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THESIS

MARKER-ASSISTED SELECTION FOR QUALITY PROTEIN IN WAXY CORN (*Zea mays* var. *ceratina*)

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Waxy or amylopectin maize (*Zea mays* var. *ceratina*) is an important staple food and vegetable in Southeast and East Asia. Its insufficient protein quality can be remedied by the *opaque-2* gene mutation, demanding the combination of two recessive endosperm quality genes, *opaque-2* (o_2o_2) and *waxy* (*wxwx*). Crosses were made between waxy and opaque-2 maize as female and male parents, respectively. In the segregating progenies of two crosses, Kwi#1 × AgQ53 and Kwi#9 × AgQ53, immediate selfing or one-time backcrossing to the waxy parent before selfing were used to achieve the combination o_2o_2wxwx, associated with marker-assisted selection of *opaque-2* by *phi057* and of *waxy* by *phi022*. Eleven of waxy-opaque-2 (o_2o_2wxwx) lines were achieved from initial backcrossing and selfing methods. Furthermore, high variation in grain quality traits is an incentive for further improvement by breeding. Consumption of high-quality protein maize will improve the diets of children, a good reason to produce double-quality vegetable waxy maize. The percentage of tryptophan content in endosperm of these lines was high about 0.95% while the normal maize was about 0.5%. Moreover, amylopectin was high as waxy maize. The results indicating that the goal of combining two quality traits within one grain was achieved. The best green weight of hybrid was about 2,127 kg/rai while the check variety was about 1,798 kg/rai. Total protein content of fresh seed was 12.3% for hybrids and 11.11% for checks. The average of tryptophan content in endosperm of F_1 hybrid was 0.68 and 0.65% from backcrossing and selfing methods, respectively while the check variety was about 0.52%.

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Bunyarit Sinkangam

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LIST OF ABBREVIATIONS

CIMMYT	=	The International Maize and Wheat Improvement Center
FAO	=	Food and Agriculture Organization of the United Nations
HQPM	=	high quality protein maize
MAS	=	marker-assisted selection
WSP	=	water soluble polysaccharides
GCA	=	general combining ability
SCA	=	specific combining ability
RFLP	=	restriction fragment length polymorphism
RKM	=	relative kernel mass
PCR	=	polymerase chain reaction
RAPD	=	random amplification of polymorphic DNA
SSR	=	simple sequence repeat
AFLP	=	amplified fragment length polymorphism
SNP	=	single nucleotide polymorphism
QTL	=	quantitative trait loci
MABC	=	marker-assisted backcross
SCLB	=	southern corn leaf blight
NCLB	=	northern corn leaf blight
SCMV	=	sugarcane mosaic virus

MARKER-ASSISTED SELECTION FOR QUALITY PROTEIN IN WAXY CORN (*Zea mays* var. *ceratina*)

INTRODUCTION

The problem of protein deficiency has found in many countries in the world particularly to non-developed and developing countries. There are wide spread of malnutrition among children and women. Most children are stunted because of malnutrition and underweight. The serious problem is lacking of high quality protein sources.

Waxy corn or glutinous corn (*Zea mays* var. *ceratina*) is one of the most popular fresh corns in Thailand and Asia regions because it has good taste, high tenderness, sticky and highly economical value. However, the protein in ordinary maize is poor nutritional value because of it deficiency of two essential amino acids, lysine and tryptophan. Both of amino acids value is helping meet the world's human and animal nutritional needs. Fortunately, breeder and biochemist at Purdue University discovered an *opaque-2* mutant gene causing high lysine and tryptophan in endosperm of maize over of normal maize (Mertz *et al*., 1964). In field corn, worldwide conversion programs produced *opaque-2* varieties and hybrids, which subsequently with extensive testing in many countries in the world. Some problems that are considered seriously, in general, have included low grain yield, non acceptability of soft lusterless texture of kernel and greater susceptibility to ear rots and greater infestation by insects. Following only a few institutes and programs showed continuing interest. CIMMYT have more effort to change in kernel phenotype from soft to hard endosperm, a combination of *opaque-2* gene and genetic modifier was considered most appropriate to rectify phenotype appearance and other agronomic problems affecting in the normal maize. Fortunately, new variety of maize was found as high quality protein maize (HQPM), which extremely proved beneficial for other QPM researches.

However, there has fewer documents about improvement of waxy corn with *opaque-2* gene. If it is possible to transfer the *opaque-2* (o_2o_2) gene to waxy corn (*wxwx*), it will be benefit for the human food. Most importantly, this study aimed to transfer a high quality protein trait from QPM to waxy corn, by integrating techniques between standard breeding programs, backcrossing and inbreeding methods accompanied with a marker-assisted selection (*MAS*), which can rapidly help to select the characters of tryptophan and waxy traits. The *opaque-2* gene was tested with the *phi057* marker (Chin *et al*., 1996) as co-dominant type, in addition, the waxy trait (*wxwx*) was tested by *phi022* (Senior *et al*., 1998) that screened for each generation to select the double homozygous recessive (o_2o_2wxwx) lines as our goal and, finally F_1 hybrids was made at the end of this program.

OBJECTIVES

1. To improve protein quality of waxy corn by transferring the *opaque-2* gene from the quality protein maize (QPM)

2. To conduct marker-assisted selection (*MAS*) for *opaque*-2 and *waxy* genes during selecting

3. To find the combining ability of the selected *waxy-opaque-2* lines

LITERATURE REVIEW

Waxy corn backgrounds

In many decade publications, the new plants as possessing a number of unique characters, no indications of these characters in any recorded form of *Zea mays* had thus far been found. Several of the unique features combine to enable the plant to resist the drying out of the silks by dry, hot winds at the time of flowering. Although the plants produced such small ears that it could find no place in direct competition with the improved varieties, the possession of this adaptation gave the new type an economic interest. Consequently, the effort has been made to combine by hybridizing the desirable characters of this small variety with those of larger and more productive types (Collins, 1914). When he found such a distinct difference in the appearance of normal and waxy maize endosperm, he suspected a difference in chemical composition, but the analysis did not yield any unusual results. The percentage of starch, oil and protein were all within the normal range.

A waxy corn selection found in China in the early 1900s was described as having an endosperm with a dull, waxy-like appearance (Collins, 1909). This endosperm trait was subsequently found to be controlled by a single recessive gene on chromosome 9 and was designated as *waxy* (*wx*). It has also been referred to as Chinese waxy (*wx-c*) due to its origin of discovery (Coe *et al*., 1988). In a review of the genetics of corn that emphasized the uniqueness of this phenotype and described the waxy trait as having a marble-like opacity with a hardness similar to normal corn. The distinctiveness of the waxy phenotype is so vivid that it is easily identified visually in most corn germplasm backgrounds. However, the moisture content of the kernel must be 16% or lower before the waxy trait can be recognized visually. Corn endosperms that are homozygous for the *wx* gene produce only the branched starch component (amylopectin) and are devoid of the linear amylose fraction. Another unique characteristic of the waxy trait is its specific staining reaction, when exposed to a dilute solution of iodine (Weatherwax, 1922). Argentine waxy (*wx-a*), an allele at the *wx* locus first reported by University of Buenos Aires, Argentina is known to

produce small amounts of amylose (<5%) and gives an intermediate staining reaction with iodine.

Bates *et al*. (1943) confirmed that endosperm starch of waxy maize consists nearly exclusively of amylopectin. The presence of amylopectin in rice had been demonstrated previously by Parnell (1921). In 1937, Sprague and other plant breeders at what was then called Iowa State College had begun a crossbreeding program to attempt to introduce the waxy trait into a regular high-yielding hybrid maize. By this time, the waxy plant no longer had the peculiar structural traits noted by Collins, probably due to years of crossing into various genetic stocks. Only the unique endosperm had been retained. At this time, waxy maize was not so important because the main source of pure amylopectin still was also found in the cassava plant, a tropical shrub with a large underground tuber (FAO, 2004).

A single recessive gene (*wx*), located on the short arm of chromosome 9, codes for the waxy endosperm of the kernel (Neuffer *et al*., 1997). This was first shown by Collins (1909); Kempton (1919). The structure of the wild-type *waxy* (wx^+) locus has been determined through DNA sequence analysis. The gene has 3718 bp (14 exons and 13 introns). Waxy endosperm is the counterpart in maize of the "glutinous" character in rice (Mangelsdorf, 1974). There is a wide range of species also presenting the waxy mutation, including rice, sorghum, millet, barley and wheat, which were characterized by starch granules staining red with iodine. In crosses between heterozygous plants for the waxy character, a small but significant deviation from an expected Mendelian ratio in self pollination is produced. Bear (1944) obtained from segregated ears on the F_1 generation, 77% of waxy kernels and 23% of non-waxy kernels. This is evidenced by the two heterozygous types, *Wx:Wx:wx* and *wx:wx:Wx*. The *waxy* gene is epistatic (Creech, 1968; Boyer *et al*., 1976) for all known other starch forming mutants genes like *dull* (*du*), *sugary-1* (su_1) and *sugary-2* (su_2) (White, 1994) it increases sugars and water-soluble polysaccharides (WSP) in a su_1 background (Andrew *et al*., 1944) and it causes dramatic increases in sugars and reduction in starch with *ae* or *aedu*.

Echt and Schwartz (1981) demonstrated that the extract from *wx-C* starch lacked a 60-kd protein that was present in the extracts from *Wx* starch. The demonstration that the *wx* locus is the structural gene for the 60-kd protein came from the observation that four of the *wx* alleles mapped by Nelson (1962) produced altered forms of this protein. The connection between the 60-kd protein and the glucosyl transferase was affirmed by showing that the amount of protein increased in triploid endosperms through the dosage series *wx:wx:wx, wx:wx:Wx, Wx:Wx:wx* and *Wx:Wx:Wx* in the same manner in which the enzymatic activity had been shown to increase (Tsai, 1974). Fedoroff *et al*. (1983) identified a restriction fragment from *Ac wx-m9,* an autonomous mutable allele resulting from the insertion of the transposable element *Ac* into a *Wx* allele. This was the initial isolation of *Ac*, which was shown to be a 4.3 kb element.

Waxy corn improvement

From the onset of many waxy inbreds development programs, the backcross method has probably been the most popular. There is one compelling reason to choose this breeding method for waxy hybrid improvement. Competition between the seed companies to provide better hybrids as fast as possible encouraged many breeders to choose the backcross breeding method. Conversion of elite dent lines provide the fastest and most positive result. However, the new hybrids are only expected to have equal but not to exceed the performance of its counterparts. And meanwhile, there are newer and better dent hybrid developments. That is the reason why another breeding method is wished. Initially, most private waxy breeding programs did not have adequate reserves of waxy converted germplasm to permit its utilization in more complex breeding schemes. Nowadays, enough germplasm material exists and a recurrent selection program would be possible. But due to the limited resources and markets for waxy maize, no long-term program has yet been conducted. Actually, it does not seem that long-term recurrent selection programs are really necessary for waxy maize breeding (Brink, 1925).

The major requirement is the presence of the *waxy* gene within the source breeding population at a frequency that would provide a reasonable level of success in recovery, assuming proper selection techniques were used. For example, in a pedigree-selection scheme performed within a breeding population derived from crossing two elite inbred sources, it is necessary that only one of the two inbred lines contribute the *waxy* gene to the population. This is the only additional genetic trait, compared with a normal dent breeding program that has to be considered in the selection process. Adequate sample size of the source breeding population is, however, of prime importance. Economics of a breeding program and the potential benefits from it have to be determined before making the final decisions on what approach to pursue. Waxy corn is considered a special type corn and does not have the market potential like normal dent, consequently, not all private seed companies involved in marketing waxy corn can justify as an extensive breeding program including substantial expenditures into population improvement *per se*. Reconsiderations, however, are warranted if a private seed company has sufficient sales potential in both the dent and waxy corn markets.

The development of waxy inbred lines by self pollination within crosses among elite germplasm sources followed by selection and testing is a common procedure in most waxy corn breeding programs. The development of elite waxy inbred lines by the backcross method has been very successful, regardless of the breeding methods used, waxy breeding programs can be enhanced by the employment of the pollen staining technique. This technique improves the efficiency of a program by eliminating pollination of plants, which do not carry the *wx* gene. This technique is more applicable to consecutive backcrossing programs designed to obtain the maximum cycles per year and eliminates the necessity in a continuous backcrossing program for using testcrosses to detect plants having the *waxy* gene. Fergason (2001) presented data that, on average, waxy hybrids were within 3.5% of the yield of its normal counterparts. Two hybrids had similar yields, and three waxy hybrids had 4.9 to 7.4% lower yields. Fergason (2001) also included data that the test weights of 14 waxy hybrids averaged 2% greater than those of its normal dent versions. Seed companies reported that yields of waxy hybrids were 95 to 97% those of normal dent

corn, and 32 waxy corn produces were reported that waxy corn hybrids had yields similar to those of normal dent corn hybrids in 1998 and 1999 (U.S. Grains Council, 2002).

The waxy corn in Thailand has imported seed for along times. All most all, it has big ear for "Kao Pod Kao Neaw" and small ear for "Kao Pod Tien". In the recent year, waxy corn breeding programs have initiated in a few parts especially in public sectors, which were open-pollinated varieties. In the current, the single cross varieties have more growing up to utilize in several locations and to pick up for developing high quality traits such as high yield, good agronomic traits, good defensive traits, high quality traits, *etc*. However, waxy corn have still discarded good germplasm for using in breeding program, thus, the import waxy corn seed to collect germplasm derived from primitive and exotic varieties always to create a new strongly genetic pattern. Amnuaysitt *et al*. (1998) created a waxy corn hybrid from 7 primitive open-pollinated varieties in diallel crossing pattern. The results showed that some varieties highly expressed GCA and SCA. They suggested that, therefore, waxy corn hybrid is able to use open-pollinated variety as the parents. Furthermore, Chiangmai Field Crop Research Center (1991) released a new waxy hybrid that was SSR TW 8801 from "Tien Hua Raw" by S_1 selection and modified mass selection. It has good agronomic characters such as yield, good stem vigor, ear stability, good bite taste, good tenderness, *etc*.

Promkam *et al.* (2003) had recorded around 30 characters in waxy corn. The highlight traits were seed colors that consist of white, yellow, purple and spotted, while days to flowering contained early type: lesser than 45 days and between 46 to 55 days, late type: more than 55 days, *etc*. Ear size consisted of small: 2.5 × 10.5 cm and big: 4.5 × 18 cm. Likewise, Thongleung *et al.* (1997) collected 136 varieties of "Kao Pod Tien" and "Kao Pod Kao Neaw", which were found in differently parts of Thailand and brought to continue successful developed an new open-pollinated varieties. The recent waxy varieties have done single cross waxy corn hybrids in many projects. Moreover, Thongleung *et al.* (2003) produced a new recommended single cross waxy corn hybrid namely, Kwsx107. Later, Thongleung *et al*. (2005)

released a single cross eight-row waxy corn hybrid as Kwpsx 7436, which had a good taste with sticky texture. It had high yield, good agronomic characteristics including a good husk cover and had moderately resistant to rust and viral diseases.

