowchong and the staff from the National Corn and Sorghum Research Center, Kasetsart University, Nakhon Ratchasima Province, for field assistance.

My graduate education was funded by Cooperative Research Network (CRN) Thailand, the financial support from this organization is gratefully acknowledged.

Finally, my deep thanks devotes to my parents, my husband “Asst. Prof. Dr. Choosak Jompuk”, my sons “Kanit” and “Kamnuan” for their love, care and support during my study.

Peeranuch Jompuk

April, 2007

# TABLE OF CONTENTS

**Page**

# LIST OF TABLES

# LIST OF TABLES (Continued)

# LIST OF FIGURES

# LIST OF FIGURES (Continued)

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ASI | anthesis-silking interval |
| bp | base pairs |
| cDNA | complementary DNA |
| cm | centimeter |
| CIMMYT | The International Maize and Wheat Improvement Center |
| CV | coefficient of variation |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleotide triphosphate |
| ELISA | enzyme-linked immunosorbent assay |
| FAO | Food and Agriculture Organization of the United Nations |
| FW | field weight |
| g | gram |
| GCA | general combining ability |
| h | hectare |
| kg | kilogram |
| LSD | least significant difference |
| MAS | marker assisted selection |
| MC | moisture content |
| mg | milligram |
| mM | millimolar |
| mm | millimeter |
| ng | nanogram |
| OPVs | open-pollinating varieties |
| QPM | quality protein maize |
| QTL | quantitative trait loci |
| RCBD | randomezed completely block design |
| RFLP | restriction fragment length polymorphism |
| SCA | specific combining ability |
| SR | shelling ratio |

## LIST OF ABBREVIATIONS (Continued)

| | |
|---|---|
| SSR | simple sequence repeat |
| t | ton |
| μM | micromolar |
| uORFs | upstream open reading frames |

# IMPROVEMENT OF QUALITY PROTEIN MAIZE USING MARKER-ASSISTED SELECTION (MAS)

## INTRODUCTION

Maize (*Zea mays* L.) is a major cereal crop for livestock feed, human consumption and several industrial uses as well. Because of all these benefits, maize has become out of the favorite crops for farmers in developing countries. Generally, maize has low protein quantity and quality. Maize kernels not only contain about 9.5 percent of average protein quantity but also two essential amino acids, lysine and tryptophan are also low in either. Both essential amino acids could not be synthesized by human and other simple-stomach animals such as pigs and poultry. Generally, half of the protein in a kernel in common maize is composed of protein types that contain almost no lysine or tryptophan (FAO, 1992).

The nutritional superiority of quality protein maize (QPM) materials, both in human and animal nutrition, especially in monogastric animals, has been clearly and repeatedly demonstrated in studies carried out in several countries around the world with infants, young children and adults (Bressani, 1992) and in animal feeding trials (Gevers and Lake, 1992). Breeders who use conventional breeding methods to accomplish QPM line conversion face two main constraints. First, breeders are unable to determine whether if young plants in the field produce ears with elevated levels of lysine and tryptophan. To identify QPM, maize grain samples must be sent to the laboratory for evaluation. To identify heterozygous plants, breeders have to do self-pollination all plants in the population and sow their seeds in the next season to observe the QPM phenotype. According to this procedure, the QPM genotypes ($o_2o_2$) from previous generation are obtained (Dreher *et al*., 2000). Molecular markers provide a potential solution to this problem. DNA markers that are linked to a particular trait in a plant genome can inform breeders with the presence of desirable bands for each plant. If the presence of the desirable alleles can be confirmed through the use of markers, breeders do not have to resort to costly and time consuming

phenotypic evaluation to determine whether or not the alleles are present. (Weber and May 1989; Melchinger, 1990) This is fruitful for breeders to discard plants non-QPM prior to pollination. The breeding population is reduced and it saves both time and money. Second, it also helps breeders select either homozygous or heterozygous plants. (Ribaut and Hoisington, 1998).

# OBJECTIVES

The objectives of this study were (1) to improve inbred lines for quality protein maize (QPM) using marker-assisted selection (MAS) and (2) to estimate general combining ability (GCA) and specific combining ability (SCA) for grain yield and some agronomic traits.

# LITERATURE REVIEWS

## Quality protein maize (QPM)

In 1963, researchers at Purdue University discovered that a recessive mutation in the gene known as *opaque-2* resulted in maize kernels with elevated levels of two essential amino acids, lysine and tryptophan. This finding was of more than academic interest. Despite its widespread use as human food and animal feed, maize does not provide an adequate amount of lysine and tryptophan, which humans and monogastric animals cannot synthesize for them. Breeders subsequently tried to incorporate the mutant *opaque-2* allele and its associated phenotype into their breeding materials, initially with limited success. Early varieties of maize with the *opaque-2* mutation suffered from numerous problems, including soft endosperm (which is unappealing to many consumers), increased susceptibility to certain grain pests and ear rots, slower drying time in the field and, perhaps most importantly, a 10-15% drop in yield (Villegas, 1994). Over time, breeders found modifier genes that restored the desirable hard endosperm phenotype in materials containing the recessive *opaque-2* mutation. These agronomically acceptable, nutritionally enhanced materials later came to be known as quality protein maize (QPM). Recognizing the potential benefits of nutritionally enhanced maize for developing countries, in the late 1960s CIMMYT launched a QPM breeding program. Over 30 years, numerous elite QPM materials were developed using conventional selection methods, including both improved open-pollinating varieties (OPVs) and elite inbred lines (CIMMYT, 1999). While the QPM materials have been undergone further improvement, CIMMYT breeders also are interested in transferring the mutant *opaque-2* allele and the associated modifiers into other elite lines that lack the QPM phenotype. Typically this is done using some type of backcross breeding scheme.

## Genetics

Several mutants have been detected over the past 30 years that can favorably modify characteristics of the maize endosperm protein by elevating levels of two

deficient amino acids, namely lysine and tryptophan. The value, use and inheritance characteristics of such genes, however, vary tremendously. The first discovered high lysine mutant was *opaque-2* ($o_2$) (Mertz *et al*., 1964). The biochemical effects of *floury-2* ($fl_2$) mutant were discovered (Nelson *et al*., 1965). Search for new and better genes has continued, and to date, additional mutants are known that can improve protein quality of corn endosperm protein. Some such mutants worth mentioning are *opaque-7* ($o_7$), *opaque-6* ($o_6$) and *floury-3* ($fl_3$) (Mertz, 1994).

Several research workers worldwide have studied the effects of high quality protein maize mutants. More studies have been conducted with $o_2$ and $fl_2$ mutants and thus much of the available information in literature is available on these two mutants. Both $o_2$ and $fl_2$ mutants change the amino acid profile, thus resulting in an increase of lysine and tryptophan. The *opaque-2* mutant still ranks at the top of all single viable mutants and normal cultivars in lysine and trytophan, although other high lysine types have been found (Mertz, 1986). Isolation and characterization of the *opaque-2* gene revealed that it encodes a transcriptional factor that regulates the expression of zein genes and a gene encoding a ribosomal inactivating protein (Schmidt *et al*., 1990; Lohmer *et al*., 1991; Bass *et al*., 1992). The *opaque-2* gene significantly reduces the level of 22-kD alpha-zeins while increasing the content of non-zein proteins particularly, EF-1 alpha, which is positively correlated with lysine content in the endosperm (Habben *et al*., 1995).

**Inheritance**

The specific chromosome and position on the chromosome is known for some of the mutants. The $o_2$ mutant is located on chromosome 7, $fl_2$ on chromosome 4, *opaque-7* on chromosome 10 and *floury-3* on chromosome 8. The mutants $o_2$ and $o_7$ are completely recessive and manifest their biochemical effects on zein synthesis only when present in homozygous recessive condition. The two floury mutants, $fl_2$ and $fl_3$, are semi-dominant and exhibit variable expression for kernel opacity and protein quality depending on the presence of one or more recessives in the triploid endosperm (Vasal, 1994).

**Pleiotropic and other secondary effects**

It is well known that new genes and gene combinations that bring about drastic alterations in either plant or kernel characteristics also produce several secondary or undesirable effects. The low prolamine or high lysine mutants are not exceptions. These mutants, besides reduced zein synthesis and altered concentration of lysine and tryptophan, also affect several other biochemical changes. In addition to biochemical traits, the high lysine mutants adversely affect a whole array of several agronomically important traits including kernel characteristics. The poor agronomic performance of QPM has been reported by several research workers. The $o_2$ and other mutants affect dry matter accumulation adversely, thus resulting in lower yields due to decreased endosperm size. The kernel phenotype is also changed to a soft chalky dull appearance not liked by many growers in the developing countries. The kernels dry slowly following physiological maturity of the grain and have a higher incidence of ear rots. Potassium, zinc and oil content may show an increase in opaque over the normal. Other changes include thicker pericarp, larger germ size, reduced cob weight, reduced color intensity in yellow corns, and reduced kernel weight and density. Such effects may, however, differ in different genetic backgrounds and sometimes within the same background, especially if the material is structured on a family basis (Vasal, 1994).

**Modifying genes**

Modifying genes or genetic modifiers are a series of genes which apparently do not have any effect of their own but they do interact and modify the expression of quality protein maize mutants. The effect could be on any trait, but more pronounced changes have been observed in regards to kernel phenotype. Though any quality protein mutant could be involved in an interaction with a modifying gene complex, the greatest effort has been spent on the *opaque-2* gene. The reasons obvious, as no other mutant offers any additional advantage over the *opaque-2* gene system. Its inheritance is simple and not complicated by any dosage effect for kernel opacity or protein quality (Vasal, 1994). The role of genetic modifiers in altering kernel

phenotype has been studied much more extensively than any other traits. The modified *opaque-2* kernels have been observed and studied by several breeders working on quality protein breeding programs (Lopes and Larkins, 1994). The pattern of kernel modification can be either regular or irregular. In regular patterns, the modified fraction increase progressively from the crown towards the base of the kernel. In irregular patterns, the translucent fraction may be present as a band, scattered, resembling a bridge and translucent base. From a practical standpoint, only regular patterns appear more important and have been emphasized by most maize breeders. Various aspects of genetic modifiers have been increased in earlier publications (Vasal *et al*., 1984) and only salient features will be discussed. Quality consideration of genetically modified *opaque-2* kernels is extremely important. Several reports indicate that soft opaque and modified opaque do not differ in protein quality. However, the CIMMYT scientists and that of other research workers are quite contrary to the above findings. Protein quality and kernel modification or vitreousness generally is negatively correlated. Exceptions, however, occur when protein quality of the samples is monitored. The results may be frustrating initially, but as the accumulation of favorable modifiers continues, fewer samples in each generation would need to be discarded. It has been amply demonstrated that good kernel modification and acceptable protein quality can be combined. Perhaps in some materials one may have to sacrifice a slight decrease. Protein content of modified kernels the vitreous fraction is generally higher in protein content compared to the soft fraction (Vasal *et al*., 1980).

Accumulation of genetic modifiers changes physical characteristics of *opaque-2* kernels with respect to vitreousness. This may or may not always be reflected in increased kernel density and weight. Variation and opportunities for selection do exist in materials undergoing such selection process resulting in better performing materials approaching standard genotypes. The expression of genetic modifiers may be affected by maternal influence. Endosperm is a triploid tissue, one may expect maternal influence since two doses of modifying alleles are contributed by the maternal parent and only one by the paternal parent. Reciprocal differences in crosses between soft opaque and modified opaque have been reported by some workers. Other factors such

as genetic background and kernel texture could also alter phenotypic manifestation of modifying genes. Flint genetic backgrounds generally exhibit a higher frequency of modified kernels (Vasal, 1994). The work at CIMMYT and at other institutes has indicated quantitative nature of modifying gene system. The additive genetic variation seems to be more important in controlling kernel vitreosity in *opaque-2* maize. Bjarnason and Vasal (1992) have reviewed several studies which show that the modified texture of modified kernels is governed by the quantitative genetic system with additive gene effects playing an important role in controlling this trait; however, in other cases either a single gene or perhaps a few genes may be involved in kernel modification. Single gene modifiers will have a definite role in conversion programs provided protein quality is maintained and that they can facilitate in overcoming problems associated with the *opaque-2* system.

**Germplasm and source materials**

The soft *opaque-2* germplasm is available in the form of broad-based populations, synthetics, $o_2$ converted lines and hybrids. The International Maize and Wheat Research Center (CIMMYT) converted several genotypes to soft *opaque-2* type, but now has only a few soft $o_2$ populations because of continuing emphasis on include Tuxpeno *opaque-2* (Population 37), CIMMYT *opaque-2* composite, Composite 1 (highland adaptation), Composite K (tropical adaptation) and Puebla *opague-2* composite (Vasal, 1994). The University of Illinois, Urbana, has developed some synthetic populations which they have used for recurrent selection studies (Dudley *et al*., 1975), including SSSS-$o_2$, disease oil synthetic (DO-$o_2$), Syn. A-$o_2$, and Syn. B-$o_2$. Another breeding population BSAA-$o_2$ was developed in Iowa by Loesch (Loesch, 1979). BSAA-$o_2$ is an improved *opaque-2* version of broadly based Iowa synthetic AA (BSAA). Superior 10% of $S_1$'s for yield, percentage protein, and percentage of lysine were recombined to form BSAA$o_2$-$C_1$. Thailand has *opaque-2* versions of Thai *opaque-2* composite and Thai *opaque-2* composite #3 (Sriwatanapongse *et al*., 1974). As regards modified *opaque-2* types, CIMMYT has perhaps the largest collection. Most of this germplasm was developed and improved over a span of almost 20 years. The materials were developed at CIMMYT include

several QPM populations and pools. These breeding populations possess different ecological adaptation, maturity grain color and texture. (Vasal, 1994).

**Combined interaction of *opaque-2* gene and genetic modifiers to develop QPM germplasm**

This approach exploits the use of the $o_2$ gene in combination with the genetic modifiers to overcome major defects in developing agronomically desirable QPM cultivars with modified texture of the grain. Recognizing the magnitude and the importance of the problems, a desired association of the *opaque-2* gene with genetic modifiers can be sought. The development of hard endosperm *opaque-2* maize was initialed from the existing germplasm in CIMMYT, which at that time possessed soft endosperm texture. Initial efforts were directed at encountering variations for partially modified ears in *opaque-2* materials during the conversion process, improvement of existing *opaque-2* composites, formation of *opaque-2* population crosses and during seed increase of *opaque-2* materials for international testing. Selected partially modified ears were shelled individually, followed by section of the best modified kernels from each ear. The selected kernels were planted on an ear to row basis. The best plants were self-pollinated and at harvest good modified ears were selected for further work. Both between and within ear selection was emphasized, coupled with laboratory analysis to build up the raw QPM germplasm that provided the future base for the QPM work. The initial effort was not only difficult, but quite tedious and at times very frustrating (Vasal, 1994).

**Recurrent selection in QPM germplasm**

Some of the QPM donor stocks described in the proceeding section seemed to be good candidates for the recurrent selection program. Full-sib recurrent selection using progeny testing data was practiced in these populations for at least two to five cycles. Upon completion of the reorganization process, all the newly formed QPM gene pools and populations, which were homozygous for the *opaque-2* gene, were subjected to intra-population improvement schemes. The QPM gene pools were

handled by a modified half-sib system. The half-sib system as periodically interrupted for an inbreeding phase to expose and discard deleterious recessives, improve inbreeding stress tolerance, identify superior $S_1$ lines for inbred development and to permit sampling of new lines from the more advanced cycles on a continuous basis. The QPM populations were improved by modified full-sib family selection (Vasal, 1994).

