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Improved Tryptophan Content in Maize with *Opaque-2* gene Using Marker Assisted Selection (MAS) in Backcross and Selfing Generations

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ABSTRACT

The low nutritive value of the maize endosperm protein is genetically controlled. However, quality protein maize (QPM) which is available today contains the *opaque-2* gene (o_2o_2) for double tryptophan content in the endosperm along with numerous modifiers for kernel vitreousness. The objectives of this study were: 1) to convert normal inbred lines to QPM inbred lines by the backcross method and marker-assisted selection (MAS) of the *opaque-2* gene and 2) to estimate the combining ability of QPM inbred lines for yield and some agronomic characters. Crosses were made between normal maize and QPM as female and male parents, respectively. These were backcrossed to the recurrent parent. By the marker phi057, it was shown that all F_1 were heterozygous (O_2o_2) while the BC_1F_1 progenies of Agron20 × Pop65C_6-46 and Agron29 × Pop65C_6-55 had 1:1 ratio of homozygous dominant (O_2O_2) to heterozygous (O_2o_2). The heterozygous offspring were self-pollinated to produce the BC_1S_1. By the marker of phi057 again, homozygous recessive (o_2o_2) plants were identified in one and six from the crosses of Agron20 × Pop65C_6-46 and Agron29 × Pop65C_6-55, respectively. The protein content in the endosperm of these seven lines ranged from 7.35 to 7.72% and the tryptophan content in the protein ranged from 0.70 to 0.84% which was in the range known for *opaque-2* maize (0.80%), twice more than in normal maize (0.41%). The diallel cross of BC_1S_2 lines was made by Griffing's Method 4 (fixed effect) and 21 F_1-hybrids were obtained. The best test hybrid yielded 7.67 t ha^{1}, which was close to the commercial single cross hybrid (KSX4452; 8.35 t ha^{-1}). One line (P1) had the best combination of general combining ability effects for grain yield; a second line (P6) was almost equal. Both were considered to be suitable as tester lines for the quality protein maize program.

Keywords: tryptophan, marker-assisted selection, *opaque-2* gene, phi057

INTRODUCTION

Maize is a major cereal crop for livestock feed and human consumption and is a raw material for several industrial uses. However, conventional maize has a low protein quality due to its deficiency in two essential amino acids—namely, lysine and tryptophan (FAO, 1992). A

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breakthrough came in the 1960s, with the discovery of the nutritional quality of the maize mutant *opaque-2* (o_2o_2) which produces higher levels of lysine and tryptophan; numerous breeding programs were initiated then to improve the protein quality in maize (Mertz *et al*., 1964; Vasal, 1994). At present, while quality protein maize (QPM) has a genotype in which the *opaque-2* gene has been associated with modifiers of grain physical quality, it still contains twice the amount of tryptophan and lysine compared with normal maize endosperm (Vasal, 1994; Babu *et al*., 2005). *Opaque-2* is a recessive gene located on chromosome 7 and the modifiers behave as a multigenic trait. Although conventional breeding procedures have been used to convert commercial lines to QPM, the procedure is tedious and time consuming. With the development of and access to reliable PCR-based allele-specific markers such as simple sequence repeat (SSR), DNA marker-assisted selection (MAS) became an attractive option for detecting simple inherited traits in the newly developed cultivars with higher yield potential (Ribaut and Hoisington, 1998; Pixley and Bjarnason, 2002; Babu *et al*., 2005). The objectives of the present study were: 1) to convert normal inbred lines to QPM inbred lines by the backcross method using marker-assisted selection (MAS) of the *opaque-2* gene and 2) to estimate the combining ability of QPM inbred lines for yield and other agronomic characters.