Quality protein maize backgrounds

Quality Protein Maize (QPM) contains nearly twice lysine and tryptophan compared to other normal maize grown in the tropics and grain yields was not lesser than traditional varieties. QPM was developed by Dr. Surinder Vasal and Dr. Evangelina Villegas at the International Maize and Wheat Improvement Center (CIMMYT) in the late 1990's. For their achievement, they won the 2000 World Food Prize (Kataki and Babu, 2002). In Central and South America, Africa, and Asia, several hundred million people rely on maize as their principal daily food, for weaning babies, and for feeding livestock. Unfortunately, maize has one significant flaw that lacks of the full range of amino acids, namely lysine and tryptophan that needed to produce proteins. Hence diets high in corn produce a condition known as wet malnutrition a person is receiving sufficient calories, but her or his body malfunctions due to a lack of protein. A chronic lack of protein in the diet leads to kwashiorkor. Thus, conventional maize is a poor quality food staple, unless consumed as part of a varied diet, which is beyond the means of most people in the developing world it typically causes malnutrition. Babies weaned on it are frequently underweight, prone to disease, and at high risk for starvation. QPM produces 70-100% more of lysine and tryptophan than the most modern varieties of tropical maize. These two amino acids allow the body to manufacture complete proteins, thereby eliminating wet malnutrition

Several mutants have been detected over the past 30 years than can favorably modify characteristics of the corn 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). Shortly thereafter 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) (McWhirter, 1971), *opaque-6* (o_6) (Ma and Nelson, 1975), *floury-3* (fl_3) (Ma and Nelson, 1975), mucronate (*Mc*) (Salamini *et al.*, 1983), and defective endosperm (*De-B30*) (Salamini *et al*., 1979). Attempts also have been made to search high lysine gene(s) but with still a high level of zein fraction. Two such mutants, *opaque* 7749 and *opaque* 7455 (o_{11}), were identified (Nelson, 1980). The *opaque* 7749 is particularly interesting since it is markedly higher in lysine than the normal counterpart, although not as high as *opaque-2*, and the prolamin fraction is quite high. The mutant 7455 was later referred to as *opaque-11*.

The effects of HQPC mutants have been studied by several research workers worldwide. Of course more studies have been conducted with o_2 and fl_2 mutants and Both o_2 and fl_2 mutants change the amino acid profile, thus resulting in an increase of lysine and tryptophan. The lysine in corn is the first and tryptophan the second limiting amino acid. In addition, other amino acids such as histidine, argenine, aspartic acid, and glycine have shown an increase. Some other amino acids like glutamic acid, alanine, and leucine are decreased compared to normal corn. A most notable decrease occurs in leucine. This is desirable because it makes the leucine-isoleucine ratio more favorable, which in turn helps to liberate more tryptophan for niacin biosynthesis. HQPC has notable benefit in combating pellagra, even though it has no more niacin than normal corn. Methionine, a sulfur containing amino acid, registers an increase in fl_2, o_7, and o_6. Other mutants, however, show no change in the content of methionine.

Quality protein maize improvement

Major emphasis in most breeding programs for protein quality improvement is based on the *opaque-2* (o_2) mutant and genetic modifiers of the endosperm textural properties of *opaque-2* corn. The implications of the *opaque-2* discovery (Mertz *et al.*, 1964) were considered remarkable. Later, however, many practical problems and limitations arose with the standard *opaque-2* (soft-endosperm) corn. The failure of o_2

corn to become more acceptable was primarily due to reduce grain yield. Kernels of o_2 are characterized by a soft, chalky, nontransparent appearance with very little hard, vitreous (horny) endosperm. This type of kernel is more prone to damage by insects and kernels rots both in the field and in storage and harvesting machinery.

Grain yields of earlier o_2 (high-lysine) hybrids were 85 to 92% of those of normal dent cross. The variation in yield of a U.S. commercial o_2 hybrid ranged from 86 to 92% of its normal counterpart. Definite improvement has been made in resistance to kernel and ear rots and some improvement in grain yield of standard o_2 germplasm. In 1982, the South African Department of Agriculture released a commercial yellow high-lysine hybrid "HL2", a modified single cross. "HL2" is at a similar yield and agronomic level as the best normal commercials hybrids. It has hard endosperm, nearly indistinguishable from normal, and it has extremely good resistance to ear rot diseases. A white high-lysine o_2 hybrid, "HL1", was released commercially in 1979. The standard o_2 corn hybrids would appear quite acceptable for more temperate areas of the world, where corn is used primarily in the feed-grain industry, provided of course that it was nutritionally efficient and economically competitive with the better yielding normal materials. It does not have good dry-milling characteristics. In some parts of the world soft-endosperm, floury corns are preferred such as in the highlands of Bolivia, Peru, and Ecuador. The appearance of the soft endosperm o_2 is unacceptable in many tropical and subtropical areas of the world from the standpoint of human preference and some association desirable characteristics.

Modifying genes or genetic modifiers are a series of genes, which apparently do not have any effect of its own but it does interact and modify the expression of quality protein corn 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 reason is 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. The role of genetic modifiers in altering kernel phenotype has been studied much more extensively than any other trait. The modified *opaque-2* kernels have been observed and studied by several breeders working on quality protein breeding programs (Paez *et al.*, 1969; Bauman and Aycock, 1971). The pattern of kernel modification can be either regular or irregular. In regular patterns, the modified fraction increases 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 corn breeders.

Various aspects of genetic modifiers have been discussed in earlier publications (Vasal, 1975; Bjarnason and Vasal, 1992) 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 (Paez *et al.*, 1969; Annapurna and Reddy, 1971). However, experience of CIMMYT scientists and that of other research workers is 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 opaque registers a slight increase (Vasal, 1975; Vasal *et al.*, 1984).

Opaque-2 as double mutant combinations

All known high lysine mutants, when tried alone exhibit soft chalky phenotype and encounter other problems presented earlier. Double mutant combinations involving high lysine mutants itself or in combination with some endosperm mutants have been attempted to alter at least unacceptable properties of standard soft opaques. Two combinations, which deserve special mention are *opaque-2 floury-2* (o_2fl_2) and

sugary-2 opaque-2 (su_2o_2). Both combinations have been produced, and experiences of those involved in exploiting these combinations are discussed below.

This combination generated interest as it produced translucent kernels (Nelson, 1966). At CIMMYT, in earlier stages, attempts were made to produce double mutant combinations in a wide array of genetic backgrounds to improve upon protein content and kernel appearance. Unfortunately, CIMMYT breeders did not encounter translucent shiny kernels resulting from the interaction of these mutant genes. Other breeding programs have also met with limited success. It was likely that translucent kernels occur in rare genetic backgrounds, but overall experience worldwide did not favor continuation of this project.

Extensive work has been done at Purdue University to exploit interaction of *opaque-2* and *sugary-2* mutants to develop phenotypically appealing HQPC. Work was also initiated at CIMMYT on the advice of Purdue University scientists. A modest effort was initiated in mid-1970 to develop this mutant combination. Much of the experience gained and available to date has come from the work of these two institutions. The translucent kernel phenotype of su_2o_2 corn had been reported by several research workers (Vasal *et al.*, 1984; Glover, 1992). In addition, the su_2o_2 segregates are either equal to or better than its counterpart o_2 kernels in protein quality. Some general conclusions, based on work conducted at Purdue University, CIMMYT, and some other research institutions, can be made on this double mutant combination.

Molecular genetic approaches to maize improvement

In agriculture, one of the main objectives of plant breeder is to improve the existing cultivars, which are deficient in one or more traits by crossing such cultivars with lines that possess the desires trait. A conventional breeding program thus involves crossing whole genomes followed by selection of the superior recombinants from among the several segregation products. Indeed, such a procedure is laborious and time consuming, involving several crosses, several generations, and careful

phenotypic selection, and the linkage drag (tight linkage of the undesired loci with the desired loci) may make it further difficult to achieve the desired objective. Advent of DNA marker technology, development of several types of molecular markers and molecular breeding strategies offered possibilities to plant breeders and geneticists to overcome many of the problems faced during conventional breeding.

The goal of most backcrossing programs is to improvement a particular strain (recurrent parent) for specific characteristics, usually a single gene, obtained from a donor parent (Tracy, 2004). In most backcross programs, the objective is to recover the recurrent parent essentially unchanged except for the introgression of the new characteristics. Backcrossing allows the plant breeder more precise control of allele frequencies than other traditional plant breeding methods. On average, with each backcross generation (BC) 50% of recurrent parents alleles are recovered (e.g., F_1-50% [recurrent parent alleles]; BC_1-75%; BC_2-87.5%; BC_3-93.25%; *etc.*). This is the theoretical average. Linkage and epistasis will result in the persistence of unwanted donor alleles and chromosome segments. Molecular markers can be used to reduce the size of donor linkage blocks and reduce the number of generations of backcrossing needed to recover the recurrent parent genotype.

Marker-assisted backcross (MABC) breeding has now become a standard application in modern plant breeding and the optimizing of MABC strategies was already reviewed (Frisch *et al*., 1999; Reyes-Vadez, 2000). The reason for the success of this strategy is two-fold. Firstly, the time required for conversion a Recurrent Line into it's Near Isogenic Line through MABC can be reduced from 6 to 3 generations. Such a significant reduction in time to market can significantly influence the success of s new variety. Secondly, a good performing variety represents a very valuable fixed combination of alleles and keeping this combination intact, when adding simply inherited traits, is very attractive for many variety improvement programs (Peleman and Rouppe van der Voort, 2003).

An incorporation of recessive resistance genes derived from exotic or un-adapted germplasm normally requires long-lasting backcrossing procedures to

combine this resistance with other agronomic traits, in particular superior yield. Incorporating recessive resistance genes by phenotypic selection takes twice as long as incorporating dominant genes, since a selfing generation is required after each backcross (BC) step for the phenotypic identification of homozygous recessive resistant plants to be used in the next BC cycle. However, by using co-dominant markers, like SSRs, heterozygous carries of the resistance encoding allele to be used for the next BC step can be detected directly in F_1, thereby saving one year for each BC cycle. The same holds true for dominant markers generating an additional fragment linked to the resistance encoding allele (Ordon *et al*., 1999).

Microsatellites, or simple sequence repeats (SSRs), consist of varying numbers of tandemly repeated units (1 to 6 base pairs each) and represent a class of repetitive DNA that is commonly found in eukaryotic genomes (Tautz and Renz, 1984). They are characterized by great abundance (Condit and Hubbell, 1991), high variability (Schug *et al*., 1998), and even distribution throughout the genomes in many species (Liu *et al*., 1996; Taramino and Tingey, 1996). Microsatellites are typically multi-allelic loci, and loci with more than five alleles are commonly observed in plants (Senior *et al*., 1998) and animals (MacHugh *et al*., 1997). In addition, automated PCR based techniques, which enable high-throughput data collection and good analytical resolution at a low cost, have been developed for microsatellites (Mitchell *et al*., 1997). Because of these qualities, microsatellites are now one of the preferred genetic markers in plants and animals. Microsatellites have been exploited as tools to measure genetic distance and diversity in evolutionary studies.

Molecular markers for QPM improvement

Although conventional breeding procedures have been used to convert commercial lines to QPM forms, the procedure is tedious and time consuming. Rapid advances in genome research and molecular technology have led to the use of DNA marker-assisted selection, which holds promise in enhancing selection efficiency and expediting the development of new cultivars with higher yield potential (Ribaut and Hoisington, 1998). While marker-assisted foreground selection (Tanksley, 1983;

Melchinger, 1990) helps in identifying the gene of interest without extensive phenotypic assays, marker-assisted background selection expedites significantly the rate of genetic gain/recovery of recurrent parent genome in a backcross breeding program. With the development and access to reliable PCR-based allele specific markers such as SSRs, and SNPs, marker-assisted selection is becoming an attractive option for simply inherited traits (Babu *et al*., 2004).

Babu *et al*. (2005) performed the marker-assisted selection for *opaque-2* gene that distinguished polymorphism could be observed between the normal and QPM inbred lines with all the three SSR markers. However, the nature of polymorphism was different with respect to *phi112*, which exhibited dominant (presence-absence) polymorphism, restricting its potential utility in identifying the three forms of genotypes for the *opaque-2* gene. Nevertheless, this marker could be of use in checking the seed purity during routine field maintenance of QPM inbred lines. Co-dominant nature of the polymorphism exhibited by *phi057* and *umc1066* enables its potential utility in *MAS* programs to successfully discriminate between homozygotes and heterozygotes. Identification of heterozygotes in the seedling stage prior to pollination aided in the rejection of non-target BC progenies resulting in substantial saving of labor and material resources. However, as demonstrated by our results, polymorphism may not be obtained for all the normal inbred lines against QPM inbred lines with these SSR markers, warranting cautious selection of QPM donors in a marker aided QPM breeding program. With respect to the gene specific markers such as *phi057* and *umc1066*, which are located within the *opaque-2* gene itself, the individual plants in any segregating population could be scored directly for the gene, eliminating the probability of occurrence of false positives and false negatives.

Yang *et al*. (2004) performed the recessive allelic variations of three microsatellite sites within the O_2 gene by using 14 inbred o_2 lines and a wild-type line in maize. Among the 15 lines, allelic variations were observed at *umc1066*, *phi057*, and *phi112* sites. Two alleles were found at the *umc1066* site a recessive allele with 2 perfect GCCAGA repeats and a dominant allele with 3 perfect repeats. Three alleles were found at the *phi057* site 2 recessive alleles with 3 and 5 perfect GCC repeats,

respectively, and another with 4 perfect repeats consistent with a dominant allele. At least 4 alleles exist at the *phi112* site among which 1 recessive allele has a 1 bp deletion, another has a 15 bp deletion, and other has no PCR products compared to the dominant allele; all the alleles have unchanged AG repeats. The *phi057* site in exon 6 was identified to be a hypervariable region in the encoding sequence of the O_2 gene, in addition to the 2 hypervariable regions in exon 1 previously reported. The primary mechanisms underlying the variations in repeat numbers and regions flanking the SSR within the O_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. The lysine content of kernel in the O_2 and o_2 lines correlates to a considerable extent with nucleotide variations at the *umc1066*, *phi057*, and *phi112* sites. They suggested that it is best to use the 3 markers together in molecular marker-assisted selection for high-lysine maize materials.

Manna *et al*. (2005) proposed that under the conditions of PCR used, SSR markers *phi057* and *phi112* are polymorphic in most of the populations used and will, therefore, constitute the framework for marker-assisted introgression of the *opaque-2* trait into suitable maize genotypes in Uganda, particularly when visual selection is not possible. Optimized phenotypic background selection may be a realistic way to obviate the need for the more expensive marker-assisted background selection. However, the opportunity for phenotypic selection might differ between years and locations. In addition, breeding regimes that select on the basis of phenotype alone might require a much larger number of backcrosses and plant populations to enhance the success of selection. The accuracy of its phenotypic selection needs to be validated using molecular markers. The reasons for the high levels of tryptophan in PED49B are not yet verified, although other potentially useful genetic systems that repress zein synthesis like the o_2 mutation might be responsible. Additional research to elucidate this is needed.

In Thailand with the *opaque-2* gene in maize, Jompuk *et al.* (2006) reported that three populations, *i.e.*, $Pop61C_1$, $Pop62C_6$ and $Pop65C_6$ from the International Maize and Wheat Improvement Center (CIMMYT) were used in their study. S_0-plants

were selected for the presence of QPM using two markers (*phi057* and *phi112*) as indicated by the amplified products of 140-160 bp. The endosperms of selected S_1-seed were further analyzed for the tryptophan and protein content. The average tryptophan contents in endosperm of QPM and non-QPM as detected by *phi057* marker were 0.66% (for QPM), 0.38% (for non-QPM), 0.38% for Suwan 1 (a non-QPM) and 0.80% for the *opaque-2* control variety. Moreover, those QPM and non-QPM plants detected by *phi112* showed the same result of total protein and tryptophan contents in endosperm. To detect heterozygote maize for backcross breeding program, *phi057* was considered more feasible than *phi112* as a marker-assisted selection (*MAS*) for *opaque-2* and to identify QPM line in the short period of time.

