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Detection of Quality Protein Maize (QPM) using Simple Sequence Repeat (SSR) Markers and Analysis of Tryptophan Content in Endosperm

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ABSTRACT

Quality protein maize (QPM) controlled by *opaque-2* (o_2) gene was detected using two simple sequence repeat (SSR) markers and further confirmed by tryptophan content in the endosperm. Three populations, i.e., Pop61C_1, Pop62C_6 and Pop65C_6 from the International Maize and Wheat Improvement Center (CIMMYT) were used. 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 phi057 marker identified 24 out of 40 Pop61C_1 plants (60%), 34 out of 35 Pop62C_6 plants (97%) and 24 out of 30 Pop65C_6 plants (80%) to be *opaque-2* positive while phi112 marker identified 34 (85%), 35 (100%) and 30 (100%) to be *opaque-2* plants from the respective populations. Since phi112 was a dominant marker not for detecting the heterozygous genotype, it could only indicate the difference between QPM inbreds and normal maize. The phi057, on the other hand, is a co-dominant marker and could identify the heterozygote of maize plants and therefore, the contamination of non-QPM presented in CIMMYT population. The endosperms of selected S_1-seed were further analyzed for the tryptophan and protein content. The three maize populations were found containing the same protein quantity, but different in tryptophan 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 content 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.

Key words: quality protein maize, *opaque-2* gene, marker-assisted selection, tryptophan, protein

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INTRODUCTION

Maize is a major cereal crop for livestock feed and for human consumption as well as a raw material for several industrial uses. However, the conventional maize contains low protein quality due to its deficiency in two essential amino acids, lysine and tryptophan (FAO, 1992). A breakthrough came in 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, thereby, setting numerous breeding programs to improve protein quality in maize (Mertz *et al*., 1964; Vasal, 1994). Unfortunately, adverse pleiotropic effects of the *opaque-2* phenotype imposed severe constraints on agronomic advancement of opaque-2 cultivars of which reduced grain yield, soft endosperm, chalky and dull kernel appearance and susceptibility to ear rots and stored grain pests were prominent (Villegas, 1994). However, these inadequate qualities of *opaque-2* maize have been corrected to a certain extent through several genetically improved programs and finally quality protein maize (QPM) is achieved (Bjarnason and Vasal, 1992; Vasal, 1994). QPM has a genotype in which the *opaque-2* gene has been incorporated along with associated modifiers and contains twice the amount of tryptophan and lysine as compared to normal maize endosperm (Babu *et al*., 2005). Since there is a significant correlation between lysine and tryptophan concentrations in opaque-2 endosperm (r=0.85) (Hernandez and Bates, 1969), tryptophan itself can be used as an indicator of essential amino acid content. The *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 but the procedure is tedious and time consuming. With the development and access to reliable PCR-based allele specific markers such as simple sequence repeat (SSR), DNA marker-assisted selection (MAS) becomes 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 objective of this study was to use phi057 and phi112 markers for quality protein maize selection and analyze tryptophan content in endosperm for early detection in breeding program use.

MATERIALS AND METHODS

Plant materials

The seeds of three quality protein maize (QPM) populations developed by the International Maize and Wheat Research Center (CIMMYT), namely Pop61C_1 (Tropical maize Early Flowering Yellow Flint; TEYF), Pop62C_6 (Tropical maize Late Flowering White Flint; TLWF) and Pop65C_6 (Tropical maize Late Flowering Yellow Flint; TLYF) were obtained from the Nakhon Sawan Field Crop Research Center, Nakhon Sawan Province. The seeds were sown at the National Corn and Sorghum Research Center, Kasetsart University, Nakhon Rachasrima Province, in March 2003. S_0-plants of preferred morphological characters, i.e., early flowering, short anthesis-silking interval (ASI), healthy plants and the other desirable agronomic traits were selected. The leaves of these S_0-plants were collected from 40, 35 and 30 plants of Pop61C_1, Pop62C_6 and Pop65C_6, respectively, and also from three control plants of Suwan 1 (a non-QPM) and opaque-2 variety (containing *opaque-2* gene). All selected and control plants were self-pollinated and mature ears were harvested to determine tryptophan and protein content in the endosperm.