**QPM improvement using conventional breeding methods**

Breeders who use conventional breeding methods to accomplish QPM line conversion face two main constraints. First, breeders looking at young plants in the field are unable to determine if they will produce ears with elevated levels of lysine and tryptophan. Therefore, either they wait until the end of the season to identify desirable plants, or they pollinate an unnecessarily large number of plants because they do not know which ones carry the mutant *opaque-2* allele. Simple inspection of mature ears will not always be sufficient to identify QPM materials. To identify QPM plants definitively, maize grain samples must be sent to the laboratory for evaluation. In the first procedure, the levels of nitrogen (a proxy for protein) and tryptophan present in the grain endosperm are directly measured. In the second procedure, enzyme-linked immunosorbent assay (ELISA) analysis is used to quantify the concentration of the EF1A protein, which is highly correlated with the level of lysine present in the grain endosperm. Since lysine and tryptophan almost always occur in a 4:1 ratio in QPM materials, only one of the two laboratory procedures is needed to determine the phenotype of the plant. Second, the mutant allele that confers elevated levels of lysine and tryptophan is recessive, so heterozygous plants containing one copy of the mutant *opaque-2* allele cannot be identified phenotypically, because they do not express altered amino acid levels. To identify heterozygous plants, breeders often self-pollinate all of the plants in a population and plant the seed from the self-pollinated plants in the next season to allow the QPM phenotype to be expressed; then they can go back and identify which plants in the previous generation carried the mutant *opaque-2* allele (Dreher *et al*., 2000).

**QPM line conversion using marker assisted selection (MAS)**

The new tools needed to reinvigorate the science of plant breeding will come from the rapidly evolving field of biotechnology. Some of these new tools allow breeders to do things that cannot be done using conventional breeding methods. For example, MAS and DNA fingerprinting techniques can potentially increase the efficiency of traditional breeding programs by speeding up the time of varietals release, reducing plant population requirements and eliminating costly field evaluation. Biotechnology-based breeding tools have many potential applications, but as with any new technology, research managers must determine the circumstances under which the benefits are likely to justify the considerable costs. Conventional breeders are forced to rely on phenotypic evaluation, which does not always accurately indicate the underlying information present in a plant's genome. Environmental influences and genetic interactions can obscure the presence or absence of specific alleles, making it difficult for breeders to identify the plants that they really seek. Even in cases in which a plant's phenotype provides reliable evidence about the plant's underlying genetic characteristics, phenotypic evaluation can be costly and/or time consuming. Molecular markers provide a potential solution to some of these problems. If the presence of the desirable alleles can be confirmed through the use of markers, breeders do not have to resort to costly and time consuming phenotypic evaluation to determine whether or not the alleles are present. Among the many different types of molecular markers that can be used in plant breeding, for reasons of cost and simplicity, researchers at CIMMYT often use a particular type of marker known as simple sequence repeat (SSR) markers. SSRs are short, repeated DNA sequences found in most plant genomes (Weber and May 1989; Melchinger, 1990). Since the number of SSRs present at a particular location in the genome tends to differ among plants, SSRs can be analyzed to determine the absence or presence of specific alleles. Primers that flank a region containing SSRs within the *opaque-2* gene are then used to amplify the sample using the polymerase chain reaction (PCR) method. When the number of repeated sequences between the flanking primers differs in the normal *opaque-2* and mutant *opaque-2* alleles, the amplified DNA fragments vary in length. By electrophoretically separating the amplified DNA

fragments on gels and analyzing them, researchers can determine if the plant possesses two copies of the normal allele, two copies of the desirable mutant *opaque-2* allele, or one copy of each allele. In the context of QPM line conversion, marker assisted selection offers the possibility of overcoming the two main constraints faced by conventional breeders. First, since marker analysis can be done using DNA samples extracted from leaf tissue of very young plants, it allows QPM plants to be identified early in the breeding cycle. This allows the breeder to discard plants that do not contain mutant *opaque-2* alleles prior to pollination, reducing the size of the breeding population and saving both time and money. Second, with molecular markers, breeders can distinguish between homozygous recessive plants that carry two copies of the mutant *opaque-2* allele and heterozygous plants that carry only one copy (Ribaut and Hoisington, 1998).

**Marker-assisted selection in crop plants**

Genetics mapping of major genes and quantitative traits loci (QTL) for many important agriculture traits is increasing the integration of biotechnology with the conventional breeding process. Exploitation of the information derived from the map position of traits with agronomical importance and of the linked molecular markers, can be achieved through marker assisted selection (MAS) of the traits during the breeding program. Application of this procedure have shown that the success of MAS depends on several factors, such as the genetic base of the trait, the degree of the association between the molecular marker and the target gene and the genetic background in which the target gene has to be transferred. MAS for simply inherited traits is gaining increasing importance in breeding program (Francia *et al*., 2005)

**Marker assisted selection for *opaque-2***

The *opaque-2* gene has been cloned by several methods since 1987 (Schmidt *et al*., 1987; Motto *et al*., 1988; Hartings *et al*., 1989; Schmidt *et al*., 1990). This has provided the foundation for the genetic, variation, evolution and application studies related to the *opaque-2* mutant. For example, Bernard *et al*. (1994) reported characterization of several mutant ($o_2$) alleles at the DNA, RNA and protein levels. They proposed the occurrence of a broad spectrum of $o_2$ mutations producing different sizes of $o_2$ proteins. They further proposed a nomenclature of $o_2$ genes based on restriction fragment length polymorphism (RFLP), transcripts and products of the various alleles. In 1994, Kata *et al.* constructed a technical system for RFLP marker-assisted selection (MAS) at the *o2* locus by using an $o_2$ cDNA as a probe. This probe hybridized to genomic DNA digested by HindIII and allowed identification of $O_2O_2$, $O_2o_2$ genotypes. Hartings *et al*. (1995a and 1995b) divided 10 recessive ($o_2$) alleles of independent origin ($o_2$-R, $o_2$-m(r), $o_2$-Columbian, $o_2$-Agroceres, $o_2$-261, $o_2$-mh, $o_2$-33, $o_2$-G$o_2$-Charentes, $o_2$-Italian, and $o_2$-Crow) into 6 polymorphic groups by means of Southern analysis with 2 molecular probes corresponding to the 5' and the 3' end of the $O_2$ cDNA. By comparing the genomic sequences of recessive alleles with that of the wild type, they found nucleotide substitution, insertion, and deletion in $o_2$ alleles.

Hamada *et al*. (1982) described a novel repeated element termed simple sequence repeat (SSR), which is a short tandem repeated DNA sequence. These sequences spread widely throughout the nuclear genome of eukaryotes. Microsatellite 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).

In 2001, the primer sequences of 3 SSR markers; phi112, umc1066 and phi057-all at the $o_2$ locus, were released at the Web site of www.agron.missouri.edu, which facilitates the study and application of the $o_2$ gene. The phi112 SSR is located between the G box and 3 upstream open reading frames (uORFs) (Schmidt *et al*., 1990) in the leader sequence of the $o_2$ gene and its mutation can affect transcription of

the *o2* gene. The umc1066 and phi057 SSRs are located in exon 1 and exon 6, respectively. These are the 2 largest exons among 6 exons within the *o2* gene. Mutations at these 2 exons will increase or decrease the number of prolines in the *o2* protein and affect the activity of the *o2* protein as a transcriptional activator because the position of the proline residue influences the direction and degree of turns in the 3-dimensional structure of the *o2* protein (Yang *et al*., 2004). 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 2 hypervariable regions in the exon 1. Therefore, using phi112, umc1066 and phi057 markers, the allelic SSR variations in the leader region and the 5' region and 3' region of the *o2* gene can represent the variation within the *o2* gene.

**Inbred lines development and evaluation**

Since the final product desired from recent maize breeding program is single cross hybrids, evaluation of inbred line performance and their combining ability with tester or other inbred lines are important. Good performance of inbred lines, i.e.: high yields, are required in order to produce single-cross seed efficiently and economically. Furthermore, a high combination ability of inbred lines is required to produce good hybrid. Several studies have shown that correlation of an inbred trait with the same trait in the hybrid is relatively high, except for yield. Sprague (1964) observed that correlation of inbred lines with mean of all their hybrids progeny is higher than correlation of inbred lines with their specific single cross hybrids. Lamkey and Hallauer (1986) observed that selection for high-yielding inbreds would tend to select lines that are above average for hybrid yields.

The QPM hybrid initiative at CIMMYT was introduced in 1985, as the QPM hybrids offered several advantages in relation to (a) exploitation of heterosis; (b) easy in maintaining seed purity in contrast to open-pollinated QPM cultivars; (c) requirement for minimum protein quality monitoring as long as the the purity of parental lines is ensured; (d) uniformity and stability in kernel modification of hybrids. The last point is particularly important since not many developing countries

have well-established laboratories to analyze protein quality (Vasal *et al*., 2001). Analysis of combining ability in QPM germplasm resulted in identification of potential parental lines in QPM hybrid breeding (Vasal *et al*., 1993a and 1993b). Concurrently, inbred line development efforts have been strengthened. Several QPM hybrid combinations were derived and tested in international testing program at multiple locations in Asia, Africa and Latin America. Some of the QPM hybrids performed equal to or better than some of the local checks included in trials. The encouraging performance of QPM hybrids in various countries stimulated intensive efforts, particularly in the last decade, to derive superior hybrid combination (Prasanna *et al*., 2001)

# MATERIALS AND METHODS

## Materials

### 1. Plant materials

The seeds of six quality protein maize (QPM) populations developed by the International Maize and Wheat Improvement Center (CIMMYT), Mexico, namely Pop61$C_1$ (Tropical maize Early Flowering Yellow Flint; TEYF), Pop62$C_6$ (Tropical maize Late Flowering White Flint; TLWF), Pop63$C_2$ (Tropical maize Late Flowering White Dent; TLWD), Pop64$C_1$ (Tropical maize Late Flowering White Dent; TLWD), Pop65$C_6$ (Tropical maize Late Flowering Yellow Flint; TLYF) and Pop66$C_0$ (Tropical maize Late Flowering Yellow Dent; TLYD) were kindly provided by the Nakhon Sawan Field Crop Research Center, Nakhon Sawan Province, Thailand. The seeds were sown at the National Corn and Sorghum Research Center, Kasetsart University, Nakhon Ratchasima Province, Thailand.

$S_0$-plants of three populations preferred morphological characters, i.e., early flowering, short anthesis-silking interval (ASI), healthy plants and the other desirable agronomic traits were selected. All selected plants were self-pollinated to produce $S_1$ lines. Selected $S_1$ lines from each population were sown in September, 2003. Ten selected plants from each $S_1$ line were self-pollinated to produce $S_2$ lines. Then, $S_2$ lines were sown in May, 2004 and selected $S_2$ plants were self-pollinated to produce $S_3$ lines.

Ten $S_3$ inbred lines with opaque-2 gene ($o_2o_2$), high tryptophan content in endosperm, synchronize the flowering date and healthy plant were selected. They were P1-P4, P5-P8 and P9-P10 inbred lines from Pop65$C_6$, Pop61$C_1$ and Pop62$C_6$, respectively. The pedigree of $S_3$ inbred lines are shown as follow:

P1 = Pop65$C_6$-$S_3$-11-1-1
P2 = Pop65$C_6$-$S_3$-37-1-1

P3 = Pop65$C_6$-$S_3$-74-4-1

P4 = Pop65$C_6$-$S_3$-82-1-1

P5 = Pop61$C_1$-$S_3$-22-4-1

P6 = Pop61$C_1$-$S_3$-63-2-1

P7 = Pop61$C_1$-$S_3$-91-4-1

P8 = Pop61$C_1$-$S_3$-100-4-1

P9 = Pop62$C_6$-$S_3$-48-1-1

P10 = Pop62$C_6$-$S_3$-99-4-2

**2. PCR Primers**

Three simple sequence repeat (SSR) markers were found for the *opaque-2* gene and form the basis for three PCR-based SSR markers mapping to the short arm of chromosome 7, bin 7.01. SSR markers, phi057 and phi112, developed at Pioneer Hibred and umc1066 developed at the University of Missouri, Columbia, USA give amplification products of about 140-160 bp (Chin *et al*.,1996). Primer sequences are shown as follow:

phi057

F 5'- CTCATCAGTGCCGTCGTCCAT-3'

R 5'- CAGTCGCAAGAAACCGTTGCC-3'

phi112

F 5'- TGCCCTGCAGGTTCACATTGAGT-3'

R 5'- AGGAGTACGCTTGGATGCTCTTC-3'

umc1066

F 5'- ATGGAGCACGTCATCTCAATGG-3'

R 5'- AGCAGCAGCAACGTCTATGACACT-3'

These primers were synthesized by KU-VECTOR Custom DNA Laboratory, Kasetsart University.

## 3. Chemicals

- Hexane
- Boric acid
- Hydrochloric acid
- Sodium hydroxide
- sodium thiosulfate
- Methyl red
- Bromocresol green
- Sodium acetate anhydrous
- Sulfuric acid
- Glacial acetic acid
- Ferric chloride hexahydrate purified
- Acetic anhydride
- DL-Tryptophan
- Papain enzyme

## 4. Equipments

- Analytical balance
- Centrifuge
- Culture tube polystyrene with screw cap, 13 x 100 mm
- Cyclone mill
- Erlenmeyer flask, 1000 ml
- Volumetric flasks, 500 ml and 2000 ml.
- Micrometric syringe pipettes 0.5 to 5.0 ml
- pH meter
- Pipet tip, 0.1-1 ml
- Pyrex graduated cylinder 10 ml, 500 ml, 1000 ml and 2000 ml
- Rainbow buffer, pH 4, pH 7 and pH 10
- Shaker, Vortex-Genie 2
- Soxhlet extraction

- Spectrophotometers
- Incubators

## Methods

### 1. Field experiment

**Season 1** (March-July, 2003)

Six QPM populations were sown about 4,000 plants in each population at the National Corn and Sorghum Research Center (Suwan Farm), Pakchong, Nakon Ratchasima Province, Kasetsart University, to select the early flowering, short anthesis-silking interval (ASI), healthy plants and the other desirable agronomic traits. Three populations were selected for QPM identification by marker-assisted selection (MAS). The selected populations were Pop61$C_1$, Pop62$C_6$ and Pop65$C_6$. With the result of MAS, all homozygous recessive plants ($o_2o_2$) were self-pollinated to produce $S_1$ lines.

**Season 2** (September, 2003-January, 2004)

Twenty-five selected $S_1$ lines from each selected population were sown two rows in each line. Ten selected plants of each $S_1$ line were self-pollinated.

**Season 3** (May-September, 2004)

Two rows of ten $S_2$ lines in each population were grown. Genomic DNAs of selected $S_2$ plants which were good agronomic characters (18, 21 and 18 plants from Pop61$C_1$, Pop62$C_6$ and Pop65$C_6$, respectively) were extracted and genotypes were identified using the useful SSR primer. The seeds of $o_2o_2$ plants were advanced to $S_3$ lines.

**Season 4** (May-September, 2005)

The ten selected $S_3$ lines ($o_2o_2$) from three populations were made a diallel cross using fixed model method IV (Griffing, 1956). Forty-five $F_1$ hybrids were obtained.