In the later generation, ten S_3 inbred lines were examined for the *opaque*-2 gene using the *phi057* marker and a diallel cross was made to study the combining ability and tryptophan content in the F_1 hybrid. The results showed that protein content in endosperm of these inbred lines ranged from 7.76 to 8.61% while those of *opaque-2* and *non-opaque-2* maize contained about 8.45 and 8.73%, respectively. However, the protein content of inbred lines, the diallel cross and check variety were not significantly different. Moreover, protein contents were not significant among the diallel cross, *opaque-2* and *non- opaque-2* hybrids. But tryptophan content in endosperm of the diallel cross was higher than non-*opaque*-2 hybrids (Jompuk *et al*., 2007).

Moreover, Jompuk *et al.* (2011) converted the normal inbred line to *opaque-2* inbred line using marker-assisted selection (*MAS*) in backcross and selfing generations in field corn. Inbred lines derived from the cross of quality protein maize (QPM) and normal maize were controlled by the *opaque-2* gene and some modifying genes, and their crosses had similar protein content in the endosperm as normal maize. However, the percent of tryptophan content in the protein was almost twice that of normal maize. Moreover, the grain yield of the best diallel cross (about 7.669 t ha^{-1}) was not significantly different from the normal maize.

MATERIALS AND METHODS

Materials

Plant materials

1. Three inbred lines of 2 groups consisted of waxy (*O_2O_2wxwx*) and quality protein maize (*o_2o_2WxWx*) sources (Table 1).

Table 1 The plant performances of three inbred lines.

Inbred line	Parental type	Genotypic character	Source
Kwi#1	recurrent	*O_2O_2wxwx*	NCSRC[1/]
Kwi#9	recurrent	*O_2O_2wxwx*	NCSRC
AgQ53	donor	*o_2o_2WxWx*	Dept. of Agronomy, KU.

[1/] National Corn and Sorghum Research Center

2. Two check varieties were included in the trials, *i.e.* Big-White (commercial hybrid) as best selling hybrid and Ratchata (open-pollinated variety).

Methods

Plant materials

Two groups of genotypes were used: the *waxy* lines, Kwi#1 and Kwi#9, from the National Corn and Sorghum Research Center (NCSRC), Kasetsart University, and the QPM inbred line, AgQ53 containing *opaque-2* gene, from the Department of Agronomy, Kasetsart University, Thailand. The selection methods were standard breeding method integrated with marker-assisted selection (*MAS*) to rapidly assist line selection. Two initial crosses were made between Kwi#1 and AgQ53 and Kwi#9 and AgQ53. The F_1 plants were either backcrossed one time to the respective waxy parent

to deliver a BC_1F_1 generation, or selfed for S_1 seed. Seedling leaf material from the S_1 and BC_1F_1 were tested with the *phi057* marker to identify the *opaque-2* gene. The waxy trait was screened by mean of I_2KI staining solution (Nelson, 1962), applied at the young pollen stage. In the generations S_2, BC_1S_1 and BC_1S_2, respectively, the *phi022* marker was utilized to select for the expression of *waxy*. For each detail of each season was described as following;

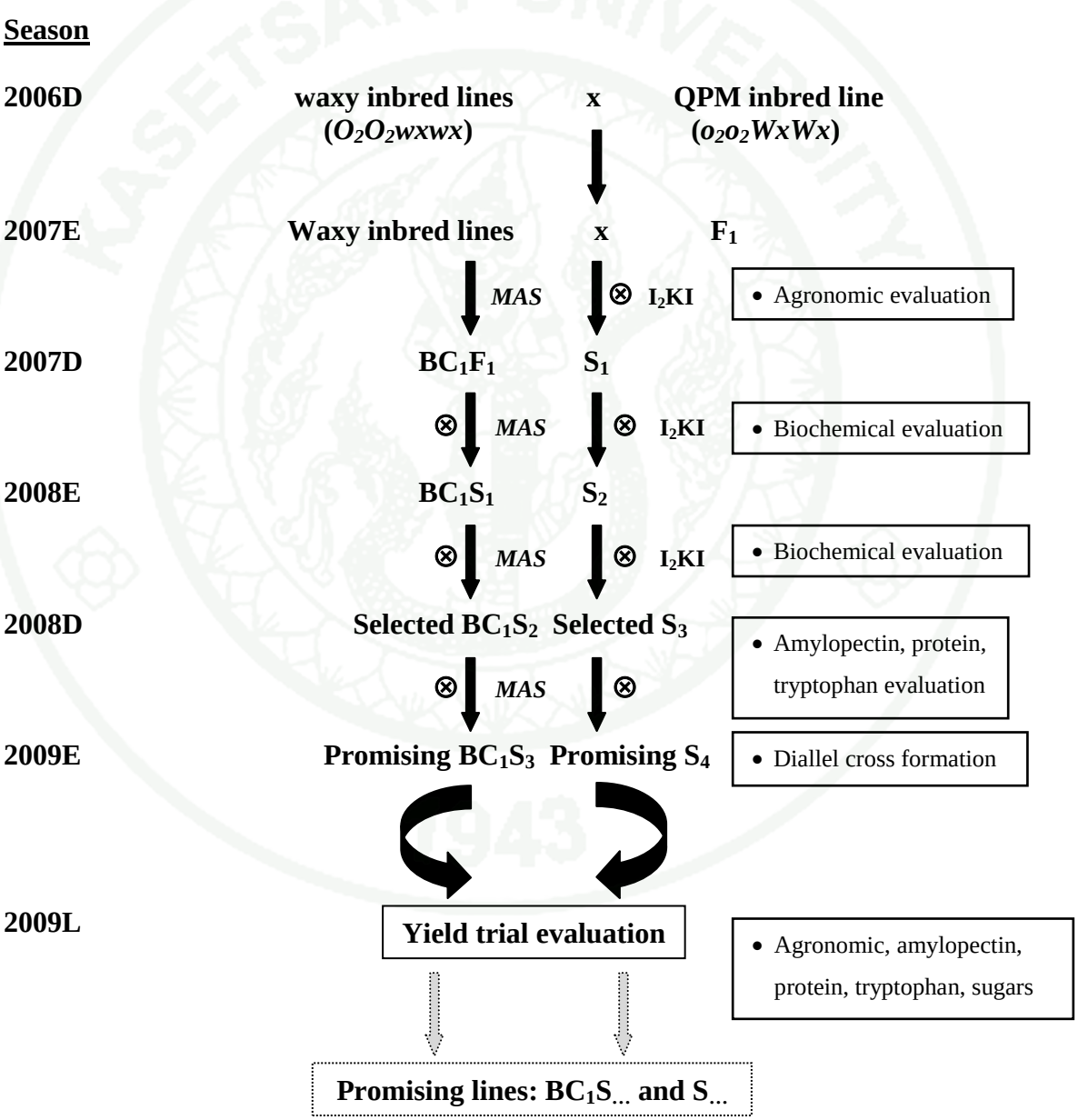

Figure 1 Breeding scheme of studying on the inheritance of high quality protein in waxy corn.

2006 Dry Season

Two initial crosses were made between Kwi#1 and AgQ53 and Kwi#9 and AgQ53. At harvested stage, ear was selected on the healthy basis ear in each cross.

2007 Early Rainy Season

The F_1 seeds were separately planted on two sides. Plant fertilities were determined by visual selection of individual line, simultaneously, discarding the weakened lines. And, the standard programs were followed on two channels, namely, the backcrossing and selfing. The F_1 plant was directly crossed back to the recurrent parents (*wxwx*) to get the BC_1F_1seed. Furthermore, S_1 seed was obtained by selfing the F_1 plant.

2007 Dry Season

The backcross (BC_1F_1) and selfing (S_1) families were grown at the National Corn and Sorghum Research Center (NCSRC). Then, the young leaf at four-week-old plants of each family was collected to extract DNA. The selected plants were determined the *opaque-2* (o_2o_2) gene by the marker, *phi057*. At the flowering stage, these selected plants were self-pollinated to produce the BC_1S_1 and S_2 from backcross and selfing plants, respectively. Moreover, iodine potassium iodide (I_2KI) was applied at the pollen to classify the expression of *waxy* gene (*wxwx*) as described by Weatherwax (1922).

2008 Early Rainy Season

The seeds of S_2 and BC_1S_1 were grown in the field to advance generation to S_3 and BC_1S_2 populations by selfing. By natural infestation of foliar plant diseases, *i.e.* downy mildew, southern corn leaf blight (SCLB) and northern corn leaf blight (NCLB), tolerant plants were selected.

2008 Dry Season

Again, the *waxy* gene determination was done by marker *phi022* and I_2KI staining solution on both families, BC_1S_2 and S_3, to get double homozygous recessive (*$o_2o_2$wxwx*) as the goal lines. Six and five homozygous recessive plants were selected from the selfed and backcross lines, respectively. Finally, eleven plants were self-pollinated to advance the generation one step further for protein, tryptophan analysis (Nurit *et al*., 2009) and amylopectin analysis as described by Juliano (1971).

2009 Early Rainy Season

The selected plants, *waxy-opaque-2* (*$o_2o_2$wxwx*) were separated into selfing and backcross groups. For selected lines in each group, a diallel cross was made using fixed model method IV (Griffing, 1956) at the National Corn and Sorghum Research Center, Kasetsart University, Nakhon Ratchasima province, Thailand. Ten F_1-hybrid were crossed from backcross group and 15 crosses were obtained from selfing group. At maturity seed, the healthy and well pollinated ears were harvested. Then, the seed from each line was analyzed for amylopectin, sugars, protein and tryptophan content. On the other hand, six and five of *waxy-opaque-2* lines were self-pollinated to advance generation to S_5 and BC_1S_4 of self and backcross group, respectively.

2009 Lately Rainy Season

The diallel cross yield trial consisted of 25 F_1 hybrids and two commercial hybrids as check varieties (Big-White and Ratchata). The experiment was conducted in a randomized complete block design (RCB), with four replications at the same research center. Each plot consisted of four 5 meter rows with 75 cm between rows and 25 cm between plants within a row. A basal fertilizer of 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 at the rate of 4 kg ha^{-1} and 4 L ha^{-1}, respectively. After 2 wk, plants were thinned to one plant per plant hill or a population size of 53,331 plants ha^{-1}. At the fourth week, 312 kg ha^{-1} of ammonium sulfate was top dressed. Agronomic traits, such as ‘days

to silking' (the number of days from planting until 50% of the plants show silks), 'days to anthesis' (the number of days from planting until 50% of the plants show shedding of pollen), plant height (distance in centimeters from the ground to the top of the tassel), ear height (distance in centimeters from ground level to the main ear-bearing node), grain moisture content at harvesting (using a moisture tester; Steinlite, SB 900) and grain yield (combine-harvested grain weight expressed in t ha^{-1} and adjusted to 15% standard moisture content) were collected.

Plant cultivation

The experiments were conducted at the National Corn and Sorghum Research Center, in Thailand from May to August in 2007 (Early Rainy Season), and from October 2009 (Late Rainy Season) to January 2010. The climate is tropical lowland, the soil a Rhodic Kandiustox Oxisol.

Extraction of genomic DNA

Young and healthy leaves of individual four-week-old plants were sampled for DNA extraction according to the method described by Agrawal *et al.* (1992). The tissue was ground into powder in liquid nitrogen. About 150 to 250 mg of ground material were transferred to 15-ml centrifuge tubes containing 6 ml extraction buffer (2x CTAB 6 ml, β-mercaptethanol 15 µl), and incubated in a water bath with gentle shaking at 60 ^{o}C for 1 h. Five ml chloroform:iso-amyl alcohol (24:1) mixture was added, mixed well with the suspended tissue, and spun at 2,500 rpm for 30 min. Without disturbing the interface, the supernatant was transferred to another 215 ml tube and 4 ml cold isopropanol was added to precipitate DNA. The DNA was put into another tube containing 70% alcohol per 1 ml and spun at 2,800 rpm for 1 min. The supernatant was removed and 1 ml washing buffer was added; the tubes were centrifuged at 2,800 rpm. The solution was decanted and incubated at 37 ^{o}C until the DNA was dry. RNase buffer (500 µl) was added. The tubes were placed in a water bath at 60 ^{o}C until the DNA had dissolved. RNase A (4 µl) was added, and the tubes were incubated at 37 ^{o}C overnight. Phenol (400 µl) was added and the tubes were

centrifuged at 12,500 rpm for 10 min. The supernatant was put into another tube, chloroform:iso-amyl alcohol (24:1) was added to the desired volume, and the tubes were centrifuged at 12,500 rpm for 10 min. The new supernatant was put into another tube and 3 M sodium acetate (30 µl) and 500 µl absolute ethanol were added; the tubes were shaken gently until DNA appeared and then were centrifuged at 5,000 rpm. The upper layer was removed, and 500 µl 70% ethanol were added; the tubes were centrifuged at 2,800 rpm. The supernatant was removed and dried. Finally, 50 µl TE buffer was added.

SSR marker assay for the *opaque-2* locus and the *waxy* locus

The *opaque-2* allele was identified by the SSR marker, *phi057* (Table 2). An initial polymorphism analysis of both parental lines had been performed. The SSR marker gave amplification products of about 140 to 160 bp (Chin *et al.*, 1996). The *waxy* allele was detected by the SSR marker *phi022.* The amplified fragment was about 142 to 157 bp. These primers were synthesized by the KU-VECTOR Custom DNA Laboratory of Kasetsart University, Thailand. Primer sequences and their repeat motifs are available in the Maize Genomic Database (http://www.maizegdb.org/).

Table 2 The SSR primer sequence of two markers.

Marker	Trait	Primer sequence	
		Forward	Reverse
phi057	*opaque-2*	CTCATCAGTGCCGTCGTCCATT	CAGTCGCAAGAAACCGTTGCC
phi022	*waxy*	GCGCACCAGCGACTGACC	GCGGGCGACGCTTCCAAAC

The amplification was performed in 20 µl containing 3 mM $MgCl_2$, 1 U Taq DNA polymerase, 0.2 mM dNTPs, 10 pM of each primer and 50 ng of template DNA and distilled water). For all the reactions, glycerol oil was added to prevent evaporation. The reactions were carried out in a thermal cycler (MJ Research), and the amplification included a cycle for initial denaturation at 94 ^{o}C for 2 min, 30 cycles at 94 ^{o}C for 30 s, annealing at 65 ^{o}C for 1 min and extension at 72 ^{o}C for 1 min. A

final extension at 72 ^{o}C for 5 min was followed by termination of the cycle at 4 ^{o}C. PCR products for *phi057* were separated in 6% polyacrylamide denaturing gel in 1xTBE buffer and stained with silver, while 4% agarose in the 1xTBE buffer was used to determine *phi022*, which was visualized under UV by staining with ethidium bromide.

Statistical analysis

Data of the quality and agronomic traits were analyzed according to a randomized complete block design (RCBD). The source of variation was shown in Table 3. Moreover, the general combining ability (GCA) and specific combining ability (SCA) were analyzed as described by Griffing (1956) and source of variation was shown in Table 4.

Table 3 Source of variation in randomized complete block design.

SOV	df
Replication	r-1
Treatment	t-1
Error	(r-1)(t-1)
Total	tr-1

Table 4 Analysis of variance for method IV giving expectations of mean squares for the assumption of model I.