DNA extraction and analysis

DNA was extracted from 150-250 mg fresh leaves of an individual selected plant (S_0) using the method described by Agrawal *et al*. (1992). Two simple sequence repeat (SSR)

markers, phi057 and phi112 (KU-VECTOR Custom DNA Laboratory, Kasetsart University) were used to detect the *opaque-2* plants. These markers give amplification product of about 140 – 160 bp (Chin *et al*., 1996). An amplification was done in the 20 µl reaction mixture containing 1x 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 theromocycler 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 phi057 marker, the amplified fragments were separated on a 6% polyacrylamide denaturing gel in 1xTBE buffer and silver-stained. For phi112 marker, PCR products were run on a 3% horizontal agarose gel in 1xTBE buffer and stained with ethidium bromide.

Trytophan and total protein analysis

Twenty-five seeds from each ear of selected S_0-plant were collected and soaked in distilled water for 25 min before removing pericarps and embryos. The endosperms were air-dried overnight and ground (to approximately 0.5 mm) in a cyclone mill and wrapped in a commercial filtered-paper envelope to defat with 100% hexane in a Soxhlet-type continuous extractor. The defated ground samples were analyzed for tryptophan content using spectrophotometer as described by Villegas and Mertz (1971) and the protein content was measured using microkjeldahl method (Bailey, 1967).

RESULTS AND DISCUSSION

Marker assisted selection for *opaque-2* gene

Using phi057 as marker, quality protein maize (QPM) were detected in 24 out of 40 $Pop61C_1$ plants (60%), 34 out of 35 $Pop62C_6$ plants (97%) and 24 out of 30 $Pop65C_6$ plants (80%), while non-QPM were detected in the remaining 16 (40%), 1 (3%) and 6 (20%) plants, respectively (Table 1). For phi112 marker, QPM were detected in 34 out of 40 $Pop61C_1$ plants (85%), all 35 $Pop62C_6$ plants (100%) and also all 30 $Pop65C_6$ plants (100%), but non-QPM were detected in only 6 of $Pop61C_1$ plants. Since these three populations were originally classified as QPM, detection of non-QPM indicated the contamination of normal maize with opaque-2 cultivars. It should be noted here that $Pop62C_6$ was a white kernel maize where contamination of yellow kernel could be

Table 1 Numbers and percentages of QPM and non-QPM plants detected by two simple sequence repeat (SSR) markers (phi057 and phi112) in three QPM populations ($Pop61C_1$, $Pop62C_6$ and $Pop65C_6$) and in two standard varieties of *opaque-2* (a QPM) and Suwan 1 (a non-QPM).

Plantvarieties	Maize types	Number of plants and percentage (%)	
		phi057	phi112
$Pop61C_1$	QPM	24 (60%)	34 (85%)
	non-QPM	16 (40%)	6 (15%)
$Pop62C_6$	QPM	34 (97%)	35 (100%)
	non-QPM	1 (3%)	0 (0%)
$Pop65C_6$	QPM	24 (80%)	30 (100%)
	non-QPM	6 (20%)	0 (0%)
Opaque-2	QPM	3 (100%)	3 (100%)
Suwan 1	non-QPM	3 (100%)	3 (100%)

selectively separated before the experiment which resulted in the distinctively high percentage of QPM. Seeds are shown as yellow kernel when white kernel maizes are contaminated with the pollen of yellow kernel variety during pollination referred to as xenia effect (Poehlman, 1987). In yellow kernel populations ($Pop61C_1$ and $Pop65C_6$), however, this selection could not be done by the apparent of kernel color.

Upon analyzing the band patterns detected by these two markers, phi112 could detect a PCR amplified product (approximately 160 bp) of Suwan 1 (non-QPM), which was not found in opaque-2 variety or other S_0-plants (QPM) (Figure 1a). Babu *et al*. (2005) showed that phi112 was a dominant marker clearly distinguished the QPM inbred lines from the normal inbred lines by the absence of 150 bp from QPM plants. Since o_2o_2 is a homozygous recessive genotype, both dominant marker and co-dominant marker should give the same identified results. However, the phi112 marker could not clearly separate the heterozygous from homozygous recessive plants (CIMMYT, 2000), which also confirmed by these results. The phi057 marker, on the other hand, could detect amplified products of 160 bp in Suwan 1 (non-QPM), 170 bp fragments in opaque-2 variety and QPM lines, and both fragments (160 bp and 170 bp) for non-QPM lines as shown in the plant number 1, 7 and 9 of $Pop65C_6$ (Figure 1b). The results agreed with Babu *et al*. (2005) on showing that phi057 gave 160 bp fragments in normal inbred lines and 170 bp fragment in QPM. Since phi057 is a co-dominant 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), it is speculated that phi057 is more closely linked to