Spacing of corn plant grown in season 1 to 4 was 75 cm between rows and 25 cm between plants within rows.

**Season 5** (October, 2005-February, 2006)

Forty-five hybrids were tested for their yielding ability and agronomic traits. The diallel cross yield trial consisted of 45 $F_1$ hybrids. Two single cross hybrids (SW4452 and NK40) and two QPM populations ($Pop62C_6$ and $Pop65C_6$) were used as check varieties. The experiment was conducted in a 7x7 simple lattice designs, with two replications. Each plot consisted of two 5-meter rows with 75 cm between rows and 25 cm between plants within rows. Basal fertilizer 15-15-15 was applied at the rate of 312 kg $ha^{-1}$ before planting. Atrazine mixed with Pendimethalin, a pre-emergence herbicide, was used a rate of 4 kg $ha^{-1}$ and 4 l $ha^{-1}$, respectively. After 2 weeks, plants were thinned to 1 plant $hill^{-1}$ or a population size of 53,331 plants $ha^{-1}$. Top-dressing was done at the $4^{th}$ week with 312 kg $ha^{-1}$ of ammonium sulfate (21-0-0). The breeding scheme of extraction QPM inbred was shown in Figure 1.

Ten $S_3$ inbred lines were sown for their yielding ability in randomized complete block design with 2 replications.

All field experiments were conducted at the National Corn and Sorghum Research Center, Pakchong, Nakon Ratchasima Province, Kasetsart University.

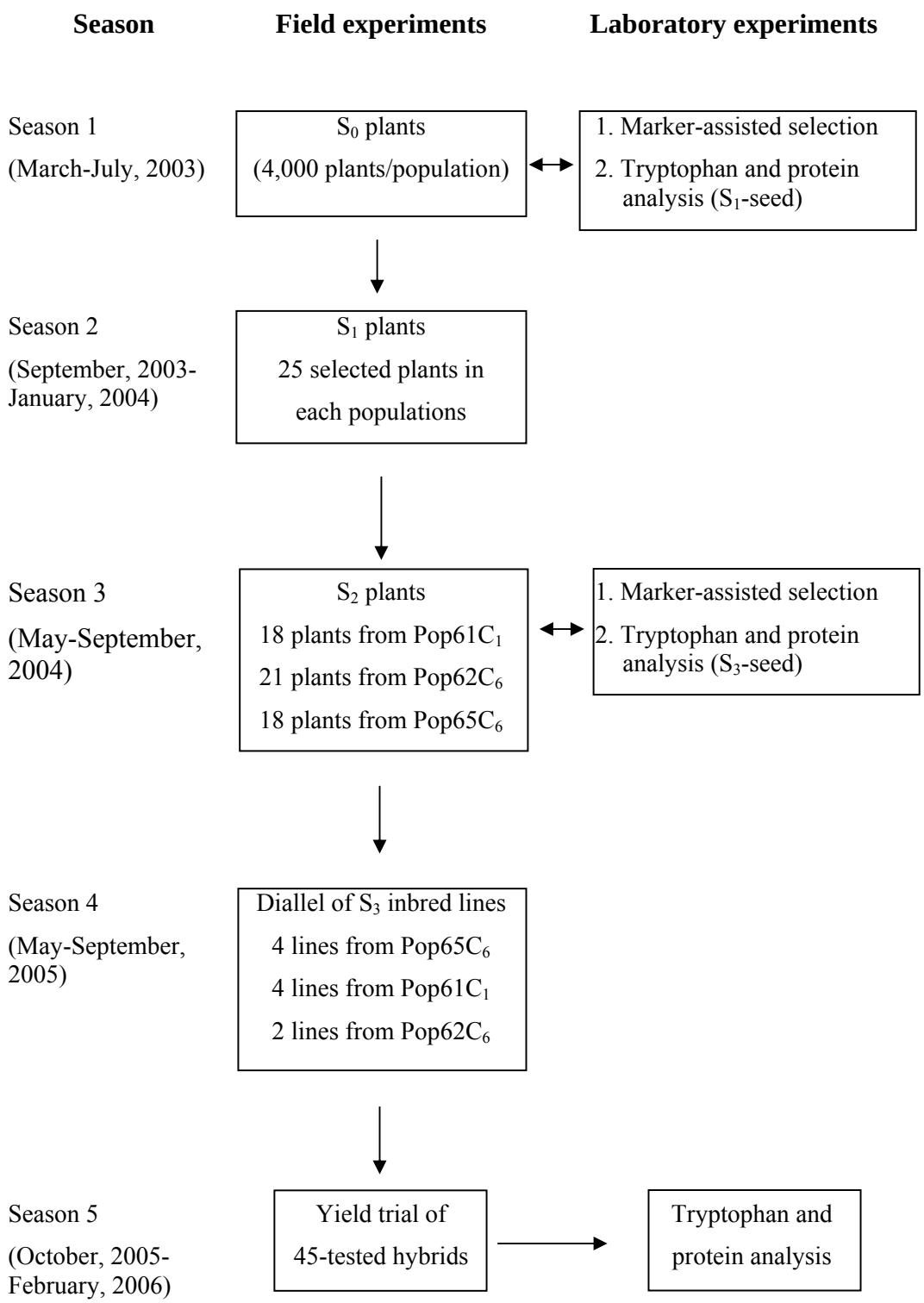



**Figure 1** Breeding scheme of extracted QPM inbred lines.

**2. Data recorded**

Days to anthesis and silking: Days to anthesis defined as numbers of days from planting to 50 percent of plants in the plot were shading of the pollens. Days to silking defined as numbers of days from planting to 50 percent of plants in the plot were displaying visible silk.

Plant and ear heights: Plant height was measured after anthesis, from the ground level to the collar of the flag leaf. Ear height was measured from the ground level to the highest ear-bearing node on 6 competitive plants.

Field weight: The weight of ears harvested from every plant with in plot. It was measured in kilogram per plot.

Grain moisture content: Five random ears of each plot were shelled and moisture content of the bulk grain samples were measured by using moisture tester.

Shelling ratio: Five random ears of each plot were weighed and then shelled. Furthermore, grains of the five samples were also weighted. Shelling ratio was calculated with the formula:

$$\text{Shelling ratio} = \frac{\text{Weight of sample grains}}{\text{Weight of sample ears}}$$

Grain yield: Grain yield was measured based upon field weight per plot (FW), grain moisture content (MC) at harvest and shelling ratio (SR). This was calculated in ton per hectare and adjusted to 15 percent standard moisture content with the formula:

$$\text{Grain yield} = \frac{\text{FW x } (100 - \text{MC})}{100 - 15} \text{ x SR x } \frac{10{,}000}{\text{PlotSize}} \text{ x } \frac{1}{1000}$$

## 3. Laboratory experiment

### Marker-assisted selection

#### $S_0$-plant

The leaves of $S_0$-plants were collected from 40, 35 and 30 plants of $Pop61C_1$, $Pop62C_6$ and $Pop65C_6$, respectively, and two plants of a non-QPM (Suwan1) and opaque-2 variety ($o_2o_2$). DNA extractions from collected leaves were done for marker-assisted selection by molecular markers. All $S_0$-plants and control plants were self-pollinated and mature ears were harvested to determine tryptophan and protein content in the endosperm.

#### $S_2$-plant

The selected $S_0$ plants containing opaque-2 were grown and self-pollinated to $S_1$ lines. In the next season, $S_2$ lines with good agronomic characters were obtained by self-pollination. The leaves of $S_2$-plants (containing opaque-2 gene in S0 plant) were collected from 18, 21 and 18 plants of $Pop61C_1$, $Pop62C_6$ and $Pop65C_6$, respectively. All selected plants were self-pollinated. The mature ears were harvested to determine tryptophan and protein content in the endosperm.

#### $S_3$-plant

Ten $S_3$ lines with good plant type were selected. They included four inbred lines (P1-P4) from $Pop65C_6$, another 4 inbred lines (P5-P8) from $Pop61C_1$ and the other 2 inbred lines (P9-P10) from $Pop62C_6$. DNA was extracted from 150-250 mg fresh leaves of each inbred line. The $o_2o_2$ of these inbred lines were confirmed by the phi057 marker.

**DNA extraction**

DNA was extracted from 150-250 mg fresh leaves of each selected line using the method described by Agrawal *et al.* (1992).

**Amplification**

The component of PCR was used as follow:

| STOCK | final concentration |
|---|---|
| ddH2O | — |
| *Taq* Buffer (10X; Mg-free) | 1X |
| MgCl2 (50 mM) | 2.5 mM |
| dNTP Mix (2.5 mM each) | 150 µM each |
| *Taq* Enzyme (5 U/µl) | 1 U |
| Glycerol (100%) (Optional) | 10 % |
| Primers, F + R (1.0 µM each) | 0.25 µM each |
| DNA (10 ng/µl) | 5.00µl |

The PCR program was set as follow:

| 1 Cycle of: | 30 Cycles of | 1 Cycle of: |
|---|---|---|
| 93°C for 1 min | 93°C for 30 sec<br>X°C for 1 min( X ranges between 50-68°C)<br>72°C for 1 min | 72°C for 5 min |

**Gel electrophoresis**

The polymorphism detected when using phi057 usually needs polyacrylamide gel to be properly resolved using 6% denaturing polyacrylamide gel (19:1). But phi112 and umc1066 is resolved using horizontal agarose (CIMMYT, 2000).

**Protein and tryptophan Determination in endosperm**

The analysis of protein and tryptophan content in the seed of $S_1$, $S_3$ lines and the derived $F_1$ hybrids were analyzed as described by Villegas and Mertz (1971).

**Sample Preparation**

1. Take a random sample of 30 seeds as representative of each ear.
2. Soak seeds in distilled water for approximately 20 minutes. Peel off the pericarp and remove the embryo with tweezers and scalpel.
3. The remaining material of the kernel is considered endosperm tissue. Air-dry the endosperm sample overnight.
4. Grind each sample of endosperm, at the 0.5 mm setting of a cyclone mill. Each sample is placed in a commercial filter paper envelope (10 x 11 cm size).
5. Defat ground samples in a Soxhlet continuous extractor with hexane for four hours. Air-dry the samples.

**Protein analysis**

The nitrogen content was measured using microkjeldahl method (Bailey, 1967) and the percentage of protein was calculated by using a factor of 6.25. The detail of chemical used sees the Appendix I.

**Procedure**

1. Place a 20 mg sample in a digestion flask. Add 1.0 g of catalyst powder mixture and 2 ml of concentrated sulfuric acid.
2. Digest 3 h, cool, add a minimum quantity of water to dissolve solids, cool, and place a thin film of Vaseline in the rim of the flask.
3. Transfer the digest to a distillation apparatus, and rinse flask five or six times with 1 to 2 ml portions of distilled water.

4. Place a 125-ml Erlenmeyer flask with 6 ml of boric acid solution and 3 drops of litmus indicator solution under a condenser with the tip extending below the surface of the solution.

5. Add 5 ml of sodium hydroxide solution to a still, and steam distill until about 50 ml of distillate collects.

6. Titrate to gray end point of first appearance of green

7. Make blank determination sousing the same quantity of reagents and the same digestion and distillation periods as for determination.

8. Calculate the percentage of nitrogen:

$$\% \text{ nitrogen} = \frac{(\text{ml HCl indetn.} - \text{ml blank}) \times \text{Normality} \times 14.007 \times 100}{\text{mg sample}}$$

**Tryptophan analysis**

Hernandez and Bates, (1969) have observed that the relationship between tryptophan and lysine in the maize endosperm protein is about 1 to 4; thus tryptophan can be used as a single parameter for maize quality evaluation. Analyzed for tryptophan content used spectrophotometer as described by Villegas and Mertz (1971)**.**

**Procedure**

1. Weigh 90 to 100 mg of finely ground defatted maize endosperm sample into a glass vial and add 4 ml of papain solution. The tubes are capped and carefully shaken; making sure that the sample is totally wetted. (Blanks must also be carried out with papain solution through this procedure.)

2. Samples are kept overnight in an incubator oven at 65 °C.

3. Hydrolysates are removed from the incubator or oven and shaken, then allowed to adjust to room temperature. When this temperature is reached, the supernatant should be clear; if not clear, centrifuge the samples.

4. One milliliter of hydrolyses is pipetted into a test tube containing 4 ml of reagent C (see Appendix II); the mixture is shaken vigorously and the color developed for 15 min at 65 C.

5. After cooling, the solutions are transferred to calibrated tubes and read at 560 nm on a spectophotometer.

6. A standard curve is prepared in a range from 0 to 40 µg $ml^{-1}$, using DL-tryptophan.

7. The tryptophan content of the sample is calculated from the standard curve and reported on a protein basis.

**4. Statistical analysis for yield trial**

The analysis of variance for a 7x7 simple lattice design of yield and characters studied was used MSTAT-C computer program (MSTAT-C, 1988). All means were adjusted for lattice blocks if the efficiency of the lattice adjustment was at least 101%, otherwise un-adjustment means were used. Differences among entries for each trait were tested and adjusted using the effective error mean square or randomized complete block error mean square; this depended upon the use of adjusted entry means from lattice analysis or unadjusted entries means from randomized complete block analysis, respectively. General form of analysis of variance for a 7x7 simple lattice design is presented in Table 1

**Table 1** General form of the analysis of variance for a 7 x 7 simple lattice design.

| Source of variation | d.f. | MS |
|---|---|---|
| Replications | r-1 | |
| Treatments | | |
| Unadjusted | $k^2$-1 | |
| Adjusted | $k^2$-1 | |
| Block within replication (adj.) | r(k-1) | Eb |
| Error | | |
| Effective | (k-1)(rk-k-1) | |
| Intrablock | (k-1)(rk-k-1) | Ee |
| Total | $rk^2$ -1 | |

Where, r = number of replications
k = number of treatments within block
Eb = interblock error mean square
Ee = intrablock error mean square

If Eb is less than or equal to Ee, the experiments were analyzed as randomized complete block design. When Eb was greater than Ee, treatments were adjusted and effective error mean square was used to calculate the statistical F-test.

The general combining ability (GCA) and specific combining ability (SCA) effects for grain yield and other desirable traits were analyzed according to Method IV, Model I diallel analysis of Griffing's approach (Griffing, 1956). The general form of the analysis of variance for Method 4 Model I is presented in Table 2

**Table 2** General form of the analysis of variance for 'Method IV Model I' diallel analysis of Griffing's approach.

| Source of variation | d.f. | MS |
|---|---|---|
| Replications | r-1 | |
| Treatments | t-1 | MSt |
| GCA | p-1 | MSg |
| SCA | p(p-3)/2 | MSs |
| Error | (r-1)(t-1) | MSe |
| Total | rt-1 | |

Where, r = replications; MSt = Treatments mean square
t = treatments; MSg = GCA mean square
p = parents; MSs = SCA mean square
MSe = Error mean square

# RESULTS AND DISCUSSION

In the first season, 6 QPM populations were grown at the National Corn and Sorghum Research Center, Kasetsart University, Nakhon Ratcharima Province, Thailand. Three populations, namely $Pop61C_1$ (Tropical maize Early Flowering Yellow Flint; TEYF), $Pop62C_6$ (Tropical maize Late Flowering White Flint; TLWF), $Pop65C_6$ (Tropical maize Late Flowering Yellow Flint; TLYF) with preferred morphological characters, i.e., early flowering, short anthesis-silking interval (ASI), healthy plants were selected for further study.