SOV	df	SS	MS	EMS
Replications	r-1			
G.C.A	n-1	S_g	M_g	$\sigma^2+r(n-2)[1/(n-1)]\sum_i g_i^2$
S.C.A	n(n-3)/2	S_s	M_s	$\sigma^2+r[2/n(n-3)]\sum_i\sum_j s_{ij}^2$
Error	(r-1)[n(n-1)/2-1]	S_e	M_e	σ^2
Total	[rn(n-1)/2]-1			

Places and Duration

Places

1. National Corn and Sorghum Research Center (NCSRC), Nakhon Ratchatchasima

2. Gamma Irradiation Service and Nuclear Technology Research Center (GISC), Kasetsart University

3. Department of Agronomy, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom

Duration

October 2006 to February 2010

RESULTS AND DISCUSSION

Results

Identification of *o_2o_2wxwx* gene in the selefing population

The F_1 plants in each cross were tested for the *opaque-2* gene and *waxy* gene by the molecular markers with the SSR markers, *phi057* and *phi022*, respectively. The F_1 plants were indeed heterozygous for these two genes, which came from the *waxy* (*O_2O_2wxwx*) and *opaque-2* (*o_2o_2WxWx*) parents. Then, self-pollinated of its plants was introduced to the S_1 generation. In the S_1 population, the leaf of 230 and 245 plants were harvested to extract DNA for marker-assisted selection (*MAS*) of *opaque-2* gene. The result showed that 42 and 35 plants were homozygous recessive in the cross of Kwi#1 × Q_{53} and Kwi#9 × Q_{53}, respectively. Then, the homozygous recessive plants of *o_2o_2* gene were self-pollinated to S_2 generation. The S_2 seeds were grown in the field for selection the unique characteristic of waxy trait by I_2KI (iodine potassium iodide) and was advanced generation to S_3 seed. In later season, three plants in each cross were identified as waxy types by *phi022 and opaque-2* by *phi057* from the cross of Kwi#1 × Q_{53} and Kwi#9 × Q_{53}. Finally, six plants were self-pollinated to advance generation one step further for protein and tryptophan analysis and given the names; As4, As5 and As6 from the cross of W_1 × Q_{53} and As7, As8 and As9 from the cross of W_9 × Q_{53} (Table 5).

Table 5 Number of plants with different genotypes detected in selfing family identify by the markers, *phi057* in S_1 plant and *phi057* and *phi022* in S_3 plants.

Cross	No of plant	Generation		
		S_1 (*phi057*)		S_3 (*phi057* and *phi022*)
		O_2O_2 and O_2o_2	o_2o_2	(o_2o_2wxwx)
$W_1 \times Q_{53}$[1/]	230	188	42	3
$W_9 \times Q_{53}$	245	210	35	3
Total	475	398	77	6

[1/] W = Kwi, Q = AgQ

Identification of the *o_2o_2wxwx* gene in the backcrossing population

Leave of each plant from BC_1F_1 population were collected to extract DNA for the detection of the *opaque-2* gene by *phi057*. Two genotypes, homozygous dominant (O_2O_2) and heterozygous (O_2o_2) were observed. Then, the heterozygous plants were self-pollinated to get BC_1S_1. In the BC_1S_1 plants, the *phi057* was applied to detect the opaque-2 gene of segregated plants. Thirty-one and 41 of homozygous recessive (o_2o_2) plants were obtained from the cross $W_1 \times Q_{53}$ and $W_9 \times Q_{53}$, respectively. From the homozygous recessive plants for the *opaque-2* gene in BC_1S_3 population, leaves were collected to test for the *wxwx* gene by *phi022* and *opaque-2 gene* by *phi 057* again. Two and three homozygous recessive plants were selected from the crosses of $W_1 \times Q_{53}$ and $W_9 \times Q_{53}$, respectively. Moreover, in BC_1S_3 generation, Ab1 and Ab3 were selected from the cross of $W_1 \times Q_{53}$, while, Ab4, Ab6 and Ab11 were selected from the cross of $W_9 \times Q_{53}$ (Table 6).

Table 6 Number of plants with different genotypes detected in backcross family identify by the markers, *phi057* in BC_1S_1 plant and *phi057* and *phi022* in BC_1S_2 plants.

Cross	No of plant	Generation		
		BC_1S_1 (*phi057*)		BC_1S_2 (*phi057* and *phi022*)
		O_2O_2 and O_2o_2	o_2o_2	(o_2o_2wxwx)
$W_1 \times Q_{53}$ [1/]	114	83	31	2
$W_9 \times Q_{53}$	133	92	41	3
Total	247	175	77	5

[1/] W = Kwi, Q = AgQ

The band patterns detected by the marker, *phi057*, amplified a product of approximately 157 to 165 bp, which clearly distinguished QPM and non-QPM. The band in lane number 5 to 10 showed the *opaque-2 gene* of selfing group while in lane number 11to 15 showed the *opaque-2 gene* of backcrossing group (Figure 2). These inbred lines had the band as same as its male parent (AgQ1, lane no. 1). But, they differed from the normal maize (Kwi#1 and Kwi#9 in lane 3 and 4, respectively).

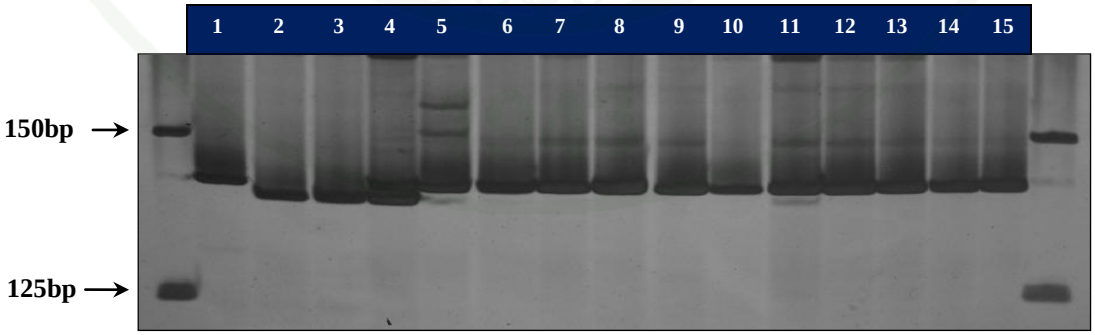

Figure 2 Polymorphism analysis using o_2o_2 SSR marker, *phi057* amplified between AgQ (1), Kwi#1 (2), Kwi#9 (3), normal (4) and new *waxy opaque-2* plants (5-15).

Moreover, the band patterns detected by marker, *phi022*, amplified a product of approximately 124 to 148 bp, which clearly distinguished waxy and non-waxy. All the new waxy opaque -2 lines (lane no. 5-11) showed the band pattern as same as the waxy parental lines, Kwi#1 and Kwi#9 (lane no. 2 and 3, respectively) (Figure 3).

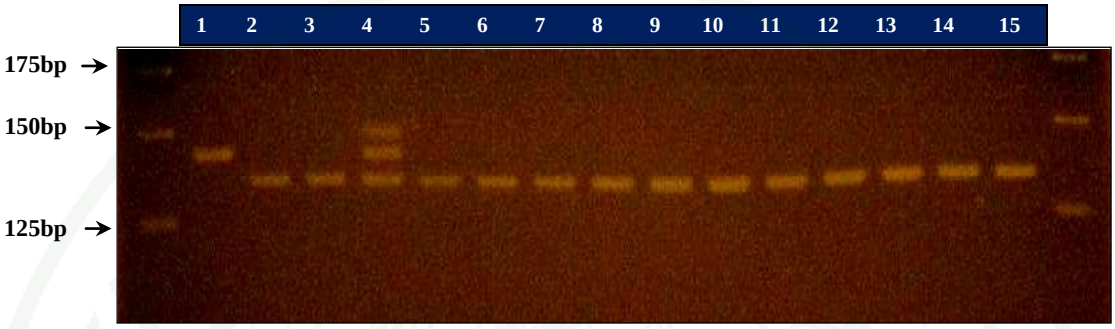

Figure 3 Polymorphism analysis using *wxwx* SSR marker, *phi022* amplified between AgQ (1), Kwi#1 (2), Kwi#9 (3), normal (4) and new *waxy opaque-2* plants (5-15).

Agronomic and biochemical characters of selected lines

Two microsatellite markers, *phi057* and *phi022* showed specific polymorphism for *opaque-2* and *waxy* genes, respectively. In the advanced generation of selfing and backcross methods, double homozygous recessive plants (o_2o_2wxwx) were obtained about 6 lines from selfing and 5 lines from backcross methods. Then, biochemical and agronomic characters were investigated as shown in Table 7.

Agronomic characters analysis

Day to tassel and silk of new *waxy-opaque-2* lines ranged from 56 to 61 days and 57 to 63 days, respectively. Plant and ear heights were different among lines, *viz.*, among 82 to 127 cm of plant and 42 to 60 cm of ear. Some economic traits, such as green weight was varying from 705 to 1,274 kg/rai. Likewise, white weight was also different varying from 255 to 715 kg/rai. The percentage of shelling was declined from 26.0 to 61.8%. Besides, the percentage of cutting ranged from 36.7 to 57.3.

Biochemical characters analysis

Amylopectin content, all of the *waxy-opaque-2* lines were about 97% which closed to standard varieties (W_1 and BW). The total sugar was rather varied from 58.33 to 88.33 mg/g. Likewise, non-reducing was 15.00 to 51.57 mg/g. The most importantly, the percentage of tryptophan in protein content was obviously differed between new *waxy-opaque-2* and normal waxy maize. Tryptophan from backcrossed lines was not only high in fresh (1.12%) but also high in dry (1.09%) endosperms. As well as, tryptophan from selfed lines were 0.82% in fresh and 0.88% in dry endosperms. More importantly, tryptophan content in endosperm of selected lines that called *waxy-opaque-2* was higher than the normal waxy (W_1 and BW) (Table 7).

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Table 7 Mean of agronomic and biochemical traits of 11 lines and 2 check varieties; trial conducted at the National Corn and Sorghum Research Center in 2009L.

Line	Tryptophan in protein (%)		Amylopectin (%)		Sugar (mg/g)		Flowering date		Height (cm)		Green weight (kg/rai)	White weight (kg/rai)	Shelling (%)	Cutting (%)	Kernel color
	Fresh kernel	Dry kernel	Fresh kernel	Dry kernel	TS[1/]	Non-RS[2/]	Tassel	Silk	Plant	Ear					
Ab_1[3/]	1.2	1.0	96.7	96.4	59.0	33.9	57	58	108	52	835	520	61.8	40.6	W
Ab_3	1.2	1.1	97.6	97.1	85.2	51.1	60	62	108	52	786	377	47.9	57.3	W
Ab_4	1.1	1.0	98.0	97.4	71.2	41.9	59	61	98	49	706	340	48.1	36.7	W
Ab_6	1.0	1.1	97.0	97.2	77.3	51.5	58	59	97	48	1051	565	54.5	47.0	W
Ab_{11}	1.1	1.2	96.0	97.6	88.3	46.1	59	62	82	41	1274	366	28.5	40.7	W
As_4[4/]	0.6	0.6	96.7	96.1	65.3	32.4	57	57	127	60	1151	518	46.8	43.7	W
As_5	0.7	0.8	96.9	97.1	58.3	15.0	56	58	125	54	1181	715	60.0	53.0	W
As_6	1.0	0.9	96.3	97.6	76.0	47.2	61	61	111	60	1075	415	38.2	45.1	W
As_7	0.9	0.9	97.7	97.1	68.5	45.1	61	62	113	59	1171	323	27.6	40.5	W
As_8	0.6	1.1	96.3	97.2	69.1	42.9	61	63	107	54	994	255	26.0	47.1	W
As_9	1.1	1.0	96.6	97.1	71.1	36.9	58	59	116	58	1070	593	55.4	49.6	W
W_1[5/]	0.5	0.7	97.6	97.5	68.1	44.7	54	54	100	44	906	905	63.3	42.8	W
BW[6/]	0.5	0.5	96.2	96.8	73.4	35.9	51	53	113	47	1674	1675	78.8	39.0	W

Table 7 (Continued)

Line	Tryptophan in protein (%)		Amylopectin (%)		Sugars (mg/g)		Flowering date		Height (cm)		Green weight (kg/rai)	White weight (kg/rai)	Shelling (%)	Cutting (%)	Kernel color
	Fresh kernel	Dry kernel	Fresh kernel	Dry kernel	TS[1/]	Non-RS[2/]	Tassel	Silk	Plant	Ear					
F-test	**	**	*	**	*	*	**	**	*	ns	**	**	**	ns	
LSD (0.05)	0.3	0.2	1.1	0.6	23.9	23.6	2	2	21	13	358	216	15.1	14.3	
CV (%)	22.1	14.3	0.7	0.2	15.6	26.1	2.1	1.9	11.3	14.6	19.9	22.0	18.3	18.9	

[1/] TS = total sugar, [2/] non-RS = non-reducing sugar, [3/] Ab = backcrossed lines, [4/] As = selfed lines, [5/] W_1 = Kwi#1, [6/] BW = big white, ns = non significant difference at $P<0.05$, *, ** = significant difference at $P<0.05$ and $P<0.01$ levels, respectively.

Yield trial of diallel cross

I. Backcrossing method

A. Yield and agronomic traits

Tasseling and silking of ten crosses and two check varieties ranged from 52 to 58 days and 52 to 59 days, respectively. Plant and ear height were obviously different among F_1 hybrids, *viz*., cross of Ab11 × Ab4 was the highest plant (180 cm), while, cross of Ab3 × Ab6 was the highest of ear (97 cm). For check variety, Big-White (BW) was the lowest of both heights. By natural disease infection, SCLB (southern corn leave blight), SCMV (sugarcane mosaic virus) and over all foliar diseases were observed. These F_1 hybrids and check varieties were good resistance. Husk cover as some quality criterion of economic yield component ranged from 4.7 to 5.0. Likewise, seedling vigor was different between F_1 hybrids and check variety. Ratchata (RAT) was a much better seedling vigor than F_1 hybrids (Table 8 and Appendix Figure 1).

B. Plant aspect and eating qualities

F_1 hybrids from backcrossed inbred lines trended to be better scored than Big-White. Ear length, ear width and tip length ranged from 13.8 to 16.1 cm, 2.9 to 3.8 cm and 12.2 to 15.1 cm, respectively. Prominently, the cross of Ab3 × Ab6 showed the highest of these three lengths. Likewise, the number of row per ear ranged from 11.5 to 15.3 rows. The average number of seed per row was about 28 seeds. For eating quality, pericarp thickness was measured for two sides; germinal and abgerminal by micrometer. The cross of Ab11 × Ab1 was the thinnest, while, Big-White was the most thickness. With the people test, flavor, tenderness and thickness were scaled from 1-5. Obviously, the cross of Ab11 × Ab6 was better eating qualities than check varieties and other crosses (Table 9 and Appendix Figure 1).

Table 8 Mean of agronomic characters of 10 F_1 hybrids of backcrossed lines and 2 check varieties.

F_1 hybrid	Days to flowering		Height (cm)		Seedling vigor (1-5)	Disease (1-5)			Husk cover (1-5)
	Tassel	Silk	Plant	Ear		Foliar disease	SCLB[1/]	SCMV[2/]	
Ab1 × Ab6	54	54	168	82	4.3	1.0	1.0	1.0	4.7
Ab3 × Ab6	55	56	178	97	4.3	1.1	1.3	1.0	5.0
Ab4 × Ab6	54	54	145	83	4.3	1.3	1.8	1.0	5.0
Ab11 × Ab6	54	54	143	89	4.0	1.0	1.0	1.0	5.0
Ab3 × Ab1	54	54	17	91	4.3	1.0	1.0	1.0	5.0
Ab4 × Ab1	54	53	140	76	4.3	1.0	1.0	1.0	5.0
Ab11 × Ab1	54	55	143	86	3.6	1.0	1.0	1.0	4.7
Ab4 × Ab3	57	59	128	74	3.0	1.1	1.3	1.3	5.0
Ab11 × Ab3	58	59	148	83	3.0	1.0	1.0	1.0	5.0
Ab11 × Ab4	56	57	180	84	3.0	1.3	1.5	1.0	5.0
BW[3/]	52	52	95	57	3.6	1.1	1.0	1.3	5.0
RAT[4/]	54	53	170	76	5.0	1.6	1.0	1.0	4.7
F-test	**	**	*	*	**	ns	ns	ns	ns
$LSD_{0.05}$	1.8	1.3	26.5	19.0	0.5	0.5	0.5	0.2	1.0
CV (%)	2.4	1.8	12.7	16.6	10.7	22.4	31.7	18.6	15.1

[1/] SCLB = southern corn leaf blight, [2/] SCMV = sugar cane mosaic virus, [3/] BW = big white, [4/] RAT = ratchata, ns = non significant difference at $P<0.05$, *, ** = significant difference at $P<0.05$ and $P<0.01$ levels, respectively.