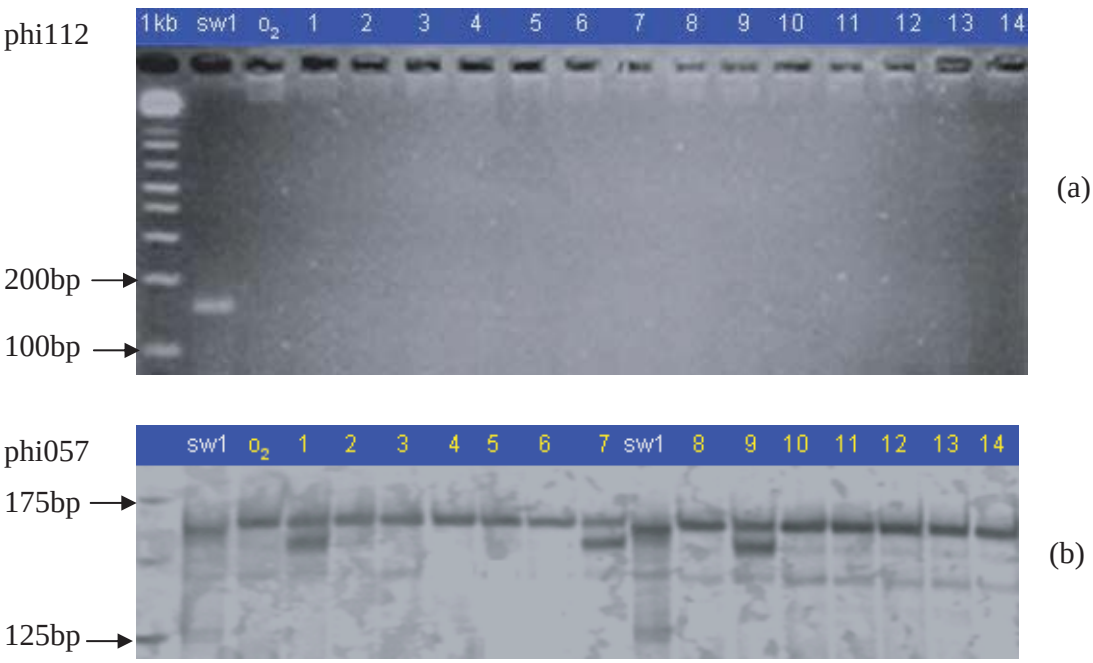

Figure 1 Identification of QPM (o_2o_2), non-QPM (O_2_) of $Pop65C_6$ S_0-plants and two standard varieties of *opaque-2* (o_2) and Suwan 1 (sw1) by (a) phi112 on 3% agarose where all S_0-plants were QPM, (b) using phi057 on 6% polyacrylamide ,non-QPM was detected on S_0-plants number 1, 7 and 9. Numbers on each column are the designated number of S_0-plants.

opaque-2 gene than phi112 marker and therefore more effective as a marker-assisted selection to discriminate between homozygote and heterozygote plants. To confirm the above results, tryptophan content in the endosperm of the heterozygote plants were determined to distinguish them from the homozygote ones.

Trytophan and total protein analysis

The QPM and non-QPM plants detected by two different markers (phi112 and phi057) were separately analyzed for protein and tryptophan content in the endosperm to compare the effectiveness of each marker in differentiating heterozygous and homozygous populations. It was interesting to find that total protein in maize endosperm of all S_0-plants (QPM, non-QPM, and controlled varieties) were non-significantly different at ~7.30% (Table 2). On the contrary, there were highly significant differences of tryptophan contents in QPM and non-QPM plants. In phi057 detected QPM and non-QPM, the average tryptophan content in maize endosperm was found to range from 0.64 to 0.67% for QPM plants, 0.36 to 0.40% for non-QPM plants, while in phi112 detected QPM and non-QPM, tryptophan content was in the range of 0.58 to 0.63% for QPM, 0.33% for non-QPM, and 0.38% for Suwan 1 and 0.80% for the opaque-2 variety for both types of markers (Table 2). These results agreed with Vasal (1994) and Prasanna *et al*. (2001) who indicated that QPM varieties had almost double amount of tryptophan compared to normal maize but were similar in overall protein content.