## Marker-assisted selection for the *opaque-2* gene of $S_0$-plants

Extracted DNA was tested with three primers (phi057, phi112 and umc1066) to identify a homozygote with the recessive gene, *opague-2* ($o_2o_2$), from $Pop61C_1$, $Pop62C_6$ and $Pop65C_6$ and two standard varieties (opaque-2 and Suwan1). The results showed that the primer phi057 and phi112 gave distinguish of QPM ($o_2o_2$) and non-QPM while it was not difference in the primer umc1066 (Figures 2). However, the primer phi057 could detect the non-QPM on plant number 9. It was suggested that the primer phi057 would be more efficient for detect opaque-2 gene than the other two primers.

Using phi057, QPM was detected in 24 out of 40 $Pop61C_1$ plants (60%), 34 out of 35 $Pop62C_6$ plants (97%) and 24 out of 30 $Pop65C_6$ plants (80%), while non-QPM was detected in the remaining 16 $Pop61C_1$ (40%), 1 $Pop62C_6$ (3%) and 6 $Pop65C_6$ (20%) plants (Table 3). For the phi112 marker, QPM was detected in 34 out of 40 $Pop61C_1$ plants (85%), all 35 $Pop62C_6$ plants (100%) and also all 30 $Pop65C_6$ plants (100%), but non-QPM was detected in only 6 $Pop61C_1$ plants. Some of those polymorphisms are shown in Figures 3, 4 and 5. Since these three populations were originally classified as QPM, detection of non-QPM indicated the contamination of normal maize with opaque-2 varieties. It should be noted here that $Pop62C_6$ is white-kernel maize, therefore contaminating yellow-kernel could be selectively separated before the experiment, which resulted in the distinctively high percentage of QPM.

Seeds are shown as yellow kernels when white-kernel maize is contaminated with the yellow-kernel pollen during pollination, which is referred to as the xenia effect (Poehlman, 1987). In yellow-kernel populations, $Pop61C_1$ and $Pop65C_6$, however, this selection could not be done by the apparent kernel color.

Upon analyzing the band patterns detected by these two markers, phi112 could detect a PCR amplified product (approximately 160 bp) of Suwan 1 (non-QPM) and some $S_0$-plants of $Pop61C_1$ (Figure 3a). But it was not found in the opaque-2 variety and other $S_0$-plants of $Pop62C_6$ (Figures 4a) and $Pop65C_6$ (Figure 5a). Babu *et al*. (2005) had shown that phi112 is a dominant marker, which clearly distinguished the QPM inbred lines from the normal inbred lines by the absence of 150 bp from QPM plants. Since $o_2o_2$ is a homozygous recessive genotype, both the dominant marker and the co-dominant marker should give the same identified results. However, the phi112 marker could not clearly separate the heterozygous from homozygous recessive plants (CIMMYT, 2000), which was also confirmed by these results. On the other hand, the phi057 marker could detect amplified products of 160 bp in Suwan 1 (non-QPM), 170 bp fragments of opaque-2 variety and QPM lines, and both fragments (160 bp and 170 bp) for non-QPM lines as shown in Figures 2b, 3b and 4b. These results agreed with Babu *et al*. (2005) in showing that phi057 gave 160 bp fragments in normal inbred lines and 170 bp fragments in QPM. Since phi057 is a co-dominant marker and could detect homozygous dominant ($O_2O_2$), heterozygous ($O_2o_2$), and homozygous recessive ($o_2o_2$) plants separately (Ribaut and Hoisington, 1998), it is speculated that phi057 is more closely linked to the *opaque-2* gene than the phi112 marker and is therefore more effective for marker-assisted selection to discriminate between homozygous and heterozygous plants. On contradictory, Yang *et al.* (2004) reported that it is the best to use the 3 markers (phi057, phi112 and umc1066) together in molecular marker-assisted selection for opaque-2 maize materials. Because of among the tested lines, allelic variations were observed at umc1066, phi112 and phi057 site. The primary mechanisms underlying the variations in repeat numbers and regions flanking the SSR within the opaque-2 gene appear to be unequal crossing over and replication slippage. Furthermore, base substitution of SSR motif can create heteroalleles and modify the repeat number of SSR. To confirm the marker-assisted

selection results, tryptophan content in the endosperm of the heterozygous plants was determined to distinguish them from the homozygous ones.

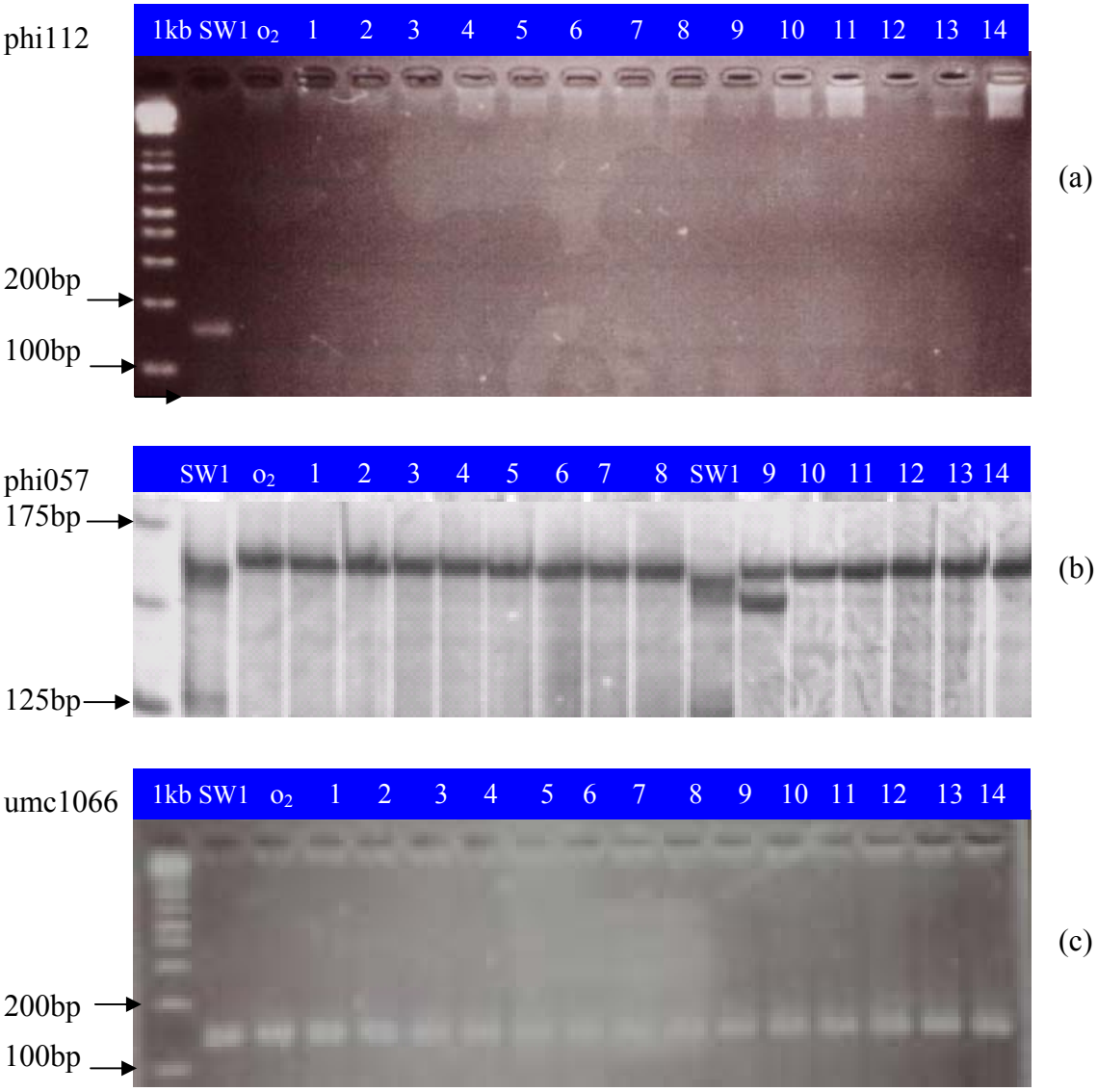



**Figure 2** Electrophoretic results of QPM ($o_2o_2$), non-QPM ($O_2\_$) of Pop62C$_6$ S$_0$-plants and two standard varieties of Opaque-2 (o$_2$) and Suwan 1 (SW1) using (a) phi112 primer on 3% agarose gel where all S$_0$-plants were QPM, (b) phi057 primer on 6% polyacrylamide gel, non-QPM was detected on S$_0$-plants sample number 9 and (c) umc1066 primer on 3% superfine agrarose gel, Numbers on each column are the designated number of S$_0$-plants.

**Table 3** Number and percentage of QPM and non-QPM plants detected by two simple sequence repeat (SSR) markers (phi057 and phi112) in the QPM Pop61$C_1$, Pop62$C_6$ and Pop65$C_6$ and in two standard varieties of Opaque-2 (a QPM) and Suwan 1 (a non-QPM).

| Populations | Maize types | Number of plants and percentage | |
|---|---|---|---|
| | | phi057 | phi112 |
| Pop61$C_1$ | QPM | 24 (60%) | 34 (85%) |
| | non-QPM | 16 (40%) | 6 (15%) |
| Pop62$C_6$ | QPM | 34 (97%) | 35 (100%) |
| | non-QPM | 1 (3%) | 0 (0%) |
| Pop65$C_6$ | QPM | 24 (80%) | 30 (100%) |
| | non-QPM | 6 (20%) | 0 (0%) |
| Opaque-2 | Opaque-2 | 3 (100%) | 3 (100%) |
| Suwan 1 | non-QPM | 3 (100%) | 3 (100%) |

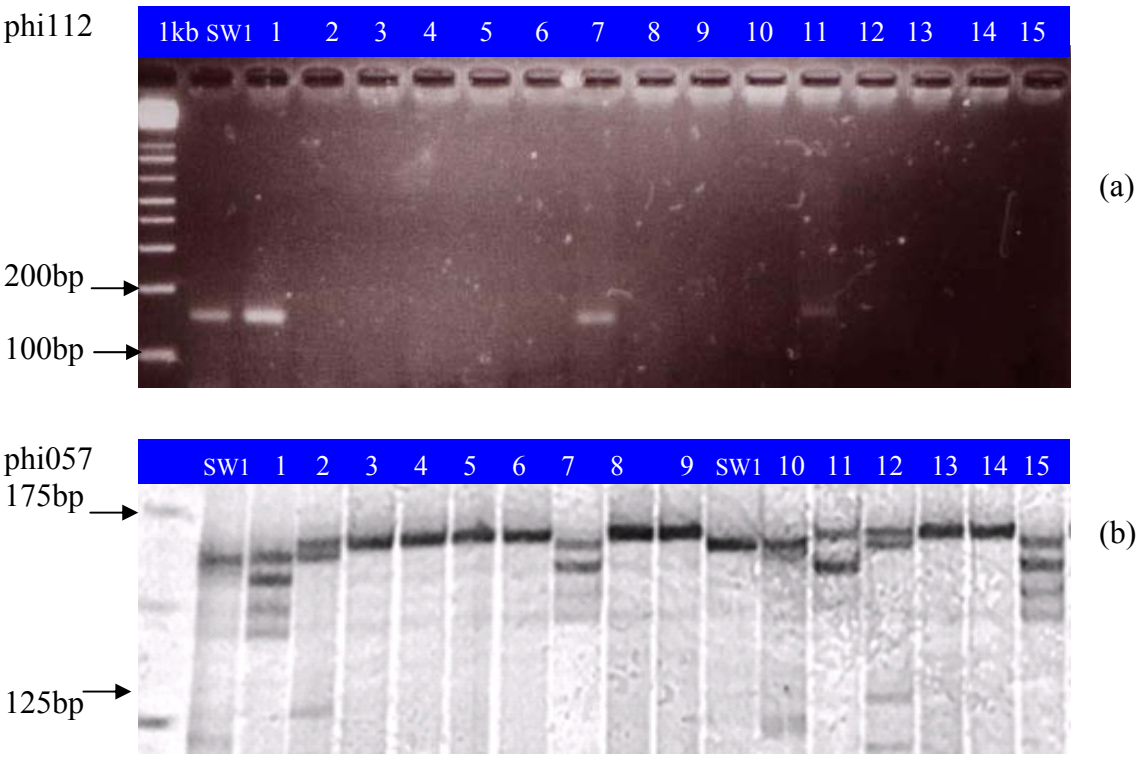



**Figure 3** Electrophoretic results of QPM ($o_2o_2$), non-QPM ($O_2\_$) of Pop61C$_1$ S$_0$-plants and Suwan 1 (SW1) using (a) phi112 primer on 3% agarose gel, non-QPM was detected on S$_0$-plants number 1, 7 and 11 (b) phi057 primer on 6% polyacrylamide gel, non-QPM was detected on S$_0$-plants sample number 1, 2, 7, 10, 11, 12 and 15. Numbers on each column are the designated number of S$_0$-plants.

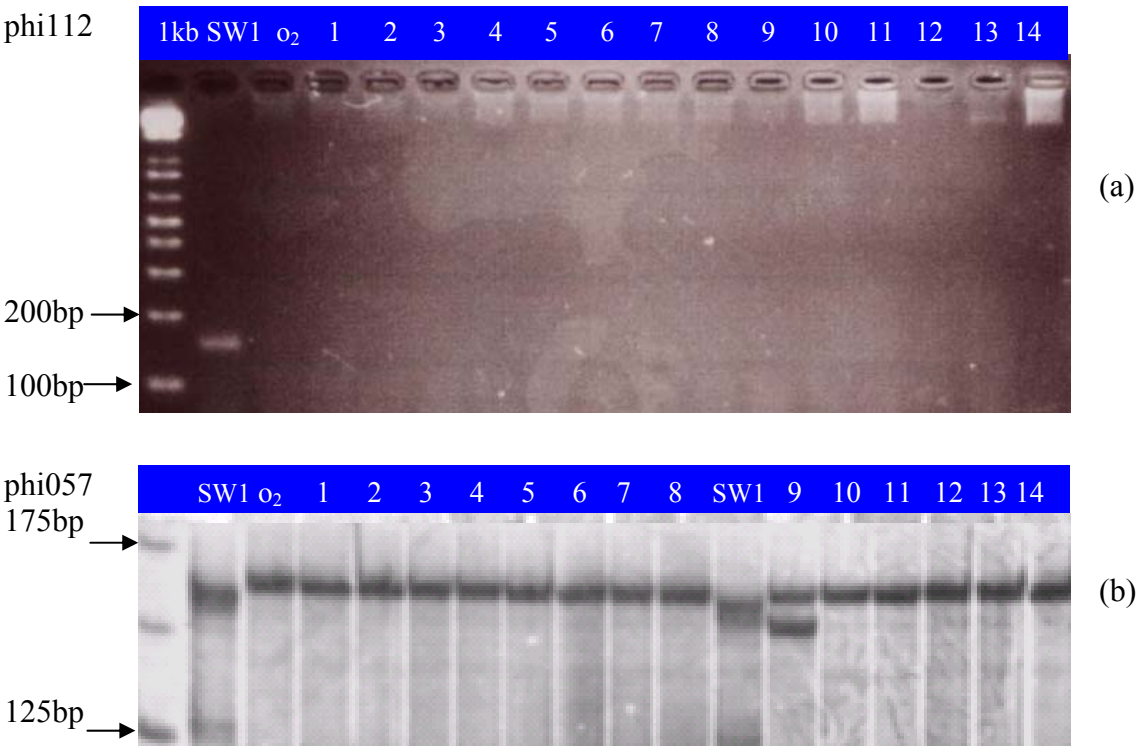



**Figure 4** Electrophoretic results of QPM ($o_2o_2$), non-QPM ($O_2\_$) of Pop62C$_6$ S$_0$-plants and two standard varieties of Opaque-2 (o$_2$) and Suwan 1 (SW1) using (a) phi112 primer on 3% agarose gel where all S$_0$-plants were QPM, (b) phi057 primer on 6% polyacrylamide gel, non-QPM was detected on S$_0$-plants sample number 9. Numbers on each column are the designated number of S$_0$-plants.