Table 9 Mean of plant aspects and eating qualities of 10 F_1 hybrids of backcrossed lines and 2 check varieties.

F_1 hybrid	Plant aspect (1-5)		Ear length (cm)			No. row per ear	No. seed per row	Pericarp thickness (micron)		Eating quality			Kernel color
	Plant	Ear	Length	Width	Tip			Ger.[1/]	Abger.[2/]	Flavor (1-5)	Tenderness (1-5)	Thickness (1-5)	
Ab1 × Ab6	3.3	3.0	14.4	2.9	13.2	13.1	27.6	251.2	211.2	3.4	3.5	1.9	W
Ab3 ×Ab6	4.3	3.3	16.1	3.8	15.1	14.7	29.1	158.3	166.1	3.6	3.8	1.8	W
Ab4 × Ab6	4.0	3.3	15.0	3.1	13.2	14.2	28.1	188.9	144.2	3.5	3.5	1.9	W/Y
Ab11 ×Ab6	4.0	3.0	14.9	3.0	13.9	15.3	27.9	203.7	176.4	4.1	4.1	1.5	W/Y
Ab3 × Ab1	4.3	3.0	15.7	3.0	14.4	12.1	29.6	169.8	157.5	3.6	3.6	1.9	W
Ab4 ×Ab1	4.0	3.7	14.6	3.0	14.1	12.2	30.1	178.8	156.6	3.8	3.9	2.1	W/Y
Ab11 × Ab1	4.3	3.3	14.6	3.0	14.5	13.4	29.6	152.3	135.9	3.7	3.8	1.8	W/Y
Ab4 × Ab3	3.0	2.3	14.7	3.2	12.4	11.5	22.5	158.5	164.8	2.8	3.3	1.8	W
Ab11 × Ab3	4.0	3.0	14.2	3.4	12.7	13.3	25.1	171.5	156.9	3.0	4.0	2.0	W
Ab11 × Ab4	4.0	3.3	13.8	3.4	12.2	13.5	23.6	191.0	246.0	3.3	3.8	1.8	W
BW[3/]	2.0	3.0	15.6	3.0	14.7	11.8	31.2	306.0	247.0	3.8	3.6	1.6	W
RAT[4/]	4.0	3.0	14.7	3.1	13.2	13.2	30.5	227.0	223.0	3.4	3.4	1.7	W

Table 9 (Continued)

F_1 hybrid	Plant aspect (1-5)	Ear length (cm)			No. row per ear	No. seed per row	Pericarp thickness (micron)		Eating quality			Kernel color
	Plant	Ear	Length	Width	Tip			Ger.[1/]	Abger.[2/]	Flavor (1-5)	Tenderness (1-5)	Thickness (1-5)	
F-test	**	ns	**	**	**	**	**	**	**	ns	ns	ns	
$LSD_{0.05}$	0.5	0.5	0.9	0.4	1.1	1.1	2.3	51.3	36.5	0.9	0.7	0.6	
CV (%)	9.4	12.8	4.6	9.3	5.9	5.7	5.9	18.6	14.3	15.8	14.1	2.4	

[1/] ger. = germinal side, [2/] abger. = abgerminal side, [3/] BW = big white, [4/] RAT = ratchata, ns = non significant difference at P<0.005, *, ** = significant difference at P<0.05 and P<0.01 levels, respectively

C. Economic yield characters

Ab11 × Ab4 was the highest of ear number per plant (averaged 1.4) and ear number per rai was also the highest (12,032 ears). Ratchata (check) was the lowest ear (7,338 ears) as same as the standard ear per rai. For standard ear weight, Ab3 × Ab6 was the highest (1,334 kg) by contrast with cross of Ab4 × Ab3 was the lowest (638 kg). Ab3 × Ab6 was the best of both green and white weights (1,931 and 1,523 kg), while, the cross of Ab4 × Ab3 was the lowest (1,262 and 754 kg). Moreover, the percentage of shelling and cutting of Ab11 × Ab3 was high (60.9%), but Ab4 × Ab6 was low (36.5%) (Table 10).

D. Combining ability of yield

Ab6 was good GCA for green weight (Table 11) and for white weight (Table 12). For SCA, the results showed that green weight of backcrossed lines was the cross of Ab4 × Ab6 and gave the highest yield (1,931 kg/rai). Likewise, white weight of Ab3 × Ab6 was good SCA and gave the highest yield (1,523 kg/rai).

Table 10 Mean of agronomic characters of 10 F_1 hybrids of backcrossed lines and 2 check varieties.

F_1 hybrid	Ear number		Standard ear		Weight (kg/rai)		Shelling (%)	Cutting (%)
	Per plant	Per rai	No. ear per rai	Weight per rai (kg)	Green	White		
Ab1 × Ab6	0.7	7438	5805	900	1552	1072	69.2	36.9
Ab3 × Ab6	1.0	8364	7339	1334	1931	1523	79.0	43.2
Ab4 × Ab6	1.0	8138	7763	1132	1763	1187	67.3	36.5
Ab11 × Ab6	1.0	9024	7563	1170	1820	1352	74.2	47.7
Ab3 × Ab1	1.0	8630	7019	1059	1810	1345	74.4	37.2
Ab4 × Ab1	0.9	8415	7820	1040	1637	1143	69.8	38.5
Ab11 × Ab1	1.1	8937	7844	1084	1734	1239	71.7	42.0
Ab4 × Ab3	0.9	8965	6924	638	1262	754	59.5	47.2
Ab11 × Ab3	1.3	11140	10040	1036	1846	1187	64.1	60.9
Ab11 × Ab4	1.4	12032	10183	930	1664	1096	65.8	54.6
BW[1/]	1.0	8707	7435	1020	1626	1243	76.0	39.8
RAT[2/]	0.9	7338	5466	938	1830	1272	69.4	46.6
F-test	**	**	*	**	**	**	**	**
$LSD_{0.05}$	1.8	1863	1926	245	227	244	7.9	8.9
CV (%)	2.4	14.9	18.1	17.1	13.3	14.5	8.1	14.4

[1/] BW = big white, [2/] RAT = ratchata, ns = non significant difference at $P<0.05$,
*, ** = significant difference at $P<0.05$ and $P<0.01$ levels, respectively.

Table 11 Means (above diagonal) and estimates of GCA (diagonal) and estimates of SCA (below diagonal) effects for green weight kg/rai for five F_1 hybrids (backcrossed lines).

Male / Female	Ab11	Ab4	Ab3	Ab1	Ab6
Ab11	-3.52	1664	1846	1734	1553
Ab4	70.58	-104.61	1262	1637	1931
Ab3	189.97	-293.08	-42.51	1810	1763
Ab1	-28.69	-24.99	86.21	64.43	1820
Ab6	-231.86	247.49	16.90	-32.53	86.20

Table 12 Means (above diagonal) and estimates of GCA (diagonal) and estimates of SCA (below diagonal) effects for white weight kg/rai for five F_1 hybrids (backcrossed lines).

Male / Female	Ab11	Ab4	Ab3	Ab1	Ab6
Ab11	38.14	1096	1187	1239	1352
Ab4	61.28	-193.23	754	1143	1187
Ab3	-57.65	-259.50	16.76	1345	1523
Ab1	-2.44	132.83	125.48	13.39	1072
Ab6	-1.19	65.39	191.67	-255.87	124.95

II. Selfing method

A. Yield and agronomic traits

Days to male and female flowerings of F_1-hybrid ranged from 53 to 63 days and 53 to 64 days, respectively. Meanwhile, the heights, two of which were not significantly differed, *viz*., cross of As4 × As5 and As4 × As7 were the highest plant and ear. For leaf diseases, there were a little different among F_1 hybrids of all diseases. Likewise, seedling vigor was ranged from 3.0 to 5.0. Husk cover was equal to 5 of all materials (Table 13 and Appendix Figure 2).

B. Plant aspects and eating qualities

F_1 hybrids from selfed inbred lines trended to be better scored than Big-White. Ear and tip length ranged from 13.5 to 16.8 cm and 11.3 to 14.9 cm, respectively. Prominently, the cross of As5 × As9 showed highest of these two lengths. Likewise, the number of row per ear ranged from 11.7 to 14.4 rows. The average number of seed per row was about 28 seeds. For eating quality, pericarp thickness was measured for two sides; germinal and abgerminal by micrometer. The cross of As5 × As8 and As6 × As7 was the thinnest. With the people test, flavor, tenderness and thickness were scaled from 1-5. Obviously, the cross of As5 × As9 was better flavor than check varieties and other crosses (Table 14 and Appendix Figure 2).

Table 13 Mean of agronomic characters of 15 F_1 hybrids of selfed lines and 2 check varieties.

F_1 hybrid	Days to flowering		Height (cm)		Seedling vigor	Disease (1-5)			Husk cover
	Tassel	Silk	Plant	Ear	(1-5)	Foliar disease	SCLB[1/]	SCMV[2/]	(1-5)
As8 × As9	58	60	144	74	3.3	1.0	1.0	1.0	5
As7 ×As9	58	60	144	75	3.7	1.2	1.0	1.3	5
As6 × As9	59	61	155	78	3.0	1.0	1.0	1.0	5
As5 × As9	55	56	153	80	4.0	1.2	1.3	1.0	5
As4 × As9	55	57	139	73	4.3	1.3	1.7	1.0	5
As7 × As8	63	64	136	66	3.0	1.0	1.0	1.0	5
As6 × As8	61	62	158	78	3.0	1.0	1.0	1.0	5
As5 × As8	55	57	156	81	4.3	1.0	1.0	1.0	5
As4 × As8	56	57	152	78	4.0	1.0	1.0	1.0	5
As6 × As7	61	64	154	70	3.0	1.0	1.0	1.0	5
As5 × As7	56	58	150	78	4.0	1.3	1.0	1.7	5
As4 × As7	55	58	165	84	4.3	1.2	1.3	1.0	5
As5 × As6	55	57	153	74	4.7	1.2	1.0	1.3	5
As4 × As6	56	58	151	71	4.0	1.5	2.0	1.0	5
As4 × As5	56	57	169	80	4.0	1.5	2.0	1.0	5
BW3/	53	53	139	74	4.3	1.3	1.0	1.7	5
RAT4/	54	53	147	77	5.0	1.0	1.0	1.0	5
F-test	**	**	ns	ns	**	ns	**	*	ns
LSD0.05	2	2	47	25	0.7	0.7	0.3	0.4	0.6
CV (%)	2.1	1.6	19.8	21	11.7	29.3	20.1	25.2	8.2

[1/] SCLB = southern corn leaf blight, [2/] SCMV = sugar cane mosaic virus, [3/] BW = big white, [4/] RAT = ratchata, ns = non significant difference at P<0.05, *, ** = significant difference at P<0.05 and P<0.01 levels, respectively.

Table 14 Mean of plant aspects and eating qualities of 15 F_1 hybrids of selfed lines and 2 check varieties.

F_1 hybrid	Plant aspect (1-5)		Ear length (cm)			No. row per ear	No. seed per row	Pericarp thickness (micron)		Eating quality			Kernel color
	Plant	Ear	Length	Width	Tip			Ger.[1/]	Abger.[2/]	Flavor (1-5)	Tenderness (1-5)	Thickness (1-3)	
As8 × As9	3.0	3.3	15.3	3.0	12.4	12.9	23.5	209.2	163.4	2.5	2.8	2.3	W
As7 × As9	3.0	2.3	15.8	3.4	13.1	13.6	19.5	173.3	189.4	2.7	3.6	2.0	W
As6 × As9	3.0	2.7	16.7	3.0	13.3	13.8	23.1	218.2	187.3	2.8	3.2	2.1	W
As5 × As9	3.7	2.7	16.8	4.1	14.9	14.3	29.8	195.5	227.2	4.0	3.7	2.0	W
As4 × As9	3.0	3.0	16.3	3.6	14.1	13.7	27.8	204.5	223.2	3.0	4.2	2.0	W
As7 × As8	3.0	2.7	14.5	3.3	12.0	14.1	21.7	221.6	171.6	2.8	3.5	2.3	W
As6 × As8	3.0	3.3	13.5	3.2	11.3	13.9	20.0	208.7	217.6	2.8	3.1	2.1	W/Y
As5 × As8	3.7	3.3	15.1	4.2	13.7	13.9	28.1	158.7	219.7	3.3	4.3	2.3	W
As4 × As8	4.0	3.3	14.6	4.0	13.9	13.6	24.8	190.7	201.7	3.2	4.0	2.3	W
As6 × As7	3.0	3.0	14.2	3.0	11.7	13.7	19.6	229.9	159.8	3.0	2.7	2.3	W
As5 × As7	3.7	3.0	16.2	3.7	13.3	13.7	27.1	182.0	216.8	3.2	3.3	2.3	W
As4 × As7	3.7	3.3	15.3	3.4	12.5	13.9	24.7	190.0	229.8	3.5	4.3	2.3	W
As5 × As6	4.0	3.3	16.2	3.9	14.0	14.2	27.2	165.7	227.9	3.2	4.1	1.8	W
As4 × As6	3.0	3.0	16.5	3.6	14.0	14.2	27.7	209.7	238.9	3.5	4.3	1.7	W
As4 × As5	3.0	3.3	15.8	4.1	13.7	14.4	27.1	185.6	232.0	3.7	4.0	2.7	W

Table 14 (Continued)

F_1 hybrid	Plant aspect (1-5)		Ear length (cm)			No. row per ear	No. seed per row	Pericarp thickness (micron)		Eating quality			Kernel color
	Plant	Ear	Length	Width	Tip			Ger.[1/]	Abger.[2/]	Flavor (1-5)	Tenderness (1-5)	Thickness (1-3)	
BW[3/]	2.0	3.7	15.0	3.0	14.0	11.7	29.5	247.4	197.0	2.7	3.4	1.7	W
RAT[4/]	4.0	3.3	14.9	3.1	13.3	13.7	29.5	272.3	166.4	2.7	2.8	2.0	W/Y
F-test	**	ns	**	**	**	**	**	*	*	**	**	ns	
$LSD_{0.05}$	0.44	0.98	1.3	0.62	1.05	0.89	3.96	51.89	51.67	0.65	0.77	0.58	
CV (%)	8.34	19.7	5.21	11.1	4.94	4.04	9.67	15.81	15.7	12.99	13.35	16.94	

[1/] ger. = germinal side, [2/] abger. = abgerminal side, [3/] BW = big white, [4/] RAT = ratchata, ns = non significant difference at $P<0.05$, *, ** = significant difference at $P<0.05$ and $P<0.01$ levels, respectively.