MATERIALS AND METHODS

Plant materials

Plant materials were crossed between normal maize inbred lines (Agron 20 and Agron 29) and QPM lines (Pop65C_6-46 and Pop65C_6-55) used as recurrent and donor parents, respectively. Two F_1 hybrids—namely, Agron20 × Pop65C_6-46 and Agron29 × Pop65C_6-55 were obtained. In the F_1 plant, marker-assisted selection was applied for the *opaque-2* gene. Then, the backcross to the recurrent parent (BC_1F_1) was carried out in each cross. Around 1,000 BC_1F_1 seeds were sown in the field for each cross at the National Corn and Sorghum Research Center, Kasetsart University, Nakhon Ratchasima province, Thailand. The leaves of BC_1F_1 plants were randomly collected at 30 d after sowing almost 100 plants of each cross. The DNA in each plant was extracted and the phi057, simple sequence repeat (SSR) marker for the *opaque-2* gene was applied to detect the heterozygous plant (O_2o_2) in this generation. Thereafter, heterozygous *opaque-2* plants were self-pollinated to obtain the BC_1S_1 seeds. Again, phi057 was applied to detect the homozygous recessive plants progeny the *opaque-2* gene (o_2o_2) in the BC_1S_1 generation. Selected plants were self-pollinated to get BC_1S_2 seeds. Finally, seven converted *opaque-2* lines were obtained:

1) P1; Agron29BC_1-17-4-11
2) P2; Agron20BC_1-13-5-11
3) P3; Agron20BC_1-13-8-17
4) P4; Agron20 BC_1-13-12-76
5) P5; Agron20BC_1-13-16-78
6) P6; Agron20BC_1-13-20-93
7) P7; Agron20BC_1-13-21-95

P1 was extracted from the cross of Agron29 × Pop65C_6-55 while P2 to P7 were extracted from the cross of Agron20 × Pop65C_6-46.

Diallel cross

Between the seven inbred lines, a diallel cross was made using fixed model method IV (Griffing, 1956) at the National Corn and Sorghum Research Center Nakhon Ratchasima province, Thailand. Twenty one combinations (F_1) were formed. At maturity, the healthy and well pollinated ears were harvested. The diallel cross yield trial consisted of 21 F_1 hybrids and two commercial hybrids as check varieties. 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.

Statistical analyses

Data from the diallel experiment were analyzed according to the randomized complete block design for all characteristics by the R computer program (R Development Core Team, 2010). Significances of hybrid, general combining ability (GCA) and specific combining ability (SCA) mean squares were estimated with F tests where three categories of significant difference were identified; that is, not significant ($P > 0.05$), significant ($P < 0.05$) and highly significant ($P < 0.01$). The GCA effects of the parents and SCA effects of the crosses were estimated following Griffing's Method IV for diallel analysis (Griffing, 1956).

Tryptophan and total protein analysis Twenty-five seeds from F_1-plants were collected and soaked in distilled water for 25 min before removing the pericarps and embryos. The endosperms were air-dried overnight and ground (to approximately 0.1 mm) in a cyclone mill (Retsch, ZM 1000) and wrapped in a commercial filtered-paper envelope to remove the fat with 100% hexane in a Soxhlet-type continuous extractor (Buchi, B-811). The ground samples with the fat removed were analyzed for tryptophan content using a spectrophotometer (Spectronic, Genesys 2) as described by Villegas and Mertz (1971) and the protein content was measured using the micro-Kjeldahl method (Bailey, 1967).

DNA extraction and analysis

DNA was extracted from 150–250 mg fresh leaves of F_1, BC_1F_1, BC_1S_1 and BC_1S_2 lines using the method described by Agrawal *et al.* (1992). A simple sequence repeat (SSR) marker, phi057 (KU-VECTOR Custom DNA Laboratory, Kasetsart University) was used to detect the *opaque-2* plants. This marker gives an amplification product of about 140–160 bp (Chin *et al.*, 1996). Amplification was carried out in the 20 μL reaction mixture containing 1 × reaction buffer, 10% glycerol, 2.5 mM $MgCl_2$, 150 μL of mixed dNTP, 0.3 μM of each primer, 1 U of Taq DNA polymerase, and 50 ng of genomic DNA. Amplifications were performed in a thermocycler (Peltier Thermal Cycle, PTC-200) programmed for the first denaturation step of 1 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 58–60 °C, 1 min at 72 °C, and a final extension of 5 min at 72 °C. For the phi057 marker, the amplified fragments were separated on a 6% polyacrylamide denaturing gel in 1 × TBE buffer and silver stained.