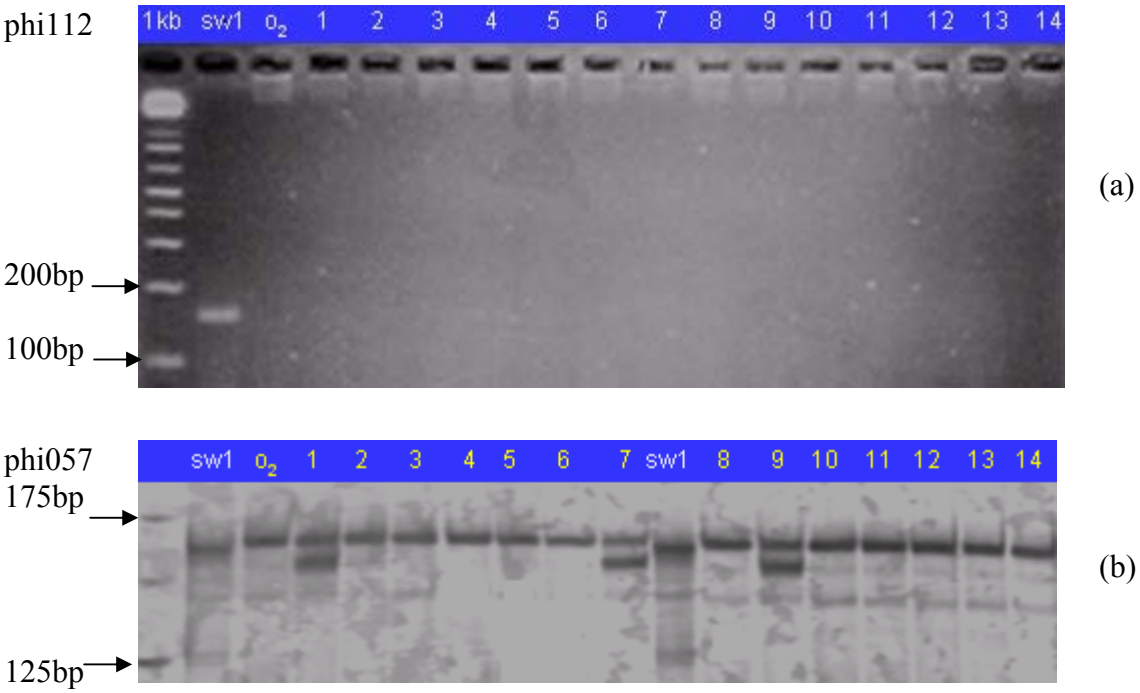



**Figure 5** Electrophoretic results of QPM ($o_2o_2$), non-QPM ($O_2\_$) of Pop65C$_6$ S$_0$-plants and two standard varieties of Opaque-2 ($o_2$) and Suwan 1 (SW1) using (a) phi112 primer on 3% agarose gel where all S$_0$-plants were QPM, (b) phi057 primer on 6% polyacrylamide gel, non-QPM was detected on S$_0$-plants sample number 1, 7 and 9. Numbers on each column are the designated number of S$_0$-plants.

## Total protein and trytophan analysis of $S_1$-seeds

The QPM and non-QPM plants detected by phi112 and phi057 were separately analyzed for protein and tryptophan content in the endosperm to compare the effectiveness of each marker in differentiating heterozygous and homozygous genotypes. It is interesting to find that the total protein contents in maize endosperm of all $S_0$-plants (QPM, non-QPM, and standard varieties) were non-significantly different (Table 4). On the contrary, there were highly significant differences in tryptophan content in QPM and non-QPM plants. In phi057-detected QPM and non-QPM, the average tryptophan content in maize endosperm was found to range from 0.64 to 0.67% for QPM plants and 0.36 to 0.40% for non-QPM plants, while in phi112 detected QPM and non-QPM, tryptophan content was in the range of 0.58 to 0.63% for QPM, 0.33% for non-QPM, 38% for Suwan1 and 0.80% for the opaque-2 variety for both types of markers (Table 4). These results agreed with Vasal (1994) and Prasanna *et al.* (2001) who indicated that QPM varieties have almost double the amount of tryptophan compared to normal maize but are similar in overall protein content.

Theoretically, phi112 should identify homozygous dominant ($O_2O_2$) and heterozygous ($O_2o_2$) plants to be non-QPM, but our results showed that some heterozygous ($O_2o_2$) plants were identified by phi112 as QPM and contained low tryptophan content as did those of non-QPM (Table 5). On the contrary, these same populations were non-QPM as detected by phi057 and contained low amounts of tryptophan as well. It is obvious that the sensitivity and accuracy of detecting QPM and non-QPM by phi112 have proven to be contradictory to the tryptophan results and support the previous speculation that the phi057 marker is more closely linked to the *opaque-2* gene than the phi112 marker. Thus, phi057 should be feasible to use as marker-assisted selection to detect the heterozygote line during the conversion of a normal elite inbred line to the QPM inbred line through backcross method without tryptophan analysis in endosperm of each backcross generation. In the case of backcross designed for transfer of the recessive gene ($o_2o_2$), identification of the heterozygote in the seedling stage prior to pollination aided to the rejection of non-

target backcross progenies (dominant homozygote) (Ribaut and Hoisington, 1998; Babu *et al.*, 2005). The phi057 could be used as a tool for early indication of three possible genotypes of the *opaque-2* plants, i.e., $O_2O_2$ and $O_2o_2$ and $o_2o_2$, especially at the seedling stage.

**Table 4** Comparison of protein and tryptophan contents in maize endosperm of $S_0$-plants of $Pop61C_1$, $Pop62C_6$ and $Pop65C_6$ and two standard varieties of Opaque-2 and Suwan 1.

| Populations | Maize types | Total protein in endosperm (%) (mean ± sd) | | Tryptophan in protein (%) (mean ± sd) | |
|---|---|---|---|---|---|
| | | phi057 | phi112 | phi057 | phi112 |
| $Pop61C_1$ | QPM | 7.46 ± 0.552 | 7.41 ± 0.481 | 0.669 ± 0.082 b | 0.584 ± 0.155 b |
| | non-QPM | 7.33 ± 0.267 | 7.41 ± 0.340 | 0.361 ± 0.058 a | 0.332 ± 0.028 a |
| $Pop62C_6$ | QPM | 7.21 ± 0.614 | 7.22 ± 0.605 | 0.642 ± 0.080 b | 0.634 ± 0.090 b |
| | non-QPM | 7.33 [1/] | no sample | 0.378[1/] a | no sample |
| $Pop65C_6$ | QPM | 7.74 ± 0.770 | 7.64 ± 0.714 | 0.665 ± 0.075 b | 0.611 ± 0.129 b |
| | non-QPM | 7.26 ± 0.086 | no sample | 0.395 ± 0.027 a | no sample |
| Opaque-2 | Opaque-2 | 7.19 ± 0.010 | 7.19 ± 0.010 | 0.803 ± 0.480 b | 0.803 ± 0.480 b |
| Suwan 1 | non-QPM | 7.33 ± 0.018 | 7.33 ± 0.018 | 0.377 ± 0.320 a | 0.377 ± 0.320 a |

$LSD_{.01}$ (tryptophan in protein) = 0.208

Means followed by the same letter in column are not significantly different at 1% level by LSD

[1/] = one sample tested

**Table 5** Comparison of tryptophan contents in the endosperm of $S_0$-plants detected by phi057 as non-QPM lines while they were detected as QPM and non-QPM lines by phi112 from $Pop61C_1$, $Pop62C_6$ and $Pop65C_6$ and two standard varieties of Opaque-2 and Suwan 1.

| Populations | Maize types | Tryptophan in protein (%) (mean ± sd) | |
|---|---|---|---|
| | | phi057 | phi112 |
| $Pop61C_1$ | QPM | no sample | 0.379 ± 0.067 |
| | non-QPM | 0.361 ± 0.058 | 0.332 ± 0.028 |
| $Pop62C_6$ | QPM | no sample | 0.378[1/] |
| | non-QPM | 0.378[1/] | no sample |
| $Pop65C_6$ | QPM | no sample | 0.395 ± 0.027 |
| | non-QPM | 0.395 ± 0.027 | no sample |
| Opaque-2 | Opaque-2 | 0.803 ± 0.480 | 0.803 ± 0.480 |
| Suwan 1 | non-QPM | 0.377 ± 0.320 | 0.377 ± 0.320 |

[1/] = one sample tested

**Marker-assisted selection for *opaque-2* gene of $S_2$-plants**

Extracted DNA was tested with the primer, phi057 to identify the homozygous recessive with the *opague-2* genotype ($o_2o_2$) from three selected populations and two standard varieties (Suwan1 and Opaque-2). With this marker, QPM was detected in 14 out of 18 $Pop61C_1$ plants (78%), 21 out of 21 $Pop62C_6$ plants (100%) and 15 out of 18 $Pop65C_6$ plants (83%) (Table 6). Some of those polymorphisms are shown in Figure 6. Theoretically, $S_2$ plants should not have any non-QPM contained in selected lines. The result showed that some pollen contamination occurred because non-QPM or yellow-kernel maize was planted close to the experiment. However, the contamination of white-kernel maize by pollination with yellow-kernel maize is easily identified, as mentioned above.

**Table 6** Number and percentage of QPM and non-QPM plants detected by simple sequence repeat (SSR) marker, phi057 in $S_2$-plants from three QPM populations (Pop61$C_1$, Pop62$C_6$ and Pop65$C_6$) and standard varieties of Opaque-2 and Suwan 1.

| Populations | Number of $S_2$-plants and percentage | |
|---|---|---|
| | QPM | non-QPM |
| Pop61$C_1$ | 14 (78%) | 4 (22%) |
| Pop62$C_6$ | 21 (100%) | 0 (0%) |
| Pop65$C_6$ | 15 (83%) | 3 (17%) |
| Opaque-2 | 5 (100%) | 0 (0%) |
| Suwan 1 | 0 (0%) | 5 (100%) |

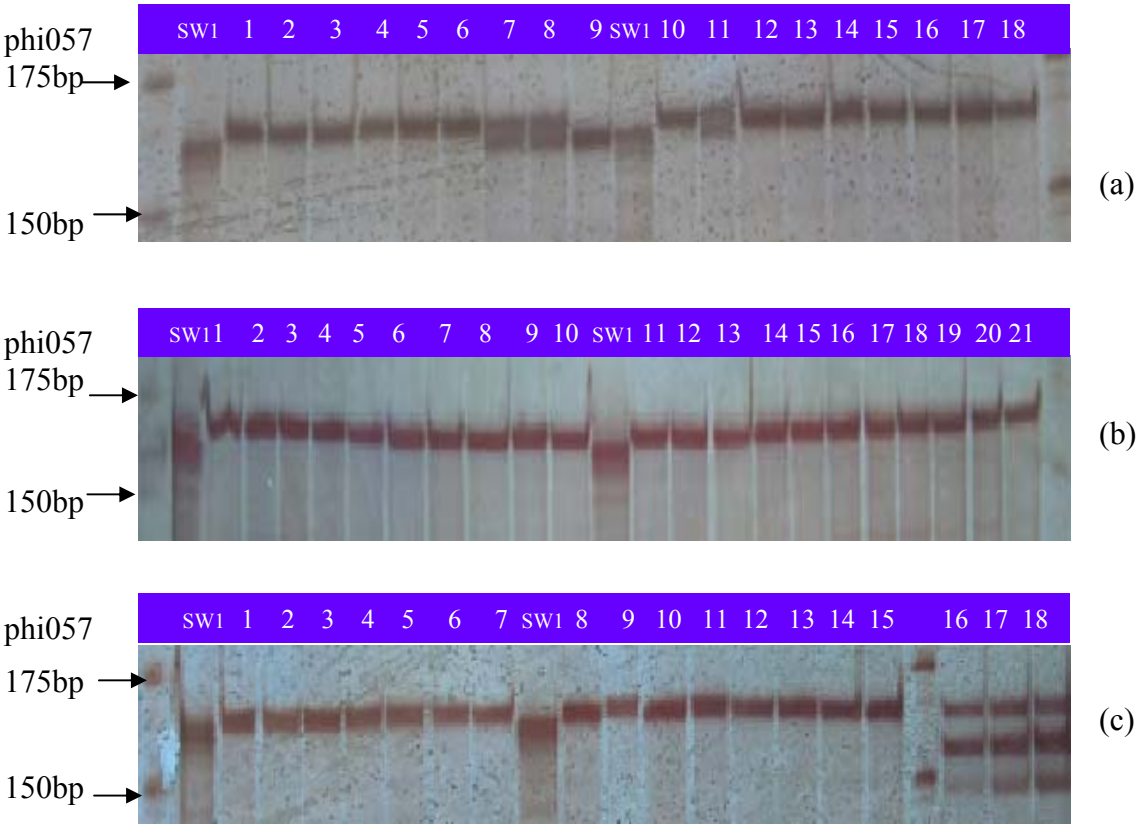



**Figure 6** Electrophoresis results of QPM ($o_2o_2$) and non-QPM ($O_2\_$) of $S_2$ plants and the standard variety (Suwan 1, SW1) from (a) Pop61$C_1$, (b) Pop62$C_6$ and (c) Pop65$C_6$ by phi057 primer on 6% polyacrylamide gel.

**Inbred line development**

$S_0$-plants of three populations preferred morphological characters, i.e., early flowering, short anthesis-silking interval (ASI), healthy plants, the other desirable agronomic traits and containing *opaque-2* gene were self-pollinated to produce $S_2$ and advanced to $S_3$-inbred lines. Ten selected $S_3$ inbred lines with good agronomic characters and high tryptophan content in endosperm were used as QPM inbred lines (Table 7). They were P1-P4, P5-P8 and P9-P10 inbred lines from Pop65$C_6$, Pop61$C_1$ and Pop62$C_6$, respectively. The pedigree of 10 inbred lines was shown in material part.

The studied agronomic traits of inbred lines, yield, moisture content, shelling ratio, ear height, plant height, days to anthesis and days to silking, were highly significant differences. The grain yield of inbred lines ranged from 1.33 to 2.45 t $ha^{-1}$. P1 gave the highest grain yield. The moisture content of inbred lines ranged from 5.1 to 9.6%. Days to anthesis and silking ranged 50-56 and 48-55 days, respectively. All the details of the other traits were shown in Table 7and the ears of 10 inbred lines were.shown in Appendix Figure 1.