C. Economic yield characters

As5 × As6 was the highest of ear number per plant (averaged 1.3 ears) and ear number per rai was also the highest (10,947 ears). Ratchata (check) was the lowest ear (6,778 ears). For standard ear weight, As5 × As9 was the highest (1,254 kg) by contrast with cross of As6 × As7 was the lowest (527 kg). As5 × As8 was the best green weight (2,126 kg), while, the cross of As6 × As9 was the lowest (1,177 kg). For the white ear weight, As5 × As9 was the best (1,447 kg), while, the cross of As7 × As8 was the lowest (629 kg). Moreover, the highest of percentage of shelling was Big-White (89%), but As7 × As8 was low (41%). The percentage of cutting of As5 × As7 was high (60%), but Big-White was low (46%) (Table 15).

D. Combining ability of yield

As5 was good GCA for green weight (Table 16) and for white weight (Table 17). For SCA, the results showed that green weight of backcrossed lines was the cross of As4 × As6, while, the cross of As5 × As8 was gave the highest yield (2,127 kg/rai). Likewise, white weight of As9 × As5 was good SCA and gave the highest yield (1,448 kg/rai).

Table 15 Mean of economic yield characters of 15 F_1 hybrids of selfed lines and 2 check varieties.

F_1 hybrid	Ear number		Standard ear		Weight (kg/rai)		Shelling (%)	Cutting (%)
	Per plant	Per rai	No ear per rai	Weight per rai (kg)	Green	White		
As8 × As9	0.9	7559	7288	735	1286	768	59.0	54.3
As7 × As9	1.0	8640	7864	817	1406	867	61.7	52.9
As6 × As9	0.9	7541	7006	606	1177	668	56.5	49.7
As5 × As9	1.2	10213	9120	1254	2059	1448	71.0	55.8
As4 × As9	0.9	7753	6328	767	1433	946	67.0	54.8
As7 × As8	0.9	8257	7748	597	1505	629	41.8	51.5
As6 × As8	1.1	8965	6852	647	1380	828	59.4	53.6
As5 × As8	1.0	8784	8306	1192	2127	1260	59.8	59.5
As4 × As8	1.0	8734	8099	1037	1776	1123	63.4	56.5
As6 × As7	1.0	8666	7019	528	1480	663	44.8	50.7
As5 × As7	0.9	8025	7311	1069	1917	1157	60.4	60.0
As4 × As7	1.2	9830	8756	984	1864	1108	60.5	56.5
As5 × As6	1.3	10947	10837	1165	1926	1181	61.3	52.1
As4 × As6	1.1	8958	7363	923	1979	1147	58.5	59.9
As4 × As5	0.9	7808	6251	848	1632	1034	63.2	57.0
BW[1/]	0.9	7197	4123	911	1572	1408	89.9	46.8
RAT[2/]	0.8	6778	5004	865	1798	1179	65.2	49.5
F-test	ns	*	**	**	**	**	**	ns
$LSD_{0.05}$	0.3	2118.3	2312	280	379	283	14.8	11.1
CV (%)	15.8	15.4	19.4	19.7	14.1	17.1	14.9	12.6

[1/] BW = big white, [2/] RAT = ratchata, ns = non significant difference at $P<0.05$, *, ** = significant difference at $P<0.05$ and $P<0.01$ levels, respectively.

Table 16 Means (above diagonal) and estimates of GCA (diagonal) and estimates of SCA (below diagonal) effects for green weight kg/rai for six F_1 hybrids (selfed lines).

Male / Female	As4	As5	As6	As7	As8	As9
As4	92.99	1632	1979	1864	1776	1433
As5	-460.09	336.99	1926	1917	2127	2058
As6	318.72	21.32	-94.87	1480	1370	1177
As7	143.26	-47.51	-52.35	-35.13	1505	1406
As8	82.91	189.24	-135.54	-59.97	-62.02	1286
As9	-84.81	297.05	-152.16	16.56	-76.65	-237.96

Table 17 Means (above diagonal) and estimates of GCA (diagonal) and estimates of SCA (below diagonal) effects for white weight kg/rai for six F_1 hybrids (selfed lines).

Male / Female	As4	As5	As6	As7	As8	As9
As4	104.00	1034	1147	1108	1123	946
As5	-342.89	284.16	1181	1157	1260	1448
As6	168.74	21.86	-113.76	663	828	668
As7	145.43	13.62	-82.41	-129.68	629	867
As8	114.14	70.71	36.95	-146.15	-83.28	768
As9	-85.42	236.70	-145.14	69.51	-75.65	-61.44

Biochemical analysis

A. Backcrossing method

The cross of Ab3 × Ab6 was the highest (91.9 mg/g) of total sugar. Likewise, the highest of reducing sugar was cross of Ab3 × Ab6 (55.6 mg/g). Simultaneously, the cross of Ab1 × Ab6 was showed the highest of non-reducing sugar (41.1 mg/g). By contrast, the cross of Ab11 × Ab4 was the lowest of these three sugars (52.1, 30.1 and 22.0 mg/g, respectively). Amylopectin of fresh kernel of F_1 hybrids ranged from 95 to 96%, while it was 97 to 98% in dry kernel. The tryptophan in protein content, all F_1 hybrids was higher than check. The cross of Ab11 × Ab6 was the highest of fresh kernel (0.81%), while, cross of Ab4 × Ab6 was the highest of dry kernel (0.92%). For check variety, Big-White (BW) was the lowest of both kernels (0.51 and 0.05%). Moreover, the protein content in endosperm was detected. The total protein of fresh kernel was ranged from 10 to 13% while it was 6 to 10% in dry kernel (Table 18).

B. Selfing method

The cross of As6 × As7 was the highest of total sugar and reducing sugar (95.6 and 54.6 mg/g) while the cross of As8 × As9 was the highest of non-reducing sugar (46.7 mg/g). The amylopection of F_1 hybrids ranged from 93 to 96% in fresh kernel and 95 to 97% in dry kernel. The tryptophan in protein content of all F_1 hybrids was higher than check. The cross of As5 × As9 was the highest of fresh kernel (0.85%), while, the cross of As7 × As8 was the highest of dry kernel (0.93%). For check variety, Big-White (BW) was the lowest of both kernels (0.44 and 0.50%). Moreover, the protein content in endosperm of fresh kernel was ranged from 8 to 13% while it was 7 to 10% in dry kernel (Table 19).

Table 18 Mean of biochemical characters of 10 F_1 hybrids of backcrossed lines and 2 check varieties.

F_1 hybrid	Sugar (mg/g)			Amylopectin (%)		Tryptophan in protein (%)		Total protein (%)	
	TS[3/]	RS[4/]	Non-RS[5/]	Fresh kernel	Dry kernel	Fresh kernel	Dry kernel	Fresh kernel	Dry kernel
Ab1 × Ab6	89.3	47.9	41.4	95.8	97.1	0.67	0.62	12.49	7.29
Ab3 × Ab6	91.9	55.6	36.3	95.8	97.1	0.56	0.85	12.20	8.00
Ab4 × Ab6	88.7	47.8	40.9	96.6	97.6	0.66	0.92	13.36	6.49
Ab11 × Ab6	59.9	31.4	28.5	95.8	97.0	0.81	0.84	13.10	9.98
Ab3 × Ab1	85.4	46.8	39.0	95.4	97.3	0.53	0.73	11.96	7.76
Ab4 × Ab1	79.9	47.0	32.9	96.2	97.7	0.78	0.77	12.75	8.45
Ab11 × Ab1	78.5	39.0	39.5	96.2	97.5	0.69	0.57	12.16	8.62
Ab4 × Ab3	58.0	35.5	22.5	96.9	97.3	0.73	0.89	13.43	9.99
Ab11 × Ab3	67.5	40.2	27.3	96.4	97.5	0.72	0.82	10.13	9.72
Ab11 × Ab4	52.1	30.1	22.0	96.6	98.4	0.72	0.90	11.45	10.97
BW[1/]	74.5	50.5	24.0	95.3	97.0	0.51	0.50	11.31	10.58
RAT[2/]	66.1	40.0	26.1	95.6	96.8	0.53	0.56	10.92	9.56

Table 18 (Continued)

F_1 hybrid	Sugar (mg/g)			Amylopectin (%)		Tryptophan in protein (%)		Total protein (%)	
	TS[3/]	RS[4/]	Non-RS[5/]	Young kernel	Mature kernel	Young kernel	Mature kernel	Young kernel	Mature kernel
F-test	**	ns	**	**	ns	**	*	ns	*
$LSD_{0.05}$	8.7	21.6	4.8	0.7	1.0	0.09	0.23	2.22	2.32
CV (%)	8.4	34.2	9.8	0.5	0.6	10.1	14.02	13.1	11.8

[1/] BW = big white, [2/] RAT = ratchata, [3/] TS = total sugar, [4/] RS = reducing sugar, [5/] non-RS = non-reducing sugar, ns = non significant difference at P<0.05, *, ** = significant difference at P<0.05 and P<0.01 levels, respectively.

Table 19 Mean of biochemical characters of 15 F_1 hybrids of selfed lines and 2 check varieties.

F_1 hybrid	Sugar (mg/g)			Amylopectin (%)		Tryptophan in protein (%)		Total protein (%)	
	TS[3/]	RS[4/]	Non-RS[5/]	Fresh kernel	Dry kernel	Fresh kernel	Dry kernel	Fresh kernel	Dry kernel
As8 × As9	95.2	49.8	46.7	93.8	96.8	0.75	0.80	8.23	10.06
As7 × As9	76.5	36.2	40.3	95.6	96.9	0.67	0.88	10.00	9.80
As6 × As9	85.5	40.0	45.5	94.9	95.7	0.58	0.77	9.83	8.57
As5 × As9	75.1	41.2	33.9	95.1	95.9	0.85	0.61	10.65	9.46
As4 × As9	66.0	38.7	27.3	95.7	96.9	0.55	0.66	12.31	10.36
As7 × As8	94.5	48.0	46.5	95.1	95.1	0.68	0.93	10.88	8.90
As6 × As8	93.0	50.0	43.0	96.4	95.4	0.76	0.8	10.64	9.53
As5 × As8	62.9	36.5	26.4	95.3	95.9	0.47	0.78	12.22	9.86
As4 × As8	74.8	43.3	31.5	94.6	96.1	0.55	0.76	13.65	8.56
As6 × As7	95.6	54.6	41.0	96.1	96.7	0.76	0.91	9.94	8.98
As5 × As7	72.1	39.3	32.8	95.8	96.7	0.63	0.79	10.75	9.15
As4 × As7	68.8	44.1	24.7	95.1	97.1	0.56	0.84	10.90	8.01
As5 × As6	69.2	46.4	22.8	95.6	96.1	0.72	0.81	10.75	8.62
As4 × As6	62.2	38.7	23.5	94.5	96.4	0.57	0.76	10.59	7.37

Table 19 (Continued)

F_1 hybrid	Sugar (mg/g)			Amylopectin (%)		Tryptophan in protein (%)		Total protein (%)	
	TS[3/]	RS[4/]	Non-RS[5/]	Fresh kernel	Dry kernel	Fresh kernel	Dry kernel	Fresh kernel	Dry kernel
As4 × As5	64.5	42.4	22.1	95.7	96.3	0.65	0.78	12.26	7.56
BW[1/]	69.7	38.4	31.3	96.2	97.3	0.44	0.50	11.31	10.58
RAT[2/]	70.8	41.9	28.9	95.8	96.9	0.63	0.56	10.92	9.56
F-test	**	ns	**	**	**	**	**	*	ns
$LSD_{0.05}$	5.2	17.9	4.3	0.8	0.8	0.16	0.18	2.11	1.86
CV (%)	4.2	26.9	7.1	0.5	0.4	15.9	11.5	11.9	9.6

[1/] BW = big white, [2/] RAT = ratchata, [3/] TS = total sugar, [4/] RS = reducing sugar, [5/] non-RS = non-reducing sugar, ns = non significant difference at $P<0.05$, *, ** = significant difference at $P<0.05$ and $P<0.01$ levels, respectively.

Discussions

The marker *phi057* revealed polymorphism in *opaque2* (Chin *et al*., 1996). The results were in agreement with those of Babu *et al*. (2005) and showed that *phi057* gave160 bp fragments in normal inbred lines and 170 bp fragments in QPM. Since *phi057* is a codominant marker and can detect homozygous dominant (O_2O_2), heterozygous (O_2o_2), and homozygous recessive (o_2o_2) plants separately (Ribaut and Hoisington, 1998). In this study, *phi057* is more closely linked to the *opaque-2*gene than is the *phi112* marker and is, therefore, more effective for MAS of homozygotes and heterozygotes. Both by selfing and backcrossing methods, vigorous double recessive inbred lines could be achieved that conformed well with respect to agronomic traits with their normal parental waxy lines. Thus it can be stated as a very positive fact for further germplasm development that this unique combination of kernel quality traits has no negative impacts on their field performance.

Founding eleven *waxy-opaque-2* lines was then directly investigated several biochemical characters. In particular, tryptophan content was an essential part of this work that performed in percentage of tryptophan in protein. The fresh kernels were appropriately harvested on 21 days after flowering showing the suitable consumption stage. Certainly, almost all of those *waxy-opaque-2* lines were really satisfied that twice as tryptophan containable as standard checks, which clearly indicated the expression of the *opaque-2* in these plant materials. Similarly, the dry kernels (harvested day) were not only superior to checks by twice but also closely related donor (AgQ53) as *opaque-2* parent. Relatively in several reports that *opaque-2* endosperm also contains a much higher level of tryptophan than original maize. These results agreed with Vasal (1994), who indicated that QPM varieties had almost double amount of tryptophan compared to normal maize but were similar in overall protein content. However, examining between both groups was differently a little tryptophan, backcrossed lines were rather considered appearing more than selfed lines.

In the part of amylopectin discrimination, it is directly affected obvious controlling by *waxy* in corn endosperms that are homozygous for the *wxwx* gene

produce only the branched starch component (amylopectin), and are devoid of the linear amylose fraction (Weatherwax, 1922; Fergason, 1994; Coe and Neuffer, 1977). By the way of *wxwx* gene on amylopectin expression, namely, it blocks the less efficient pathway of amylase synthesis, it seems to be sufficient potential of the amylopectin synthetic pathway to compensate for the lost synthesis due to *wxwx*. Therefore, amylopectin synthesis in *waxy* kernels is actually increased (Boyer and Hannah, 2001). By the results of *waxy-opaque-2* lines were confidentially stood on principle of *waxy* gene expression, namely, the whole line carried ranging from 96 to 98%. Surely, it is not only importantly appeared trait in waxy corn property but also corresponded with fundamental above 95% of amylopectin, moreover, undisturbed in epistasis reaction (Yeh *et al*., 1981). Likewise, this section is devoted to waxy corn, no attempt will be made to elucidate on the various genetic interactions involving the *wxwx* gene and other mutants located at other loci in the corn genome. Plenty of research has shown that the *wxwx* gene is epistatic to all known endosperm mutants, thus accounting for the absence of amylose starch when the *wxwx* gene is present (Creech, 1968; Boyer *et al*., 1976). Consequently, all of *waxy-opaque-2* lines in this work were conclusively approve of a good many reasoning earlier. More convince when comparing with AgQ53 as normal (72.9%), which the starch in normal corn was 75% amylopectin and 25% amylose (Kramer and Whistler, 1949).