RESULTS AND DISCUSSION

Polymorphism analysis for the *opaque-2* gene

With the primer phi057, amplified products of the F_1 hybrid in the crosses of Agron20 × $Pop65C_6$-46 and Agron29 × $Pop65C_6$-55 were detected with polymorphism similar to both

opaque-2 and non *opaque-2* maize (SW1) which indicated that the F_1 hybrid was heterozygous for the *opaque-2* gene. In BC_1F_1 plants, there were two types of the amplified product, a homozygous dominant (O_2O_2) and a heterozygous dominant (O_2o_2). There were 49 homozygous and 48 heterozygous plants in the cross of Agron20 × Pop65C_6 46. Likewise, there were 48 homozygous and 52 heterozygous plants in the cross of Agron29 × Pop65C_6 55. These results showed that the segregated plants with the *opaque-2* gene between homozygous dominant and heterozygous plants were in ratio of 1:1 as tested by a chi-square test (Table 1).

To obtain the BC_1S_1 seeds, BC_1F_1 plants heterozygous with the *opaque-2* gene were self-pollinated. Theoretically, the segregated plants should be in the ratio $1O_2O_2$: $2O_2o_2$:$1o_2o_2$ in the BC_1S_1 population. In this study, 70 plants in each cross were collected and detected for the *opaque-2* gene by the marker phi057. There were three types of segregated plants, with 8 being homozygous dominant, 56 being heterozygous and 6 being homozygous recessive in the cross of Agron20 × Pop65C_6-46. Likewise, there were 5 homozygous dominant, 64 heterozygous and 1 homozygous recessive in the cross of Agron29 × Pop65C_6-55 (Table 2). However, this segregated plant ratio differed from the theory using a chi-square test. This result may have been affected by either the small sample size or as a random result of selected plants from the field.

With the primer phi057, the amplified products of seven inbred lines were detected with polymorphism similar to *opaque-2* but different from non *opaque-2* maize (SW1). The phi057 marker could detect amplified products of 160 bp in non *opaque-2* and 170 bp fragments in *opaque-2* maize (Babu *et al*., 2005; Jompuk *et al*., 2006). The phi057 is a co-dominant marker and could detect homozygous dominant (O_2O_2), heterozygous (O_2o_2) and homozygous recessive (o_2o_2) plants separately which is useful for marker-assisted selection for the *opaque-2* gene (Ribaut and Hoisington, 1998). These results showed that the seven inbred lines contained the *opaque-2* gene, which was different from the normal maize. Then, the seven recessive homozygous of *opaque-2* gene plants were self-pollinated to produce the BC_1S_2 lines. The protein content in the endosperm of these inbred lines ranged from 7.35 to 7.72% while *opaque-2* and non-*opaque-2* maize contained 7.76 and 7.64%, respectively. However, the protein contents among all tested maize lines were not significantly different (Table 3). Moreover, the tryptophan content in the endosperm

Table 1 Genotype of *opaque-2* in BC_1F_1 identified by the marker phi057.

Cross	No. of plants	Homozygous dominant (O_2O_2)	Heterozygous (O_2o_2)	Chi-square test ($1O_2O_2 : 1O_2o_2$)
Agron20 × Pop65 C_6-46	97	49 (50.5%)	48 (49.5%)	ns
Agron29 × Pop65 C_6-55	100	48 (48%)	52 (52%)	ns

ns = no significant difference ($P > 0.05$).

Table 2 Genotypes of *opaque-2* in BC_1S_1 identified by the marker phi057.

Cross	No. of samples	Homozygous dominant (O_2O_2)	Heterozygous (O_2o_2)	Homozygous recessive (o_2o_2)	Chi-square test ($1O_2O_2$: $2O_2o_2$: $1o_2o_2$)
Agron20 (BC_1S_1)	70	8	56	6	**
Agron29 (BC_1S_1)	70	5	64	1	**
Expected	70	17.5	35	17.5	-

** highly significant difference ($P < 0.01$).

of selected inbred lines was not significantly different from *opaque-2* gene samples though it was greater than in the normal maize. The results agreed with Jompuk *et al*. (2006) and Vasal (1994) who indicated that the protein content in maize endosperm was the same for normal and *opaque-2* maize. However, the tryptophan content in the protein of maize containing the *opaque-2* gene was almost double that in Agron20 normal maize (Prasanna *et al*., 2001).

Analysis of variance

Significant differences between treatments were observed for grain yield, 1,000 seed weight, moisture content, ear height, plant height and days to male and female flowering but not for shelling percentage. Significant differences between GCA effects were observed for all traits except shelling percentage, whereas SCA effects were significantly different for grain yield, 1,000 seed weight, moisture content, ear height and female flowering (Table 4).