With the primer, phi057, the amplified products of 10 inbred lines were detected as the same as with Opaque-2 maize but they were different to non-QPM (SW1) (Figure 7). The protein content in endosperm of these inbred lines ranged from 7.76 to 8.61% while Opaque-2 and non-QPM maize contained about 8.45 and 8.73 %, respectively. However, the protein content of all tested maize was not significantly different among them (Table 7). The results agree with Vasal (1994) who indicated that the protein content in maize endosperm was the same as normal and Opaque-2 maize.

The tryptophan content in protein of inbred lines ranged from 0.73 to 0.83% while Opaque-2 and non-QPM maize contained about 0.82 and 0.42%, respectively (Table 7). The results showed that tryptophan content in protein of Opaque-2 and inbred lines had almost doubled in amount compared to normal maize (Prasanna *et al*., 2001).

The phi057 primer could generate products of 160 bp in non-opaque-2 and 170 bp fragments in opaque-2 (Babu *et al*., 2005). Since phi057 is a co-dominant marker and could detect homozygous dominant ($O_2O_2$), heterozygous ($O_2o_2$) and homozygous recessive ($o_2o_2$) plants. This is useful for marker-assisted selection for the *opaque-2* gene (Ribaut and Hoisington, 1998). Our results showed that 10 inbred lines were *opaque-2*, which was different from normal maize (Suwan 1).

**Table 7** Means of grain yield, protein, tryptophan in protein content and studied traits of 10 inbred lines, standard varieties (Opaque-2 and Suwan 1)

| Maize | Yield (ton $ha^{-1}$) | Protein (%) | Tryptophan in protein (%) | Moisture content (%) | Shelling (ratio) | Ear height (cm) | Plant height (cm) | Days to anthesis (d) | Days to silking (d) |
|---|---|---|---|---|---|---|---|---|---|
| P1 | 2.45 | 8.13 | 0.79 | 9.60 | 0.85 | 46 | 110 | 56 | 53 |
| P2 | 2.09 | 7.76 | 0.80 | 7.60 | 0.83 | 47 | 103 | 56 | 54 |
| P3 | 2.21 | 8.40 | 0.74 | 6.40 | 0.83 | 60 | 115 | 55 | 55 |
| P4 | 1.94 | 7.87 | 0.73 | 7.50 | 0.79 | 45 | 113 | 56 | 53 |
| P5 | 1.58 | 8.44 | 0.82 | 6.40 | 0.83 | 60 | 115 | 50 | 48 |
| P6 | 1.71 | 8.16 | 0.83 | 8.00 | 0.82 | 87 | 150 | 51 | 49 |
| P7 | 1.33 | 8.26 | 0.79 | 7.10 | 0.77 | 48 | 109 | 50 | 48 |
| P8 | 1.81 | 8.07 | 0.79 | 8.70 | 0.75 | 66 | 123 | 51 | 49 |
| P9 | 2.27 | 8.61 | 0.77 | 8.70 | 0.78 | 55 | 123 | 54 | 52 |
| P10 | 2.32 | 7.97 | 0.75 | 5.10 | 0.74 | 67 | 114 | 55 | 53 |
| Opaque-2 | - | 8.45 | 0.82 | - | - | - | - | - | - |
| Non-QPM (SW1) | - | 8.73 | 0.42 | - | - | - | - | - | - |
| F-test[1/] | ** | NS | ** | ** | ** | ** | ** | ** | ** |
| $LSD_{.01}$[2/] | 0.434 | - | 0.132 | 0.73 | 0.08 | 4.22 | 6.92 | 4.44 | 3.91 |

[1/] and [2/] = test among inbred lines, - = not determine, NS = non significant differences, * ,** = significant difference at 0.05 and 0.01 levels, respectively.

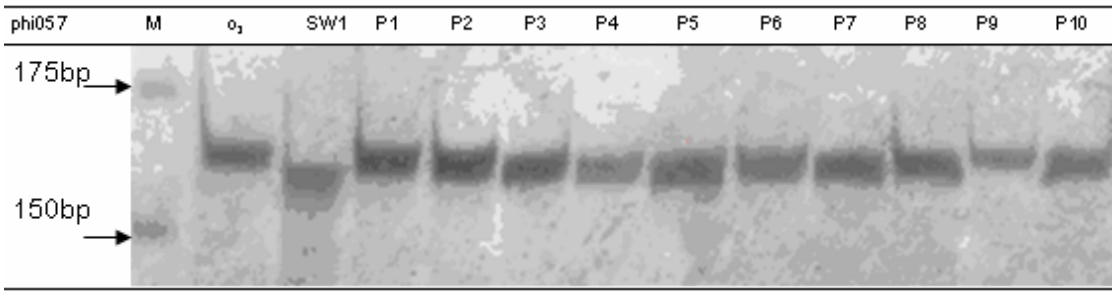



**Figure 7** Polymorphism of 10 inbred lines ($S_3$), Opaque-2 ($o_2$) and non-QPM (SW1) using phi057 on 6% polyacrylamide gel.

**Analysis of variance**

Forty-five hybrids were tested for their yielding ability and agronomic traits. The diallel cross yield trial consisted of 45 $F_1$ hybrids. Two single cross hybrids (SW4452 and NK40) and two QPM populations ($Pop62C_6$ and $Pop65C_6$) were used as standard varieties. The experiment was conducted in a 7x7 simple lattice designs, with two replications. From the analysis of variance, the result showed significant differences among treatments of grain yield, tryptophan content in protein, ear height, plant height, and days to anthesis and silking were observed. But protein content in the endosperm, moisture content and shelling ratio were no significantly different (Table 8).

Significant differences among the tested hybrids, QPM population and non-QPM (SW4452 and NK40) were observed for days to anthesis and silking, and plant and ear height. Tested hybrids, on average, flowered four days earlier (57 vs 61 days), were shorter (130 cm vs 161 cm), had lower ear placement (64 cm vs 78 cm) than non-opaque-2 checks (Tables 8 and 9).

There was not significantly different among tested hybrids, QPM population and non-QPM for grain moisture content and shelling ratio. On average, tested hybrids were 8.17% and 0.78, QPM population was 8.70% and 0.83, and non-QPM was 8.05% and 0.84 for the grain moisture content and shelling ratio, respectively (Tables 8 and 9).

## Combining ability analysis

Significant differences between general combining ability effects (GCA effects) were observed only for grain yield, whereas specific combining ability effects (SCA effects) were significant for grain yield and days to anthesis (Table 8).

### Grain yield

Mean grain yield was 4.48 ton $ha^{-1}$ for the tested hybrids, 1.97 ton $ha^{-1}$ for inbred lines, 3.30 ton $ha^{-1}$ for QPM population and 6.61 ton $ha^{-1}$ for non-QPM, the standard varieties (SW4452 and NK40) (Table 9). By contrast comparison, grain yield was significantly different between $F_1$ hybrids and QPM population, $F_1$ hybrids and non-QPM, and QPM population and non-QPM. There were also significant differences within the $F_1$ hybrids (Table 8). GCA and SCA effects of these traits were significantly different. The mean grain yield, GCA effects and SCA effects of the diallel cross are shown in Table 10. The ratio of GCA: SCA (2.32*) was significantly different at a probability of 0.05, which showed that additive gene effects played an important role for this trait. These results agree with Sriwatanapongse *et al*. (1974) who indicated that additive gene effects had a more important role than dominant gene effects on grain yield of opaque-2 maize. The tested hybrids gave the yellow kernel except the cross between P9 and P10 which gave the white kernel. Because both P9 and P10 came from the white kernel population ($Pop62C_6$). The color of hybrid kernel between white and yellow kernel inbred lines was yellow because of xenia effect (Pohlman, 1987).

Grain yield of tested hybrids ranged from 2.45 to 6.10 ton $ha^{-1}$ (Table 10 and Appendix Table 1). The highest grain yield was the cross of P9 x P4 where these inbred lines were extracted from different populations. Moreover, days to anthesis and silking of these two inbred lines were synchronizing (Table 7). On the other hand, the lowest grain yield was the cross of P6 x P5 where these inbred lines came from the same population. Inbred P10 had the best combination of GCA effects for grain yield. Moreover, inbred lines, P9, P1, P8 and P7 also gave positive GCA effects. Therefore

P9 and P10 can be used as tester lines for the quality protein maize program or the positive-GCA-effect lines could be used as the lines to form new quality protein maize populations. Grain yield of the tested hybrids was significantly different from the commercial hybrids (single cross hybrid varieties) (Tables 8 and 9). However, the top ten crosses (yield $\geq$ 5.22 ton $ha^{-1}$, Table 11) were no different from commercial hybrid checks. Grain yield results indicated that a single cross hybrid of the *opaque-2* gene with vitreous endosperm could possibly be grown in Thailand. QPM is likely to gain wider acceptance if hybrids are produced that have agronomic performance similar to normal hybrids and retain an enhanced nutritional quality (Babu *et al.*, 2005). Moreover, previous and recent studies have reported yields of CIMMYT QPM hybrids competitive with the best locally available normal-endosperm cultivars for many tropical sites (Bjarnason and Vasal, 1992; Pixley and Bjarnason, 1993 and 2002). Data from QPM trials in Brazil, Ghana, Guatemala and South Africa have also documented similar yields of QPM relative to test available normal-endosperm maize checks (Mertz, 1992). QPM hybrids have several advantages over the open-pollinated QPM varieties such as more uniform and stable endosperm modification and less monitoring required for ensuring protein quality in seed production.

There is an increasing number of elite, exotic, QPM inbred lines being developed outside Thailand. Therefore, characterization and selection for adaptation of these tropical, yellow, QPM inbred lines could enhance protein quality, increase genetic variability for quality, improve productivity and be a source of valuable genes for abiotic and biotic stress resistance in Thailand. However, if these inbred lines could not adapt to our environments the backcross method could be used to transfer the *opaque-2* gene and marker-assisted selection could be applied for this trait.

**Table 8** Mean square of grain yield and studied traits of $F_1$-hybrid; a comparison between $F_1$-hybrids and QPM populations and non-QPM (single cross hybrids); trial conducted at the International Corn and Sorghum Research Center in October, 2005.

| Source of variation | d.f | MS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Grain Yield (ton $ha^{-1}$) | Protein (%) | Tryptophan in protein (%) | Moisture content (%) | Shelling (ratio) | Ear height (cm) | Plant height (cm) | Day to anthesis (d) | Day to silking (d) |
| Replications | 1 | 0.092$^{NS}$ | 0.168$^{NS}$ | 0.003$^{NS}$ | 12.715 | 0.024 | 0.2$^{NS}$ | 864.1** | 18.9** | 1.0$^{NS}$ |
| Treatments | 48 | 2.255** | 0.939$^{NS}$ | 0.023* | 1.433$^{NS}$ | 0.008$^{NS}$ | 167.4** | 23736.5** | 5.8** | 6.0** |
| $F_1$-hybrid ($F_1$) | 44 | 1.292** | - | 0.017$^{NS}$ | - | - | 146.4* | 256.0** | 4.7** | 5.2* |
| $F_1$-vs QPM pop. | 1 | 5.296** | - | 0.001$^{NS}$ | - | - | 355.2* | 7849.4** | 0.8$^{NS}$ | 0.1$^{NS}$ |
| $F_1$ vs non-QPM | 1 | 26.419** | - | 0.378** | - | - | 1187.8** | 4578.8** | 63.0** | 58.2** |
| QPM pop. vs non-QPM | 1 | 28.125** | - | 0.207** | - | - | 140.3$^{NS}$ | 154.9$^{NS}$ | 40.5** | 32.0** |
| GCA | 9 | 1.541** | - | - | - | - | 204.8** | 308.4** | 6.9** | 6.5** |
| SCA | 35 | 0.664** | - | - | - | - | 39.4$^{NS}$ | 81.6$^{NS}$ | 1.20** | 1.6$^{NS}$ |
| Blocks/reps. (adj) | 12 | 0.098 | 0.875 | 0.005 | 0.858 | 0.006 | 88.422 | 48.175 | 5.224 | 5.973 |
| Error | 36 | 0.211 | 0.522 | 0.012 | 1.020 | 0.005 | 70.236 | 113.559 | 0.971 | 1.842 |
| Total | 97 | | | | | | | | | |
| CV (%) | | 10.126 | 9.66 | 12.59 | 12.09 | 9.40 | 13.15 | 7.41 | 1.89 | 2.57 |
| LSD: 0.05 | | 0.932 | - | 0.20 | - | - | 17.43 | 19.82 | 2.19 | 2.98 |
| : 0.01 | | 1.125 | - | 0.27 | - | - | 23.37 | 26.45 | 2.94 | 3.99 |

- = no determine, $^{NS}$ = non significant differences, *, ** = significant difference at 0.05 and 0.01 levels, respectively.

**Table 9** Mean of yield and other traits of the tested $F_1$-hybrid, inbred lines, QPM population and non-QPM.

| Maize | Mean ± sd[1/] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Yield (ton $ha^{-1}$) | Protein (%) | Tryptophan in protein (%) | Moisture content (%) | Shelling (ratio) | Ear height (cm) | Plant height (cm) | Days to anthesis (d) | Days to silking (d) |
| $F_1$-hybird | 4.48± 0.92 | 7.84± 0.91 | 0.81± 0.12 | 8.17± 1.13 | 0.78± 0.08 | 64±10.50 | 130± 13.66 | 57± 1.89 | 57± 2.01 |
| Inbreds | 1.97± 0.36 | 7.88± 0.69 | 0.73± 0.07 | 7.5± 1.33 | 0.8± 0.04 | 58± 13.05 | 117± 13.05 | 53± 2.59 | 51± 2.63 |
| QPM pop.[2/] | 3.30± 0.07 | 8.18± 0.17 | 0.82± 0.02 | 8.70± 1.97 | 0.83± 0.02 | 74± 4.54 | 175± 7.38 | 57± 1.73 | 57± 1.63 |
| Non-QPM[3/] | 6.61± 0.01 | 7.76± 0.80 | 0.42± 0.21 | 8.05± 0.80 | 0.84± 0.04 | 78± 10.09 | 161± 9.37 | 61± 0.41 | 61± 0.75 |

[1/] = Standard deviation

[2/] = Population of quality protein maize

[3/] = Single cross hybrids

**Table 10** Mean grain yield of the tested hybrid in tons per hectare (below the diagonal), general combining ability (on the diagonal line) and specific combining ability (above the diagonal) in the diallel cross.

| Parents | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 |
|---|---|---|---|---|---|---|---|---|---|---|
| P1 | **0.29** | -1.26 | 0.63 | 0.93 | 1.11 | -0.97 | -0.65 | 0.20 | -0.54 | 0.37 |
| P2 | 3.08 | **-0.44** | -0.20 | 0.36 | -0.01 | -0.18 | -0.51 | 1.33 | 0.40 | 0.08 |
| P3 | 5.38 | 3.82 | **-0.03** | -0.89 | 0.51 | 0.44 | 0.90 | -1.50 | 0.29 | -0.17 |
| P4 | 5.67 | 4.37 | 3.53 | **-0.04** | -1.36 | -0.43 | -0.31 | 0.42 | 1.21 | -0.04 |
| P5 | 5.58 | 3.72 | 4.65 | 2.87 | **-0.31** | -0.85 | -0.53 | 0.69 | 0.27 | 0.07 |
| P6 | 3.12 | 2.99 | 4.02 | 3.14 | 2.45 | **-0.87** | 0.21 | -0.34 | 0.93 | 1.01 |
| P7 | 4.29 | 3.70 | 5.52 | 4.30 | 3.81 | 3.99 | **0.16** | 0.72 | -0.01 | 0.27 |
| P8 | 5.22 | 5.62 | 3.20 | 5.10 | 5.10 | 3.51 | 5.61 | **0.24** | -1.21 | -0.32 |
| P9 | 4.69 | 4.90 | 5.20 | 6.10 | 4.89 | 4.99 | 5.00 | 3.96 | **0.45** | -1.25 |
| P10 | 5.70 | 4.68 | 4.84 | 4.95 | 4.79 | 5.17 | 5.47 | 4.95 | 4.24 | **0.55** |