Theoretically, phi112 should identify homozygous dominant (O_2O_2) and heterozygous (O_2o_2) plants to be non-QPM but these results showed that some heterozygous (O_2o_2) plants identified by phi112 as QPM contained low tryptophan content as those of non-QPM (Table 3). On the contrary, these same populations were non-QPM as detected by phi057 and contained low amount of tryptophan as well. It was obvious that the sensitivity and accuracy of detecting QPM and non-QPM by phi112 were proven to be contradict to the tryptophan results and supported the previous speculation that phi057 marker was more closely linked to *opaque-2* gene than phi112 marker. Thus phi057 should be feasible to use as marker-assisted selection to detect the heterozygote line during the conversion of normal

Table 2 Comparison of protein and tryptophan contents in maize endosperm of three populations ($Pop61C_1$, $Pop62C_6$ and $Pop65C_6$) and two standard varieties of *opaque-2* and Suwan 1.

Plant varieties	Maize types	Total protein in endosperm (%) (mean ± sd)		Tryptophan in protein (%) (mean ± sd)	
		Phi057	Phi112	Phi057	Phi112
$Pop61C_1$	QPM	7.46 ± 0.552	7.41 ± 0.481	0.669 ± 0.082 b	0.584 ± 0.155 b
	non-QPM	7.33 ± 0.267	7.41 ± 0.340	0.361 ± 0.058 a	0.332 ± 0.028 a
$Pop62C_6$	QPM	7.21 ± 0.614	7.22 ± 0.605	0.642 ± 0.080 b	0.634 ± 0.090 b
	non-QPM	7.33 [1/]	NS	0.378[1/] a	NS
$Pop65C_6$	QPM	7.74 ± 0.770	7.64 ± 0.714	0.665 ± 0.075 b	0.611 ± 0.129 b
	non-QPM	7.26 ± 0.086	NS	0.395 ± 0.027 a	NS
Opaque-2	QPM	7.19 ± 0.010	7.19 ± 0.010	0.803 ± 0.480 b	0.803 ± 0.480 b
Suwan 1	non-QPM	7.33 ± 0.018	7.33 ± 0.018	0.377 ± 0.320 a	0.377 ± 0.320 a

LSD.01 (tryptophan in protein) = 0.208

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

NS = no sample tested, [1/] = one sample tested

Table 3 Comparison of tryptophan contents in the endosperm of S_0-plants detected by phi057 as non-QPM lines while phi112 detected as QPM and non-QPM lines from three populations ($Pop61C_1$, $Pop62C_6$ and $Pop65C_6$).

Plantvarieties	Maizetypes	Tryptophan in protein (%) (mean ± sd)	
		Phi057	Phi112
$Pop61C_1$	QPM	NS	0.379 ± 0.067
	non-QPM	0.361 ± 0.058	0.332 ± 0.028
$Pop62C_6$	QPM	NS	0.378[1/]
	non-QPM	0.378[1/]	NS
$Pop65C_6$	QPM	NS	0.395 ± 0.027
	non-QPM	0.395 ± 0.027	NS
Opaque-2	QPM	0.803 ± 0.480	0.803 ± 0.480
Suwan 1	non-QPM	0.377 ± 0.320	0.377 ± 0.320

NS = no sample tested, [1/] = one sample tested

elite inbred line to QPM inbred line through backcross method without tryptophan analysis in endosperm of each backcross generation. In case of backcross designed for transferring recessive gene (o_2 gene), identification of heterozygote in the seedling stage prior to pollination aided in the rejection of non-target backcross progenies (dominant homozygote) (Ribaut and Hoisington, 1998; Babu *et al.*, 2005). The phi057 would be used as a tool for early indication of three possible genotypes of the *opaque-2* plants, i.e., O_2O_2 and O_2o_2 and o_2o_2, especially at seedling stage.

CONCLUSION

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

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