Combining ability analysis

Grain yield

Mean grain yield (t ha^{-1}) was 4.680 for the diallel cross (F_1) and 8.21 for the check (SW4452) without the *opaque-2* gene. The GCA and SCA effects of these traits were significantly different. The mean grain yield, GCA effects and SCA effects of the diallel cross are shown in Tables 4 and 5. The grain yield of the diallel cross ranged from 2.081 to 7.669 t ha^{-1} (Table 5). The highest grain yield was the cross of P1 × P4 where these inbred lines were extracted from different crosses. On the other hand, the lowest grain yield was observed for the cross of P5 × P7 where these inbred lines came from the same cross. Inbred P1 had the best combination of GCA effects for grain yield. Moreover, inbred P6 also gave positive GCA effects. Therefore P1 and P6 could be used as tester lines for the quality protein maize. The grain yield of the diallel cross was significantly different from the commercial hybrids (single cross hybrid varieties) shown in Tables 4 and 5. However, the average yield of the top three crosses (7.599 t ha^{-1}) was not highly significantly different from that of the commercial hybrid checks (8.210 t ha^{-1}). The grain yield results indicated that a single cross hybrid of the *opaque-2* gene with vitreous endosperm could possibly be grown in Thailand. Moreover, the previous and recent studies have reported yields of CIMMYT-QPM hybrids that are competitive with the best locally available normal-endosperm cultivars for many tropical sites (Bjarnason and Vasal, 1992; Pixley and Bjarnason, 1993, 2002). In addition, data from QPM trials in Brazil, Ghana, Guatemala and South Africa also documented similar yields of QPM relative to the test of available normal-endosperm maize checks (Mertz, 1992). QPM hybrids have several advantages over the open-pollinated QPM varieties such as more uniform and stable endosperm modification, and less monitoring is required to ensure protein quality in seed production.

Protein and tryptophan content

The protein contents in the endosperm of the diallel cross samples were not significantly difference among themselves or from the check varieties (data not shown). The protein content ranged from 6.44 to 7.85% for the diallel crosses. The average protein content was 7.07% for the diallel crosses, 8.22% for *opaque-2* and 7.68% for non *opaque-2* (Agron20) maize. There was no significant difference between the general and specific combining ability of the protein content in the endosperm. The tryptophan content in the maize endosperm was not significantly different between the diallel cross and *opaque-2* gene maize and the average tryptophan contents were 0.74 and 0.78% in the protein of the diallel cross and the *opaque-2* gene maize, respectively (Tables 6 and 7). The tryptophan content was higher in the diallel cross (0.74%) than in the non *opaque-2* maize

Table 3 Mean values for grain yield, protein, tryptophan in protein content and studied traits of seven inbred lines and check varieties.

Line	Yield (t ha^{-1})	Protein (%)	Tryptophan in protein (%)	Moisture content (%)	Shelling (%)	Plant height (cm)	Ear height (cm)	Time to male flowering (d)	Time to female flowering (d)
P1	1.175	7.35	0.80	18.86	75.79	120	50	55	54
P2	1.516	7.42	0.72	18.68	70.08	104	41	59	60
P3	1.782	7.63	0.74	20.09	77.38	87	35	57	58
P4	2.154	7.47	0.75	21.27	76.86	119	58	59	60
P5	1.415	7.72	0.81	20.20	73.91	93	39	60	62
P6	0.739	7.64	0.70	20.75	70.60	110	50	62	63
P7	1.456	7.40	0.74	19.08	76.41	100	36	57	59
Opaque-2	-	7.76	0.80	-	-	-	-	-	-
Agron 20	-	7.64	0.41	-	-	-	-	-	-
F test	**	ns	**	ns	*	**	**	**	**
LSD: 0.05	-	-	-	-	4.81	-	-	-	-
LSD: 0.01	0.111	-	0.151	-	-	18.64	7.68	3.89	4.57

- = not determined.
ns = no significant difference ($P > 0.05$).
* = significant difference ($P < 0.05$).
** = highly significant difference ($P < 0.01$).

Table 4 Mean square of grain yield and studied traits of F_1-hybrid; a comparison between F_1-hybrids and a check (Suwan 4452) maize, GCA and SCA.