**Table 11** Means of grain yield, protein, tryptophan in protein content and studied traits of top 10 $F_1$ hybrids.

| $F_1$ hybrids[1/] | Yield (ton $ha^{-1}$) | Protein (%) | Tryptophan in protein (%) | Moisture content (%) | Shelling (ratio) | Ear height (cm) | Plant height (cm) | Days to anthesis (d) | Days to silking (d) |
|---|---|---|---|---|---|---|---|---|---|
| P9 x P4 | 6.10 | 8.55 | 0.794 | 8.00 | 0.89 | 73 | 145 | 59 | 58 |
| P10 x P1 | 5.70 | 7.15 | 0.756 | 9.15 | 0.81 | 68 | 128 | 57 | 57 |
| P4 x P1 | 5.67 | 6.15 | 0.847 | 7.00 | 0.77 | 62 | 116 | 60 | 60 |
| P8 x P2 | 5.62 | 6.85 | 0.848 | 7.80 | 0.88 | 64 | 138 | 56 | 56 |
| P8 x P7 | 5.61 | 7.10 | 0.729 | 8.95 | 0.86 | 56 | 129 | 56 | 56 |
| P5 x P1 | 5.58 | 7.82 | 0.783 | 8.15 | 0.81 | 73 | 133 | 58 | 57 |
| P7 x P3 | 5.52 | 8.24 | 0.782 | 8.35 | 0.84 | 51 | 122 | 56 | 57 |
| P10 x P7 | 5.47 | 8.04 | 0.948 | 7.15 | 0.82 | 59 | 127 | 59 | 59 |
| P3 x P1 | 5.38 | 8.34 | 0.660 | 8.15 | 0.77 | 73 | 128 | 58 | 60 |
| P8 x P1 | 5.22 | 7.40 | 0.867 | 8.90 | 0.80 | 78 | 146 | 58 | 58 |

[1/] = Days to harvesting were 127 days.

**Protein and tryptophan contents in the tested hybrids**

The protein contents in endosperm of the tested hybrids had no significant differences between them and also no significant differences from the standard varieties (Table 8). The protein content ranged from 6.15 to 9.18% for the tested hybrids (Table 12). Average protein content was 7.84 % for the tested hybrids, 8.18 % for QPM population and 7.76 % for non-QPM. There is no significant difference between general and specific combining ability of protein content in endosperm. Tryptophan content in maize endosperm was not significantly different from the tested hybrids and QPM population and average tryptophan content was 0.81 and 0.82 % of protein in the tested hybrids and QPM population, respectively (Table 9). But tryptophan content was higher in the tested hybrids (0.81%) than non-QPM (0.42%) (Tables 8 and 9). These results show that QPM varieties have almost double the amount of tryptophan compared to normal maize, but are similar in overall protein content. Moreover, the parental lines had the same tryptophan content as the tested hybrids.

**Table 12** Percentage of protein in maize endosperm of the tested hybrids (below the diagonal) and of tryptophan in protein of maize endosperm (above the diagonal) in the diallel cross.

| Parents | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 |
|---|---|---|---|---|---|---|---|---|---|---|
| P1 | | 0.792 | 0.660 | 0.847 | 0.783 | 0.645 | 0.786 | 0.866 | 0.648 | 0.756 |
| P2 | 8.64 | | 0.928 | 0.835 | 0.869 | 0.783 | 0.724 | 0.847 | 0.830 | 0.743 |
| P3 | 8.34 | 6.82 | | 0.786 | 0.545 | 0.928 | 0.781 | 0.825 | 0.636 | 0.882 |
| P4 | 6.15 | 8.10 | 9.18 | | 0.794 | 0.889 | 0.738 | 0.862 | 0.793 | 0.868 |
| P5 | 7.82 | 7.96 | 8.49 | 8.14 | | 0.819 | 0.901 | 0.868 | 0.857 | 0.783 |
| P6 | 8.80 | 7.58 | 8.39 | 7.48 | 8.74 | | 0.883 | 0.770 | 0.760 | 0.905 |
| P7 | 7.88 | 7.74 | 8.24 | 8.68 | 8.48 | 8.58 | | 0.729 | 0.871 | 0.948 |
| P8 | 7.40 | 6.85 | 8.12 | 7.23 | 9.07 | 7.71 | 7.09 | | 0.780 | 0.890 |
| P9 | 7.80 | 7.73 | 8.51 | 8.55 | 8.00 | 7.83 | 6.89 | 7.88 | | 0.976 |
| P10 | 7.15 | 6.98 | 7.12 | 6.82 | 8.15 | 6.51 | 8.04 | 7.27 | 7.74 | |

# CONCLUSION AND RECCOMMENTDATION

## Conclusion

Checking authenticity of quality protein maize (QPM) is crucial for the breeding program, especially for the ambiguous, heterozygote plants which could be easily crossed or contaminated in nature. Choosing an appropriate marker, in this case phi057, which could clearly identify the differences between three genotypes of *opaque-2* plants (also confirmed by the amount of tryptophan in the endosperm), could lead to a more reliable and faster approach to evaluating the results of the identified plants. Furthermore, phi057 would be applied as marker-assisted selection for improvement of QPM inbred lines, which could potentially enhance the efficiency of QPM breeding and obtain the QPM hybrid varieties in a short period of time.

There were the same amount of total protein contents in the endosperm of QPM and normal maize. On the contrary, there were highly significant differences in tryptophan content between QPM and normal maize endosperm where QPM had almost double the amount of tryptophan compared to normal maize.

Inbred lines derived from QPM populations are controlled by the *opaque-2* ($o_2o_2$) and some modifying genes, and their crosses have protein contents in endosperm the same as normal maize. But the percentage of tryptophan content in protein is almost two times that of normal maize. Moreover, grain yield of the best $F_1$ hybrid, P9 x P4 is about 6.10 t $ha^{-1}$ which is not significantly different from normal maize (commercial single cross hybrids; 6.61 t $ha^{-1}$). The results show that QPM hybrids could possibly be grown in Thailand without any difference in grain yield, but with more nutritive grain.

## Recommendation

Practically, inbred lines from QPM populations could be extracted using the classical method and *opaque-2* gene of inbred lines would be examined by the molecular marker, phi057, in advanced generations before making a diallel cross or forming the population. Moreover, vitreous kernels must be observed to keep modifying genes avoiding the opaque kernel and soften endosperm. In this studied the germplasm source for QPM population improvement would be $S_3$-inbred lines P1, P7, P8, P9 and P10 inbred lines. However, directly used exotic germplasm must be awareness on the adaptation to biotic and abiotic stresses in Thailand. So, the best way of QPM breeding program is line conversion using adapted inbred lines as recurrent parents and QPM inbred lines as donor parent. The classical backcross method associated with marker-assisted selection would be employed to convert the normal inbred lines to QPM inbred line.

# LITERATURE CITED

Agrawal, G.K., R.N. Pandey and V.P. Agrawal. 1992. Isolation of DNA from *Choerospondias asillaris* leaves. **Biotech. Biodiv. Lett. 2: 19-24.**

Babu, R., S.K. Nair, A. Kumar, S. Venkatesh, J.C. Sekhar, N.N. Singh, G. Srinivasan, H.S. Gupta. 2005. Two-generation marker-aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). **Theor. Appl. Genet.111: 888-897.**

Bailey, J.L. 1967. **Techniques in Protein Chemistry**. Elsevier Publishing Co., Amsterdam, New York.

Bass, H.W., C. Webster, G.R. Obrian, J.K. Roberts and R.S. Boston. 1992. A maize ribosome-inactivating protein is controlled by the transcriptional activator *opaque-2*. **The Plant cell 4: 225-234**.

Bernard, L., P. Ciceri, A. Viotti. 1994. Molecular analysis of wild-type and mutant alleles at the *opaque-2* regulatory locus of maize reveals different mutations and types of $O_2$ products **Plant Mol. Boil. 24: 949-959.**

Bjarnason, M. and S.K. Vasal. 1992. Breeding quality protein maize (QPM), pp181-216. *In* **Plant Breeding Reviews vol. 9**. John Wiley & Sons, Inc., New York.

Bressani, R. 1992. Nutritional value of high-lysine maize in humans, pp. 205-225. *In* **Quality protein maize**. E.T. Mertz (ed.) American Assoc. of Cereal Chem., St. Paul, MN.

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

CIMMYT. 1999. Quality protein maize: food of the poor becomes inexpensive, accessible protein source, pp. 38-39. *In* **CIMMYT annual report 1998-99**: science to sustain people and the environment. Mexico.

_____ 2000. SSR markers for *Opaque-2*. **CIMMYT molecular genetics service laboratory.** Mexico City, Mexico.

Davierwala, A.P., W. Ramakrishna, P.K. Ranjekar and V.S. Gupta. 2000. Sequence variations at a complex microsatellite locus in rice and its conservation in cereals. **Theor. Appl. Genet. 101: 1291-1298**.

Dreher, K., M. Morris, M. Khairallah, J.M. Ribaut, S. Pandey and G. Srinivasan. 2000. Is marker-assisted selection cost-effective compared to conventional plant breeding methods?: the case of quality protein maize. 25 p.

Dudley, J.W., D.E. Alexander and R.J. Lambert. 1975. Genetic improvement of modified protein maize, pp.120-135. *In* **High-quality protein maize**, Hutchinson Ross, Publishing, Stroudsburg.

FAO. 1992. Maize in human nutrition. **FAO food and nutrition series, No. 25.** Rome, Italy.

Francia, E., G. Tacconi, C. Crosatti, D. Barabaschi, D. Bulgarelli, E. Dall'Aglio and G. vale. 2005. Marker assisted selection in crop plants. **Plant Cell,Tissue and Organ Culture 82: 317-342**.

Gevers, H.O. and J.L. Lake. 1992. Development of modified *opaque-2* maize, p. 49. *In* **Quality protein maize**. E.T. Mertz (ed.) American Assoc. of Cereal Chem., St. Paul, MN.

Griffing, B. 1956. Concept of general and specific combining ability in relation to diallel crossing system. **Aust. J. Biol. 9: 463-493**.

Habben, J.E., G.L. Morro, B.G. Hunter, B.R. Hamaker and B.A. Larkins. 1995. Elongation factor- 1 alpha concentration is highly correlated with the lysine content of maize endosperm. **Proc. Natl. Acad. Sci. USA 92: 8640-8644.**

Hamada, H., M.G. Petrino and T. Kakunaga. 1982. A novel repeated element with Z-DNA-forming potential is widely found in evolutionarily diverse eukaryotic genomes. **Proc. Natl. Acad. Sci. USA 79: 6465-6469**.

Hartings, H., N. Lazzaroni, V. Rossi and M. Motto. 1995a. Molecular analysis of opaque2 alleles. **Maize Genet. Coop. Newslett. 69: 18-19**.

_____,_____, _____, G.R. Riboldi, R.D. Thompson, F. Salamini and M. Motto. 1995b. Molecular analysis of opaque2 alleles from *Zea mays* L. reveals the nature of mutational events and the presence of a hypervariable region in the 5' part of the gene. **Genet. Res. 65(1): 11-19**.

_____, M. Maddaloni, N. Lazzaroni, N.D. Fonzo, M. Motto, F. Salamini and R. Thompson. 1989. The $O_2$ gene which regulates zein deposition in maize encodes a protein with structural homologies to transcriptional activators. EMBO J. 8: 2795-2801.

Hernandez, H.H. and L.S. Bates. 1969. A modified method for rapid tryptophan analysis of maize. **CIMMYT Res. Bull. 13.** CIMMYT, Mexico City, Mexico.

Jompuk, P., W. Wongyai, C. Jampatong and S. Apisitvanich. 2006. Detection of quality protein maize (QPM) using simple sequence repeat (SSR) markers and analysis of tryptophan content. **Kasetsart J. (Nat.Sci.) 40: 768-774.**

Kata, S.R., B.H. Taylor, A.J. Bockholt and J.D. Smith. 1994. Identification of *opaque-2* genotypes in segregating populations of quality protein maize by analysis of restriction fragment length polymorphism. **Theor. Appl. Genet. 89: 407-412**.

Lamkey, K.R. and A.R. Hallauer. 1986. Performance of high x high, high x low, and low x low cross of lines from the BSSS maize aynthetic. **Crop Sci. 26: 1114-1118**.

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

Loesch, P.J.Jr. 1979. Registration of $BSAAo_2(s)$ $C_1$ maize germplasm. **Crop Sci.19:755.**

Lohmer, S., M. Maddaloni, M. Motto, N. Di Fonzo, N. Hartings, F. Salamini and R.D. Thompson. 1991. The maize regulatory locus *opaque-2* encodes a DNA-binding protein which activates the transcription of the *b-32* gene. **EMBO J. 10: 617-624**.

Lopes, M.A. and B.A. Larkins. 1994.Genetic analysis of endosperm modification in quality protein maize, pp. 149-174. *In* **Quality protein maize**: 1964-1994. B.A. Larkins and E.T. Mertz (eds.) Proceedings of the international symposium on quality protein maize.December 1-3, 1994, EMBRAPA/CNPMS. Sete Lagoas, Minas Gerais, Brazil.

Melchinger, A.E. 1990. Use of molecular markers in breeding for oligogenic disease resistance. **Plant Breeding 104:1-19.**

Mertz, E.T. 1986. Genetic and biochemical control of grain protein synthesis in normal and high lysine cereals. **World Review of Nutrition and Dietetics 48:222-262.**

_____ 1992. **Quality proitein maize**. Am. Assoc. Cereal Chem., St. Paul, MN.

_____ 1994. Thirty year of *opaque-2* maize, pp. 79-88. *In* Quality protein maize: 1964-1994. B.A. Larkins and E.T. Mertz (eds.) Proceedings of the international symposium on quality protein maize. December 1-3, 1994, EMBRAPA/CNPMS. Sete Lagoas, Minas Gerais, Brazil.

Motto, M., M. Maddaloni, G. Ponziani, M. Brembilla, R. Marotta, N.D. Fonzo, C. Soave, R.D. Thompson and F. Salanimi. 1988. Molecular cloning of the $o_2$-m5 allele of *Zea mays* using transposon marking. **Mol. Gen. Genet. 212: 488-494.**

MSTAT-C. 1988. **MSTAT-C**; a microcomputer program for the design, management, analysis of agronomic research experiments. Michigan State University. 402 p.

Nelson, O.E., E. T. Mertz and L.S. Bates. 1965. Second mutant gene affecting the amino acid pattern of maize endosperm proteins. **Science 150:1469-1470**.

Pixley, K.V. and M.S. Bjarnason. 1993. Combining ability for yield and protein quality among modified-endosperm opaue-2 tropical maize inbreds. **Crop Sci. 33: 1229-1234**.