Waxy corn and its double mutant may offer a desirable increase sugars for fresh eating and may possibly be used in other food preparations, where waxy starch properties are desirable. In this research was separately determined a sugar by two forms; total and non-reducing sugar for the purpose of detecting an available sugar form of both. Namely, total sugar is normally consisting of reducing mostly as monosaccharide units and non-reducing as polysaccharide units, which importantly part lifting sweetness (Nelson, 1980; Creech, 1965 and 1968). However, both sugars were directly analyzed that significantly showed varied in all *waxy-opaque-2* lines accompanied by two standard check varieties that ranged approximately from 58 to 88 mg/g of the total sugar. Meanwhile, non-reducing sugar was directly varied depending on total sugar fraction that similarly undifferentiated appearance, *viz*., approximately ranging from 15 to 51 mg/g. In corresponding with Andrew *et al*. (1944) found that

wxwx gene had an effect of slight increasing the levels of reducing sugar and water soluble polysaccharides compared to the normal. Moreover, in as much as waxy corn has effectively contributed lifting up some carbohydrate fractions, namely, dextrin and erythrodextrin, which occur as successive steps in the transition from glucose to starch and from starch to glucose (Weatherwax, 1922). The result obtained in our study was also supported by Brink (1928) concluded that *waxy* gene exerts its differential effect subsequent to the synthesis of the sugar molecules. Furthermore, Kuntasakul (1978) analyzed total sugar content in kernels of different genotypes that found the *opaque-2* mutant contained the lowest amount. The non-*opaque-2* single mutants including *wxwx* had higher total sugar content than the normal. Importantly, all the *opaque-2* double mutant combinations together with *waxy-opaque-2* contained a higher total sugar level that its *opaque-2* counterparts.

During the growing season 2009/2010, new F_1 hybrids were grown cooperated with standard check varieties; Big-White and Ratchata. Indifferently from inbred trials, namely, in either of flowerings was directly had much trouble influencing by the cold weather when they were sown in 2009L (winter season). Affecting in the season, there was clearly prolonged a flowering on the average in backcrossed lines were 55 and 56 days in company with selfed lines were 57 and 59 days, respectively. In other words, it seems to be more prolonged in selfed lines. The measurement of the plant heights was alike but a little different in the ear height. Meanwhile, shortening the ear height of selfed lines was somewhat lower than accumulating in backcrossed approximately 10 cm.

On the other hand , the important of agronomic characters, estimating keep an eye on plant aspects was rather satisfied of both aspects; plant and ear aspect by reason of whole the targeted lines was in moderation scoring ranging from 3 to 4. However, comparing among group was clearly maintained that found better in backcrossed lines of couple aspects. In other words, either of aspects was probably gave responsible influencing from recurrent parents by backcrossing method. On the contrary in the ear sizes, selfed lines were found superior to backcrossed all ear sizes; ear length, ear width and tip length, *etc.* However, all of the targeted lines were

moderate to large of ears following the standard unit criterions of waxy corn in Thailand (National Corn and Sorghum Research Center, 2008). Likewise, the number of row per ear was also better. Nevertheless, counting the number of seed per row was thoroughly fascinated because backcrossed lines were better. To be precise the seed decoration was better to stand in a row than selfed, which might loosen or untightened. Absolutely, accumulating of the economic yields was somewhat affected (Corcuera and Naranjo, 2003). Moreover, to determine some of eating qualities was emphatically insisted on exhibiting on backcrossed lines as better than selfed. Namely, measuring the pericarp thickness was much thinner for both sides, likewise, the three of eating qualities was the most suitable to consume or used for food production (Table 9 and 14). In conclusion, the authenticity of backcrossing method was directly appreciated favoring to receive truly of *waxy* genotypes from the recurrent parents, which were crossed for two times more than selfing as one time.

The potential yield was calculated as the product of weights per rai; dehusked and husked, which were the main aims of this part for the purpose of selecting these single-cross hybrids as well as differently comparing among groups. The results obtained through both weights were seemed to be excellent in backcrossed lines, which approximately performed and equal to 1,700 and 1,190 kg per rai (on the average) while, whole selfed line was finished insisting in 1,662 and 988 kg per rai, respectively. In the same way, the percentage of shelling is also estimated. Similarly to the previous results, the backcrossed lines were more averaged than selfed lines; 70 and 59%, respectively. Differentiation in the percentage of cutting, namely, the backcrossed lines were 44.5%, while, the selfed lines were 55%. Actually, estimating this trait was directly measured by kernels cutting, therefore, it was one of the most criterions for the industry as well as for human consumption. Possibility in the selfed was found a big kernel superior to backcrossed lines.

The results from combining ability indicated that Ab6 was the highest GCA, likewise, As5 was also the best general combiner for these traits. Moreover, these two lines had capable to create hybrid not only found the highest of GCA effect but also tightly correlated all characters. By the way to be precise of GCA effects have more

strongly affected some of yield than SCA effects similar results of the modified *opaque-2* selection by agronomic yields were more effected GCA than SCA (Vasal *et al*., 1979 and 1993).

The protein content of maize is comparable with most other cereals, generally varying between 7 and 18% for different varieties (Bressani and Mertz, 1958). Variability in the protein content of maize is a function of environmental conditions, particularly nitrogen fertilizer. By theoretically, the protein is characterized by high levels of glutamic acid and leucine. As in most other cereals, lysine and tryptophan are limiting amino acid in maize. This is due to the fact that the major storage protein of maize is a prolamine fraction-zein, which forms up to 50 to 60% of the storage protein in maize endosperm (Watson, 2003; Lawton and Wilson, 2003). F_1 hybrids in this work confronted acceptable ranging protein as above, approximately 6 to 13%. If so the tryptophan in protein slightly unexhibited interferential by protein fraction clearly found in fresh kernels, namely, *opaque-2* mutants have evaluated lysine and tryptophan content, which was associated with reduced zein synthesis in maize endosperm. Reduction in zein synthesis was compensated by simultaneous increase in albumin, globulin and glutelin synthesis as whole total protein (Mertz *et al*., 1964; Paez and Zuber, 1973). A few works of combining single mutanted genes, *waxy-opaque-2* had been reported previously by Corcuera *et al*. (2004) in kernel quality, evolution cycle length and yield of waxy, high protein quality and double recessive inbreds developed in Argentina revealed that four of *wx*/o_2 obtained the percentage of protein ranged 8 to 12%. Likewise, Kuntasakul (1978) concluded that two genotypes of *wx*/o_2 harvested at 21 days after pollination were exhibited 11 and 12% of protein content.

Surprisingly, the evaluation of the protein content was performed exclusively discriminated for two kinds of kernel; fresh and dry, which somewhat differently changed a protein levels from fresh to dry. Indicating determination of its protein almost hybrids was truly decreased on the average of groups, *viz*., 12.39 and 8.73% as well as 10.91 and 8.90% for backcrossed and selfed, respectively. The total protein deterioration was convinced reducing in kernel from fresh to dry. Therefore, it might

perform in changing protein content to decrease from the early stage to seed dried. Studies of Evan (1941) found that the percentage of protein on dry of corn kernel decreased as the seed dried and reached a constant value at about 43 days after silking. In addition, Bressani and Conde (1961) reported that the protein content changed from 17.6% on the 10 days to 18.6% on the 16 days, and decreased to 11.0% on the 65 days after flowering. Likewise, Kuntasakul (1978) concluded that the percentage of protein was approved of decreasing of double mutated as two *waxy-opaque-2* genotypes at 21 days for 12.03 and 11.58%, and at 42 days for 9.28 and 9.67%, respectively,

CONCLUSION AND RECOMMENDATION

Conclusion

1. Tryptophan content in waxy corn was increased through backcrossing and selfing with *opaque-2* maize. Two SSR markers; *phi057* and *phi022* exclusively detected these genotypes and clearly amplify two fragments, *i.e.*, *opaque-2* (o_2o_2) and *waxy* (*wxwx*). Double homozygous recessive genotype (o_2o_2wxwx) was finally achieved. Separating between homozygous and heterozygous genotypes in both loci could be precise by performed. Expression of waxy gene could be confirmed by dying with I_2KI.

2. Six *waxy-opaque-2* (o_2o_2wxwx) lines appeared clearly in S_3 generation of selfing and five lines in BC_1S_2 generation. The genotype can be maintained by selfing in the advanced generation in S_4 and BC_1S_3. Several biochemical investigations were performed, i.e. the tryptophan in protein content (1.02 to 1.24% for fresh seed and 1.02 to 1.18% for dry seed of backcross population; 0.58 to 1.12% for fresh seed and 0.6 to 1.09% for dry seed of selfed population; the amylopectin content (96 to 98% for fresh seed and 96.4 to 97.6% for dry seed of backcrossed population; 96.3 to 97.7% for fresh seed and 96.1 to 97.6% for dry seed of selfed population, total sugar (59 to 88.33 mg/g of backcross population and 58.33 to 71.1 mg/g of self population and non-reducing sugar (33.91 to 51.57 mg/g of backcross population and 15 to 47.2 mg/g of selfed population), respectively. Tryptophan in *waxy-opaque-2* was higher than normal and comparable to donor parent (AgQ53).

3. Waxy corn lines with economical yield can be developed by were both backcross/self and immediate self methods. In a cross among backcross lines of Ab3 × Ab6 gave high green weight (1,931 kg/rai) and white weight (1,523 kg/rai). Hybrids from the self lines As5 × As8 gave high green weight (2,127 kg/rai), while white weight was detected from the crosses of As5 × As9 (1,448 kg/rai). The eating quality was found best in the cross Ab11 × Ab6 and As5 × As9. Ab6 and Ab5 were also high in GCA for yield.

Recommendation

This experiment was successful in introgressing an *opaque-2* gene from QPM maize into waxy maize to create *waxy-opaque-2* (o_2o_2wxwx) lines and to use these materials to develop into F_1 hybrids. However, the lines were still lacking in a number of agronomic and quality traits. F_1 seed production of these waxy corns must be done in an isolated location from normal maize to present unwanted pollination from other corn. Further study is required to clarify this phenomenon to understand the mechanism of both genes acting together.

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.

Amnuaysitt, K., P. Sangajit and N. Sriramoon. 1998. **The Potential of Open Pollinated Primitive Waxy Corns for Hybrid Improvement.** Rajamangkara Institute of Technology, Phitsanuloke Campus, Phitsanuloke, Thailand. (Mimeographed)

Andrew, R.H., R.A. Brink and N.P. Neal. 1944. Some effects of the waxy and sugary genes on endosperm development in maize. **J. Agric. Res.** 69: 355-371.

Annapurna, S. and G.M. Reddy. 1971. Modified opaque maize for possible applied use. **Curr. Sci.** 40: 581-582.

Babu, R., S.K. Nair, A. Kumar, S. Venkatesh, J.C. Sekhar, N.N. Singh, G. Srinivasan and 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.

Babu R, S.K. Nair, B.M. Prasanna and H.S. Gupta. 2004. Integrating marker-assisted selection in crop breeding-prospects and challenges. **Curr. Sci.** 87: 607-619.

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

Bates, L.L., D. French and R.E. Rundle. 1943. Amylose and amylopectin content of starches determined by their iodine complex formation. **J. Am. Chem. Soc.** 65: 142-148.

Bauman, L.F. and H. Aycock. 1971. Selection for modifier genes to improve performance of opaque-2 genotypes, p. 139-143. *In* **Proceeding of the 25th Corn and Sorghum Research Conference.** Amer. Seed Trade Assoc., Washington, D.C.

Bear, R.P. 1944. Mutations for waxy and sugary endosperm in inbred lines of dent corn. **J. Am. Soc. Agron.** 36: 89-91.

Bjarnason, M. and S.K. Vasal. 1992. Breeding of quality protein maize (QPM), pp. 181-216. *In* J. Janick, ed. **Plant Breeding Review. Vol 9.** John Wiley and Sons, Inc., New York.

Boyer, C.D. and L.C. Hannah. 2001. Kernel mutants of corn, pp. 1-31. *In* A.R. Hallauer, ed. **Specialty Corns. 2nd ed**. CRC Press, Inc., Boca Raton, Florida.

Boyer, C.D., D.L. Garwood and J.C. Shannon. 1976. Interaction of the amylose extender and waxy mutants of maize: Dosage effects. **J. Hered.** 67: 209-214.

Bressani, R. and R. Conde. 1961. Changes in the chemical composition and in the distribution of nitrogen of maize at different stages of development. **Cereal Chem.** 38: 76-84.

Bressani, R. and E.T. Mertz. 1958. Studies on corn proteins. IV. Protein and amino content of different corn varieties. **Cereal Chem.** 35: 227.

Brink, R.A. 1925. Mendelian ratios and the gametophyte generetion in angiosperms. **Genetics.** 10: 359-388.

Brink, R.A. 1928. **Dynamic of the Waxy Gene in Maize: the Nature of Waxy Starch**. The University of Wisconsin, Madison, Wisconsin.

Chiangmai Field Crop Research Center. 1991. SSR TW 8801 waxy corn variety. **Field Crop Research Annual Report**. Department of Agriculture, Chiangmai, Thailand.

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

Coe, E.H.Jr. and M.G. Neuffer. 1977. The genetics of corn, pp. 111-223. *In* G.F. Sprague, ed. **Corn and Corn Improvement. 2nd ed**. The American Society of Agronomy, Madison, Wisconsin.

Coe, E.H.Jr., M.G. Neuffer and D.A. Hoisington. 1988. The genetics of corn, pp. 81-258. *In* G.F. Sprague and J.W. Dudley, eds. **Corn and Corn Improvement. 3rd ed.** The American Society of Agronomy, Madison, Wisconsin.

Collins, G.N. 1909. A new type of Indian Corn from China. **Bureau of Plant Industry Bull.** 161: 1-30.

Collins, G.N. 1914. Inheritance of endosperm texture in sweet x waxy hybrids of maize. **The American Naturalist**. 48: 584-594.

Condit, R. and S.P. Hubbell. 1991. Abundance and DNA sequence of two-base repeat regions in tropical tree genomes. **Genome.** 34: 66-71.

Corcuera, V.R. and C.A.Naranjo. 2003. Yield of waxy and high quality protein maize hybrids developed in Argentina. **Maize Genet. Coop. Newslett.** 77: 46-48.

Corcuera, V.R., E.A. Bernatene and C.A. Naranjo. 2004. Quality maize hybrids developed in Argentina: chemical evaluation of endosperm. **Maize Genet. Coop. Newslett.** 78: 29-32.

Creech, R.G. 1965. Genetic control of carbohydrate synthesis in maize endosperm. **Genetics.** 52: 1175-1186.

Creech, R.G. 1968. Carbohydrate synthesis in maize. **Adv. Agron.** 20: 275-322.

Echt, C. and D. Schwartz. 1981. Evidence for the inclusion of controlling elements within the structural gene at the waxy locus in maize. **Genetic** 99: 275-284.

Evan, J.W. 1941. Changes in the biochemical composition of the corn kernel during development. **Cereal Chem.** 18: 468-473.

FAO. 2004. **Global Cassava Market Study**. Available Source: http://www.fao.org/docrep/007/y5287e/y5287e00.htm, January 14, 2006.

Fedoroff, N., S. Wessler and M. Shure. 1983. Isolation of the transposable maize controlling elements *Ac* and *Ds.* **Cell.** 35: 235-242.

Fergason, V. 1994. High amylose and waxy corn, pp. 56-77. *In* A.R. Hallauer, ed. **Specialty Corns.** CRC Press, Inc., Boca Raton, Florida.

Fergason, V. 2001. High amylose and waxy corns, pp. 63-84. *In* A.R. Hallauer. ed. **Specialty Corns. 2nd ed**. CRC Press, Inc., Boca Raton, Florida.

Frisch, M., M. Bohn and A.E. Melchinger. 1999. Comparison of selection strategies for marker-assisted backcrossing of a gene. **Crop Sci.** 39: 1295-1301.

Glover, D.V. 1992. Corn Protein-Genetics, breeding, and value in foods and feeds, pp. 111-121. *In* E.T. Mertz, ed. **Quality Protein Maize**. Am. Assoc. Cereal Chem., Inc., St. Paul, Minnesota.