Source of variation	d.f	Yield (t ha^{-1})	1,000 seed weight (g)	Moisture (%)	Shelling (%)	Ear height (cm)	Plant height (cm)	Time to male flowering (d)	Time to silking (d)
Replications	3	0.721	1002	20.25	12.23	151.3	64	1.12	2.74
Treatments	20	11.513**	6742**	12.42**	9.52^{NS}	451.9**	1368.7**	24.44**	39.13**
F_1 versus check	1	72.117**	55869**	6.701^{NS}	55.18*	6707**	9521**	0.78^{NS}	4.38^{NS}
GCA	6	8.918**	5346.38**	5.33**	1.72^{NS}	299.73**	929.68**	19.28**	26.51**
SCA	14	0.289**	116.71**	2.15**	2.67^{NS}	32.95*	90.39^{NS}	0.47^{NS}	2.61**
Error	60	0.717	189	3.02	10.46	64.4	231.9	1.26	1.19
Total	83	84962	1797	5.91	10.30	160.9	500	6.84	10.39
CV (%)		18.10	5.62	7.54	4.03	14.31	12.19	2.06	1.99
LSD$^{1/}$: 0.05		-	-	-	-	-	-	-	-
: 0.01		1.59	25.86	3.27	-	15.10	28.65	2.11	2.05

d.f. = degrees of freedom; - = not determined; ^{NS} = no significant difference ($P > 0.05$); * = significant difference ($P < 0.05$); ** = highly significant difference ($P < 0.01$).
$^{1/}$ = LSD of treatment at the 0.05 or 0.01 level.

Table 5 Mean grain yield of the tested hybrid in t ha^{-1} (below the diagonal), general combining ability (on the diagonal line in bold) and specific combining ability (above the diagonal) in the diallel cross.

Parents	P1	P2	P3	P4	P5	P6	P7
P1	**2.729**	0.356	-0.688	0.474	-0.590	-0.242	0.691
P2	7.572	**-0.193**	-0.121	0.251	0.070	-0.363	-0.194
P3	5.989	3.635	**-0.731**	0.156	0.432	-0.059	0.279
P4	7.669	4.525	3.892	**-0.213**	-0.357	-0.381	-0.143
P5	6.338	4.076	3.903	3.629	**-0.481**	1.062	-0.617
P6	7.557	4.514	4.280	4.475	5.651	**0.390**	-0.017
P7	6.598	2.791	2.726	2.823	2.081	3.552	**-1.501**

Mean grain yield = 4.680 t ha^{-1}.
SW4452 grain yield = 8.210 t ha^{-1}.

Table 6 Percentage of protein in maize endosperm of tested hybrids (below the diagonal) and percentage of tryptophan in protein of maize endosperm (above the diagonal) in the diallel cross.

Parents	P1	P2	P3	P4	P5	P6	P7	Mean (%)
P1	-	0.77	0.61	0.75	0.69	0.66	0.77	
P2	7.51	-	0.72	0.73	0.73	0.79	0.83	
P3	6.95	6.54	-	0.78	0.78	0.83	0.69	
P4	7.21	7.26	6.86	-	0.62	0.72	0.80	
P5	7.33	7.29	6.44	7.36	-	0.76	0.73	
P6	7.85	6.52	6.62	7.59	6.56	-	0.72	
P7	7.45	6.68	6.40	7.34	7.29	7.36	-	0.74
Mean (%)							7.07	

Table 7 Percentage of protein and tryptophan in protein in maize endosperm of normal maize (Agron 20) and quality protein maize (QPM).

Inbred	Protein (%)	Tryptophan in protein (%)
Agron20	7.68	0.42
QPM	8.22	0.78
t test	Ns	**

ns = no significant difference ($P > 0.05$); ** = highly significant difference ($P < 0.01$).

(0.42%) as shown in Tables 6 and 7. These results showed that QPM varieties had almost double the amount of tryptophan compared to normal maize, but they had a similar content of overall protein (Jompuk *et al*., 2006, 2007). Moreover, the parental lines had the same tryptophan content when compared with their diallel cross.

CONCLUSION

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 percentage 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} in P1 × P4,) was not significantly different from the normal maize (8.21 t ha^{-1} in commercial single cross hybrids). Inbred P1 had the best combination of GCA effects for grain yield. The P6 inbred line also had positive GCA effects. Therefore, P1 and P6 inbred lines could be used as tester lines or as QPM germplasm for the quality protein maize breeding program. In conclusion, QPM hybrids could possibly be grown in Thailand without any difference in grain yield and to greater advantage in terms of grain nutrition compared with normal maize.

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