_____ 2002. Stability of grain yield, endosperm modification, and protein quality of hybrid and open-pollinated quality protein maize (QPM) cultivars. **Crop Sci. 42: 1882-1890.**

Poehlman, J.M. 1987. **Breeding Field Crops**. Van Nostrand Reinhold, New York.

Powell, W., G.C. Machray and J. Provan. 1996. Polymorphism revealed by simple sequence repeats. **Trends Pl. Sci. 1: 215-222**.

Prasanna. B.M., S.K. Vasal, B. Ksssahun and N.N. Singh. 2001. Quality protein maize. **Curr. Sci. 81: 1308-1319.**

Ribaut, J.M. and D.A. Hoisington. 1998. Marker-assisted selection: new tools and strategies. **Trends in Pl. Sci. 3(6): 236-239.**

Schmidt, R.J., F.A. Burr and B. Burr. 1987. Transposon tagging and molecular analysis of the maize regulatory locus *opaque-2***. Science. 238: 960-963.**

_____,_____, M.J. Aukerman and B. Burr. 1990. Maize regulatory gene opaque-2 encodes a protein with a "leucine-zipper" motif that binds to zein DNA. **Proc. Natl. Acad. Sci. USA 87: 46-50.**

Sprague, G.F. 1964. Early testing and recurrent selection. pp. 400-417. *In* J.W. Gowen (ed.). **Heteosis.** Hafner publ. Company, New York.

Sriwatanapongse, S., E.C. Johnson, S.K. Vasal and E. Villegas. 1974. Inheritance of kernel vitreosity in *opaque-2* maize. SABRAO. J. 6:1-6.

Vasal, S.K. 1994. High quality protein corn, pp. 79-121. *In* **Specialty Corns**. A.R. Hallauer (ed.) CRC Press, Boca Raton, Florida.

_____ 2001. High quality protein corn, pp. 85-129. *In* **Specialty Corns**. A.R. Hallauer (ed.) CRC Press, Boca Raton, Florida.

_____, E. Villegas, M. Bjarnason, B. Gelaw and P. Goertz. 1980. Genetic modifiers and breeding strategies in developing hard endosperm *opaque-2* materials, pp. 37-73. *In* Improvement of quality traits of maize for grain and silage use. W.G. Pollmer and R.H. Phillips (eds.) Martinus Nijhoff. London, UK.

_____, _____, C.Y. Tang, J. Werder and M. Read. 1984. Combined use of two genetic systems in the development and improvement of quality protein maize. Kulturpflange 32:171-185.

_____, G. Srinivasan, S. Pandy, C.F. Gonzalez, J. Crossa, D.L. Beck. 1993a. Heterosis and combining ability of CIMMYT's quality proteinmaize germplasm I: lowland tropical. **Crop Sci. 33: 46-51.**

_____ 1993b. Heterosis and combining ability of CIMMYT's quality proteinmaize germplasm II: sub tropical. **Crop Sci. 33: 51-57.**

Villegas, E. 1994. Factors limiting quality protein maize (QPM) development and utilization, pp. 79-88 *In* **Quality protein maize**: **1964-1994**. B A. Larkins and E.T. Mertz (eds.) Proceedings of the international symposium on quality protein maize. December 1-3, 1994, EMBRAPA/CNPMS. Sete Lagoas, Minas Gerais, Brazil.

_____ and E.T. Mertz. 1971. Chemical screening methods for maize protein quality at CIMMYT. **CIMMYT Res. Bull. 20**. CIMMYT, Mexico City, Mexico.

Weber, J.L. and P.E. May. 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. **American Journal of Human Genetics 44:388-396.**

Yang, W.P., A.C. De oliverira, I. Godwin, K. Schertz and J.L. Bennetzen. 1996. Comparison of DNA marker technologies in characterizing plant genome diversity: variability in Chinese sorghums. **Crop Sci. 36: 1669-1676**.

_____, Y.G. Zheng, S. Ni and J. Wu. 2004. Recessive allelic variations of three microsatellite site within the $o_2$ gene in maize. **Plant Molec. Biol. Rept. 22: 361-374**.

# APPENDIX

**Appendix 1** Reagents for protein analysis

1. Sulfuric acid: nitrogen free.
2. Catalyst mixture: by the ratio of 100 $K_2SO_4$ : 10 $CuSO_4$. : 1 Se metal
3. Sodium hydroxide solution (dissolve 100 g of NaOH in distilled water and dilute to 100 ml).
4. Boric acid solution (4%).
5. Indicator solution methyl red-bromocresol green (mix 1 part 0.2% methyl red in ethanol with 5 parts 0.2% bromocresol green in ethanol).
6. Hydrochloric acid solution: 0.02 N.

**Appendix 2** Reagents for tryptophan determination

**Reagent A**: 270 mg of $FeCl_3$. $6H_2O$ dissolved in 1 liter of glacial acetic acid in a volumetric flask. Each bottle of acetic acid must be tested for color development in the presence of tryptophan.

**Reagent B**: 30N Sulfuric acid. Dissolve 833.3 ml concentrated sulfuric acid in distilled water and diluted to 1 liter. Slowly add concentrated sulfuric acid to the distilled water in a volumetric flask, on iced water with constant magnetic mixing.

**Reagent C**: Prepare a volume to volume mixture of reagents A and B, 1 hour prior to use. This solution will contain glyoxylic acid which is an impurity of acetic acid and is also formed on mixing acetic acid containing ferric chloride with sulfuric acid. This glyoxylic acid in the presence of tryptophan (indole group) produces the color.

**Reagent D**: 0.1N Sodium acetate buffer, pH 7.0 – Dissolve 8.203 g sodium acetate in distilled water and dilute to 1 liter in a volumetric flask. The pH should be adjusted to 7.0 with glacial acetic acid.

**Reagent E**: Papain solution: Dissolve 4 mg papain enzyme per ml 0.1 N sodium acetate buffer, pH 7.0. This solution should be prepared just minutes before use.

**Reagent F**: Standard tryptophan solution of 100 μg per ml – Dissolve 50 mg tryptophan in distilled water and dilute to 500 ml in a volumetric flask (for preparation of the standard curve).

**Appendix Table 1** Means of grain yield, protein, tryptophan in protein content and studied traits of $F_1$ hybrids.

| $F_1$ hybrids | Yield (ton $ha^{-1}$) | Protein (%) | Tryptophan in protein (%) | Moisture content (%) | Shelling (ratio) | Ear height (cm) | Plant height (cm) | Days to anthesis (d) | Days to silking (d) | Kernel color |
|---|---|---|---|---|---|---|---|---|---|---|
| P2 x P1 | 3.08 | 8.64 | 0.792 | 9.15 | 0.75 | 66 | 130 | 59 | 60 | yellow |
| P3 x P1 | 5.38 | 8.34 | 0.660 | 8.15 | 0.77 | 73 | 128 | 58 | 60 | yellow |
| P4 x P1 | 5.67 | 6.15 | 0.847 | 7.00 | 0.77 | 62 | 116 | 60 | 60 | yellow |
| P5 x P1 | 5.58 | 7.82 | 0.783 | 8.15 | 0.81 | 73 | 133 | 58 | 57 | yellow |
| P6 x P1 | 3.12 | 8.81 | 0.645 | 8.00 | 0.70 | 59 | 117 | 58 | 57 | yellow |
| P7 x P1 | 4.29 | 7.89 | 0.786 | 7.00 | 0.80 | 62 | 117 | 58 | 58 | yellow |
| P8 x P1 | 5.22 | 7.40 | 0.867 | 8.90 | 0.80 | 78 | 146 | 58 | 58 | yellow |
| P9 x P1 | 4.69 | 7.80 | 0.648 | 8.20 | 0.79 | 83 | 147 | 56 | 56 | yellow |
| P10 x P1 | 5.70 | 7.15 | 0.756 | 9.15 | 0.81 | 68 | 128 | 57 | 57 | yellow |
| P3 x P2 | 3.82 | 6.82 | 0.928 | 7.10 | 0.79 | 57 | 117 | 57 | 57 | yellow |
| P4 x P2 | 4.37 | 8.10 | 0.835 | 7.05 | 0.80 | 71 | 130 | 57 | 57 | yellow |
| P5 x P2 | 3.72 | 7.96 | 0.870 | 8.35 | 0.73 | 66 | 135 | 56 | 56 | yellow |
| P6 x P2 | 2.99 | 7.58 | 0.783 | 8.45 | 0.66 | 57 | 114 | 53 | 54 | yellow |
| P7 x P2 | 3.70 | 7.74 | 0.724 | 7.90 | 0.68 | 65 | 138 | 55 | 56 | yellow |
| P8 x P2 | 5.62 | 6.85 | 0.848 | 7.80 | 0.88 | 64 | 138 | 56 | 56 | yellow |

**Appendix Table 1** (continued).

| $F_1$ hybrids | Yield (ton $ha^{-1}$) | Protein (%) | Tryptophan in protein (%) | Moisture content (%) | Shelling (ratio) | Ear height (cm) | Plant height (cm) | Days to anthesis (d) | Days to silking (d) | Kernel color |
|---|---|---|---|---|---|---|---|---|---|---|
| P9 x P2 | 4.90 | 7.74 | 0.830 | 8.30 | 0.79 | 61 | 125 | 57 | 56 | yellow |
| P10 x P2 | 4.68 | 6.98 | 0.743 | 6.60 | 0.75 | 57 | 128 | 57 | 58 | yellow |
| P4 x P3 | 3.53 | 9.18 | 0.786 | 7.60 | 0.78 | 70 | 139 | 57 | 58 | yellow |
| P5 x P3 | 4.65 | 8.50 | 0.546 | 7.70 | 0.78 | 59 | 145 | 55 | 57 | yellow |
| P6 x P3 | 4.02 | 8.39 | 0.928 | 7.65 | 0.81 | 59 | 114 | 55 | 57 | yellow |
| P7 x P3 | 5.52 | 8.24 | 0.782 | 8.35 | 0.84 | 51 | 122 | 56 | 57 | yellow |
| P8 x P3 | 3.20 | 8.12 | 0.826 | 6.90 | 0.72 | 77 | 137 | 57 | 58 | yellow |
| P9 x P3 | 5.20 | 8.52 | 0.636 | 9.75 | 0.86 | 75 | 157 | 57 | 57 | yellow |
| P10 x P3 | 4.84 | 7.12 | 0.882 | 9.15 | 0.78 | 57 | 134 | 57 | 58 | yellow |
| P5 x P4 | 2.87 | 8.14 | 0.794 | 8.15 | 0.69 | 64 | 137 | 57 | 58 | yellow |
| P6 x P4 | 3.14 | 7.49 | 0.889 | 8.55 | 0.64 | 63 | 118 | 58 | 59 | yellow |
| P7 x P4 | 4.30 | 8.69 | 0.739 | 7.25 | 0.69 | 57 | 132 | 60 | 60 | yellow |
| P8 x P4 | 5.10 | 7.23 | 0.862 | 7.05 | 0.82 | 80 | 143 | 60 | 59 | yellow |
| P9 x P4 | 6.10 | 8.55 | 0.794 | 8.00 | 0.89 | 73 | 145 | 59 | 58 | yellow |
| P10 x P4 | 4.95 | 6.82 | 0.868 | 7.05 | 0.67 | 68 | 128 | 59 | 59 | yellow |

**Appendix Table 1** (continued).

| $F_1$ hybrids | Yield (ton $ha^{-1}$) | Protein (%) | Tryptophan in protein (%) | Moisture content (%) | Shelling (ratio) | Ear height (cm) | Plant height (cm) | Days to anthesis (d) | Days to silking (d) | Kernel color |
|---|---|---|---|---|---|---|---|---|---|---|
| P6 x P5 | 2.45 | 8.74 | 0.819 | 8.90 | 0.80 | 75 | 144 | 55 | 55 | yellow |
| P7 x P5 | 3.81 | 8.48 | 0.901 | 8.55 | 0.76 | 52 | 124 | 57 | 57 | yellow |
| P8 x P5 | 5.10 | 9.07 | 0.868 | 9.05 | 0.88 | 66 | 132 | 58 | 58 | yellow |
| P9 x P5 | 4.89 | 8.01 | 0.857 | 10.80 | 0.81 | 69 | 137 | 57 | 57 | yellow |
| P10 x P5 | 4.79 | 8.16 | 0.783 | 8.65 | 0.81 | 55 | 128 | 56 | 56 | yellow |
| P7 x P6 | 3.99 | 8.58 | 0.883 | 9.25 | 0.81 | 49 | 114 | 56 | 56 | yellow |
| P8 x P6 | 3.51 | 7.71 | 0.771 | 9.20 | 0.76 | 57 | 108 | 55 | 54 | yellow |
| P9 x P6 | 4.99 | 7.84 | 0.760 | 8.45 | 0.77 | 64 | 122 | 57 | 56 | yellow |
| P10 x P6 | 5.17 | 6.52 | 0.906 | 7.60 | 0.81 | 48 | 108 | 55 | 53 | yellow |
| P8 x P7 | 5.61 | 7.10 | 0.729 | 8.95 | 0.86 | 56 | 129 | 56 | 56 | yellow |
| P9 x P7 | 5.00 | 6.89 | 0.871 | 8.55 | 0.67 | 60 | 125 | 60 | 60 | yellow |
| P10 x P7 | 5.47 | 8.04 | 0.948 | 7.15 | 0.82 | 59 | 127 | 59 | 59 | yellow |
| P9 x P8 | 3.96 | 7.88 | 0.780 | 7.85 | 0.88 | 73 | 140 | 59 | 58 | yellow |
| P10 x P8 | 4.95 | 7.28 | 0.891 | 8.60 | 0.77 | 76 | 142 | 58 | 59 | yellow |
| P10 x P9 | 4.24 | 7.74 | 0.976 | 7.90 | 0.76 | 59 | 121 | 58 | 56 | white |

P1 = Pop65$C_6$-$S_3$-11-1-1

P2 = Pop65$C_6$-$S_3$-37-1-1

P3 = Pop65$C_6$-$S_3$-74-4-1

P4 = Pop65$C_6$-$S_3$-82-1-1

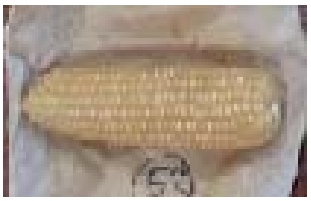
P5 = Pop61$C_1$ -$S_3$-22-4-1

P6 = Pop61$C_1$ -$S_3$-63-2-1

P7 = Pop61$C_1$ -$S_3$-91-4-1

P8 = Pop61$C_1$ -$S_3$-100-4-1

P9 = Pop62$C_6$ -$S_3$-48-1-1



P10 = Pop62$C_6$ -$S_3$-99-4-2

**Appendix Figure 1** Ears and pedigrees of 10 $S_3$ QPM inbred lines

# CURRICULUM VITAE

**NAME** : Ms. Peeranuch Jompuk

**BIRTH DATE** : April 29, 1967

**BIRTH PLACE** : Nakhon Ratchasima, Thailand

| **EDUCATION** | **: YEAR** | **INSTITUTE** | **DEGREE** |
|---|---|---|---|
| | 1989 | Kasetsart University | B.S. (Agriculture) |
| | 1993 | Kasetsart University | M.S. (Agriculture) |

**POSITION** : Assistant Professor

**WORK PLACE** : Department of Applied Radiation and Isotope,
Faculty of Science, Kasetsart University, Thailand

**SCHOLARSHIP** : Thai Government Scholarship 2003-2006 for Cooperative Research Network