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

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.

Jompuk, P., W. Wongyai, S. Apisitvanich and C. Jampatong. 2007. Combining ability of inbred lines derived from quality protein maize populations. **Kasetsart J. (Nat. Sci.)** 41: 433-441.

Jompuk, C., P. Cheuchart, P. Jompuk and S. Apisitwanich. 2011. Improved tryptophan content in maize with *opaque-2* gene using marker assisted selection (MAS) in backcross and selfing generations. **Kasetsart J. (Nat. Sci.)** 45: 666-674.

Juliano, B.O.A. 1971. Simplified assay for milled-rice amylose. **Cereal Science Today** 16: 334-340.

Kataki, P.K. and S.C. Babu. 2002. **Food Systems for Improved Human Nutrition: Linking Agriculture, Nutrition, and Productivity**. Haworth Press, Binghamton, New York.

Kempton, J. H. 1919. Inheritance of waxy endosperm in maize. **USDA. Bull**. 754.

Kramer, H.H. and R.L. Whistler. 1949. Quantitative effects of certain genes on the amylose content of corn endosperm starch. **Agron. J.** 41: 409-411.

Kuntasakul, P. 1978. **Interaction of the Opaque-2 Gene with Other Endosperm Mutant Genes in Maize.** M.S. Thesis. Kasetsart University, Bangkok.

Lawton, J.W. and C.M. Wilson. 2003. Proteins of the kernel, pp. 313-354. *In* **The Chemistry of Cereal Proteins. 2nd ed**. CRC Press, Inc., Boca Raton, Florida.

Liu, Z.W., R.M. Biyashev and M.A. Saghai Maroof. 1996. Development of simple sequence repeat DNA markers and their integration into a barley linkage map. **Theor. Appl. Genet.** 93: 869-876.

Ma, Y. and O.E. Nelson. 1975. Amino acid composition and storage proteins in two new high-lysine mutants in maize. **Cereal Chem.** 52: 412-419.

MacHugh, D.E., M.D. Shriver, R.T. Loftus, P. Cunningham and D.G. Bradley. 1997. Microsatellite DNA variation and the evolution, domestication and phylogeography of taurine and zebu cattle (*Bos taurus* and *Bos indicus*). **Genetic** 146: 1071-1086.

Mangelsdorf, P.C. 1974. **Corn, Its Origin, Evolution and Improvement**. Cambridge, Massachusetts, The Belknap Press of Harvard University Press.

Manna, R., D.K. Okello, J. Imanywoha, K. Pixley and R. Edema. 2005. Enhancing introgression of the opaque-2 trait into elite maize lines using simple sequence repeats (SSR). **African Crop Science Journal** 13: 215-226.

McWhirter, K.S. 1971. A floury endosperm, high lysine locus on chromosome 10. **Maize Genet. Coop. Newslett.** 45: 184.

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

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

Mitchell, S.E., S. Kresovich, C.A. Jester, C. Javier Hernandez and A.K. Szewc-McFadden. 1997. Application of multiplex PCR and fluorescence- based, semi-automated allele sizing technology for genotyping plant genetics resources. **Crop Sci.** 37: 617-624.

National Corn and Sorghum Research Center. 2008. **Experimental Detail of Pre-Commercial Waxy Corn Varieties of Public and Private Section Cooperative Hybrid Waxy Corn Trial.** National Corn and Sorghum Research Center, Nakhon-Ratchasima, Thailand. (Mimeographed)

Nelson, O.E. 1962. The waxy locus in maize. I. Intralocus recombination frequency estimates by pollen and conventional analyses. **Genetic** 47: 737-742.

Nelson, O. E. 1966. Opaque-2, floury-2 and high protein maize, pp. 156-186. *In* E.T. Mertz and O.E. Nelson, eds. **Proceeding of the High Lysine Corn Conference**. Corn Industries Research Foundation, Washington, D.C.

Nelson, O.E. 1980. Genetic control of polysaccharide and storage protein synthesis in the endosperms of barley, maize, and sorghum. **Advance in Cereal Science and Technology** 3: 41-71.

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

Neuffer, M.G., E.H. Coe and S.R. Wessler. 1997. **Mutants of Maize**. Cold Spring Harbor Laboratory, New York.

Nurit, E., T. Axel, P.V. Kevin and R.P. Natalia. 2009. Reliable and inexpensive colorimetric method for determining protein-bound tryptophan in maize kernels. **J. Agric. Food Chem.** 57: 7233-7238.

Ordon, F., A. Schiemann, B. Pellio, V. Dauck, E. Bauer, S. Streng, W. Friedt and A. Graner. 1999. Application of molecular markers in breeding for resistance to the barley yellow mosaic virus complex. **J. Plant Dis. and Protect.** 106: 256-264.

Paez, A.V., J.L. Helm and M.S. Zuber. 1969. Lysine content of opaque-2 maize kernels having different phenotypes. **Crop Sci.** 9: 251-252.

Paez, A.V. and M.S. Zuber. 1973. Inheritance of test-weight components in normal, Opaque-2, and Floury-2 Corn (*Zea mays* L.). **Crop Sci.** 13: 417-419.

Parnell, F.R. 1921. Note on the detection of segregation by examination of the pollen of rice. **Genetic** 11: 209-212.

Peleman, J.D. and J. Rouppe van der Voort. 2003. Breeding by design. **Trends Pl. Sci.** 8: 330-334.

Promkam, V., S. Wayoopak, P. Sornwat, T. Manootirapan and B. Poosri. 2003. The data base of Kao Pod Tien and Kao Pod Kao Neaw characters, pp. 30-37. *In* Kasetsart University and Department of Agriculture, ed. **The 31st Thailand National Corn and Sorghum Program Annual Report**. Kasetsart University, Bangkok, Thailand.

Rayes-Valdes, M.H. 2000. A model for marker-based selection in gene introgression breeding programs. **Crop Sci.** 40: 91-98.

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

Salamini, F., N. Di Fonzo, E. Gentinetta and C. Soave. 1979. A dominant mutation interfering with protein accumulation in maize seeds, p. 97-108. *In* **Seed Protein Improvements in Cereals and Grain Legumes. Vol. I**. IAEA, Vienna.

Salamini, F., N. Di Fonzo, E. Fornasari, E. Gentinetta, R. Reggiani, C. Soave and M.C. Mucronate. 1983. A dominant gene of maize which interacts with opaque-2 to suppress zein synthesis. **Theor. Appl. Genet.** 65: 123-128.

Schug, M.D., C.M. Hutter, K.A. Wetterstrand, M.S. Gaudette, T.F.C. Mackay and C.F. Aquadro. 1998. The mutation rates of di-, tri- and tetra-nucleotide repeats in *Drosophila melanogaster*. **Mol. Biol. Evol.** 15: 1751-1760.

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

Tanksley, S.D. 1983. Molecular markers in plant breeding. **Plant Mol. Bio.** 1: 3-8.

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

Tautz, D. and M. Renz. 1984. Simple sequences are ubiquitous components of eukaryotic genomes. **Nucleic Acids Res.** 12: 4127-4138.

Thongleung, S., Y. Chuthatong, T. Pothisoong and S. Srichomporn. 2003. Kwsx 107: a new recommended single cross waxy corn hybrid, pp. 413-422. *In* Kasetsart University, ed. **Proceeding of 41st Kasetsart University Annual Conference**, Thailand 3-7 February, 2003, Subject:plants and Agricultural Extension and Communication. Kasetsart University, Bangkok, Thailand.

Thongleung, S., Y. Chuthatong, S. Srichomporn and T. Pothisoong. 2005. Kwpsx 7436: a single cross eight-row waxy corn hybrid, pp. 585-590. *In* Kasetsart University, ed. **Proceeding of 43rd Kasetsart University Annual Conference**, Thailand 1-4 February, 2005, Subject: plants. Kasetsart University, Bangkok, Thailand.

Thongleung, S., Y. Chuthatong, S. Srichomporn, T. Pothisoong and S. Thongdaeng. 1997. The collection and improvement of Kao Pod Tien and Pao Pod Kao Neaw. *In* Suwanwajokkasikit Field Crop Research Station, Inseechadrastitya Institute, National Corn and Sorghum Research Center, Kasetsart University, ed. **National Corn and Sorghum Research Center Annual Report.** Kasetsart University, Pakchong, Nakhon ratchasrima, Thailand.

Tracy, W.F. 2004. Breeding: the backcross method, pp. 237-240. *In* R.M. Goodman, ed. **Encyclopedia of Crop Science**. Marcel Dekker, Inc., New York.

Tsai, C.Y. 1974. The function of the Waxy locus in starch synthesis in maize endosperm. **Biochem. Genet.** 11: 83-96.

US Grain Council. 2002. **Value Enhanced-Corn (VEC) Quality Report 2001-2002**. Available Source: http://www.vegrains.org/documents/ 2002veg_report /toc/tablecont.html , January 30, 2006.

Vasal, S.K. 1975. Use of genetic modifiers to obtain normal-type kernels with the opaque-2 gene, p. 197. *In* L.F. Bauman, P.L. Crane, D.V. Glover, E.T. Mertz, D.W. Thomas, A. Carballo, M. Colegrove, C. De Leon, P.R. Goldsworthy, G.R. Granados, M.G. Gutierrez, E.C. Johnson, A.Y. Kato, A.C. Ortega, A.F.E. Palmer, E.W. Sprague, S.K. Vasal, W.D. Villena and A. Violic, eds. **High-Quality Protein Maize**. Dowden, Hutchinson and Ross, Inc., Stroudsburg, Pennsylvania.

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

Vasal, S.K., G. Srinivasan, S. Pandey, F.C. Gonzalez, J. Crossa and L.B. David. 1993. Heterosis and combining ability of CIMMYT's quality protein maize germplasm: I lowland tropical. **Crop Sci.** 33: 46-51.

Vasal, S.K., E. Villegas and R. Bauer. 1979. Present status of breeding quality protein maize, pp. 127-150. *In* **International Symposium on Seed Protein Improvement in Cereals and Grain Legumes.** IAEA, Vienna.

Vasal, S.K., E. Villegas and C.Y. Tang. 1984. Recent advances in the development of quality protein maize at the Centro Internacional de Mejoramiento de Maízy Trigo, p. 167. *In* **Cereal Grain Protein Improvement**. IAEA, Vienna.

Watson, S.A. 2003. Description, development, structure, and composition of the corm kernel, pp. 69-106. *In* P.J. White and L.A. Johnson, eds. **Corn: Chemistry and Technology. 2nd ed**. CRC Press, Inc., Boca Raton, Florida.

Weatherwax, P. 1922. A rare carbohydrate in waxy maize. **Genetic** 7: 568-572.

White, P.J. 1994. Properties of corn starch, pp. 29-54. *In* A. Hallauer, ed. **Specialty Corns**. CRC Press, Inc., Boca Raton, Florida.

Yang, W., Y. Zheng, N.I. Shen and J. Wu. 2004. Recessive allelic variations of three microsatellite sites within the o2 gene in maize. **Plant Mol. Boil.** 22: 361-374.

Yeh, J.Y., D.L. Garwood and J.C. Shannon. 1981. Characterization of starch from maize endosperm mutants. **Starch** 33: 222-230.

APPENDIX

Appendix 1 Staining pollen grains

Maize pollen grains carrying the *waxy* allele can be identified by means of an iodine potassium iodide solution containing 1% potassium iodide (w/v) and 0.3% iodine (w/v) (Nelson, 1962). Pollen of the ordinary maize starch type stains a deep blue, while pollen of waxy maize stains reddish brown. Thus it was easy to distinguish the two types of pollen. After adding the solution to fresh pollen, the change in color was observed under a microscope.

Appendix 2 Analysis of total sugars

Seeds of each selected plant were harvested at 21 days after flowering. The seeds were chopped, crushed, and percolated through a cotton membrane. The white suspension was poured into tubes and centrifuged at 1,200 rpm at 4 ^{o}C for 20 min. The supernatant was passed through the filter membrane, and the filtrate was used to determine the percent of ^{o}brix with a hand-held refractometer to achieve the appropriate dilution. Thereafter 0.05 ml of a filtered sugar suspension was used for serial dilution; 15 ml of distilled water were added and mixed well in another tube. One ml of solution was placed in a tube, and 0.5 ml 0.1 N HCL was added; the solution was boiled at 100 ^{o}C for 15 min. After cooling, the solution was treated according to the Nelson's reducing-sugar method (Nelson, 1980). Finally, the sample was measured spectrophotometrically at 500 nm; the resulting absorbance value was compared with the standard D-glucose solution as mg glucose per ml.

Reagents of alkalic copper reagent for sugar analysis

1. Dissolve anhydrous sodium carbonate (Na_2CO_3) 25 g in distilled water 250 ml and add sodiumtartrate ($C_4H_4KNaO_6.4H_2O$) 12 g.

2. Solution I: Dissolve 10% copper sulfate 40 ml and add sodium bicarbonate ($NaHCO_3$) 16 g.

3. Solution II: Dissolve anhydrous sodium sulfate (Na_2SO_3) 180 g in distilled water 500 ml.

4. Iodine solution: Dissolve iodine 0.2 g and Potassium iodide 2.0 g. in distilled water 80 ml and dilute to 100 ml.

Appendix 3 Starch analysis

A small sample of seeds was ground in a fine powder; 0.1 g was removed and put into a 100 ml tube. One ml 95% ethyl alcohol was added and gently mixed. Nine ml of NaOH solution was added and spun for 10 minutes with a magnetic stirrer. The concentrate was then adjusted by adding distilled water to a volume of 100 ml. Five milliliters were put into another 100 ml tube containing 70 ml distilled water, 1 ml glacial acetic acid and 2 ml iodine. Distilled water was added to give 100 ml. The absorbance was measured spectrophoto-metrically at 620 nm after blanking with 1 ml acetic acid and 2 ml of iodine solution in 97 ml distillate water. The absorbance was compared with a standard graph to calculate the percent of amylose (Juliano, 1971).

Reagents for amylose analysis

1. NaOH 1 N solution: Dissolve NaOH 40.0 g in distilled water 800 ml and dilute to 1 liter.

2. Glacial acetic acid 1 N solution: Dissolve CH_3COOH 60 ml in distilled water 800 ml and dilute to 1 liter.

3. Iodine solution: Dissolve iodine 0.2 g and Potassium iodide 2.0 g. in distilled water 80 ml and dilute to 100 ml.

Appendix 4 Analysis of tryptophan and total protein

Twenty-five seeds per plant were soaked in distilled water for 25 min before removing pericarps and embryos. The endosperms were air-dried overnight, ground in a cyclone mill and wrapped in a commercial filter-paper envelope to remove the fat with 100% hexane in a Soxhlet-type continuous extractor. The ground samples were then analyzed for tryptophan content using a spectrophotometer as described by

Nurit *et al*., (2009), the protein content was measured according to the micro-Kjeldahl method (Bailey, 1967).

$$\% \text{ Tryptophan} = \frac{\text{OD560}}{\text{standard curve slope}} \times \frac{\text{hydrolysis volume}}{\text{sample weight}} \times 100$$

And the protein content was measured using the micro-kjeldahl method (Bailey, 1967) that calculated as follow;

$$\% \text{ Total nitrogen} = \frac{(\text{ml std. } H_2SO_4 \text{ samples} - \text{ml std. } H_2SO_4 \text{ blank}) \times 0.05018 \times 0.014 \times 50 \times 10^6)}{\text{weight (g)} \times 10 \times 100}$$

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.

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.

Appendix Figure 1 Plant aspects of F_1 hybrids of backcrossed lines.

Appendix Figure 2 Plant aspects of F_1 hybrids of selfed lines.

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