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Marker-assisted introgression of *opaque* 2 *gene* into elite maize inbred lines

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Abstract

Marker assisted backcrossing can greatly accelerate the introgression of modified *opaque2* gene into elite inbred lines. This study aimed to transfer *opaque* 2 gene into elite maize inbred lines using SSR markers. Two backcross maize (white and yellow) populations were developed by crossing quality protein (QP) line with non-QP lines and subsequently backcrossed to non-QP lines until BC_3 generation. Among the three SSR markers (*phi 057, umc 1066, and phi 112*) used for opaque2 gene selection, only SSR *phi 057* marker was employed in segregating populations owing to its good discriminatory power in the survey of parental polymorphism. Using marker assisted selection, we transferred *opaque 2* gene into USM elite breeding lines. Of the 26 converted QPM lines (16 white, 10 yellow) selected from the BC_3F_3 populations, seven lines (4 white, 3 yellow) were finally selected as quality protein inbred lines, having satisfied the minimum standard criteria for protein quality. These converted QPM lines could be used as parent lines in the development of QPM varieties.

Key words: backcrossing, corn breeding, elite inbred lines, QPM lines (quality protein maize), and ssr marker.

Introduction

Maize plays a very important role in human and animal nutrition. Superior QPM hybrids are extremely valuable for the white-maize food industry and yellow maize for the animal feed industry. In the Philippines, 80% of the maize produced is used as feed for swine and poultry. Eleven percent (11%) and 13 % are used as human food and for other industrial purposes, respectively (Gerpacio 2001). The development of well-adapted QPM germplasm will have tremendous value for the food and feeds industry. Moreover, it does provide a nutritionally balanced source of protein. The *opaque 2 (o2)* mutation increases the lysine and tryptophan contents in maize endosperm by decreasing the synthesis of zein proteins and increasing the level of other lysine-rich proteins. Scientists in CIMMYT and South Africa systematically introgressed *mo2* (*o2* modifiers) genes into *o2* germplasm and developed hard endosperm *o2* populations to produce so-called "Quality Protein Maize" (QPM). The potential of QPM to dramatically improve nutrition was recognized in the award of the World Food Prize in 2000 (Cordova 2001). Three SSR markers, phi57, phi112, and umc1066, identified as internal repetitive sequences within the *o2* gene, were used successfully to convert normal maize lines into QPM and CIMMYT (Dreher et al 2003). The markers could be used in confirming the homozygous *o2* gene, thus avoiding the need to determine lysine content in QPM breeding program. Moreover, the marker-assisted breeding strategy obviates the need for selfing and lysine examination after each backcross, thus markedly shortening the breeding duration. With the new research thrust focusing on maize hybrid breeding program of the University of Southern Mindanao Agricultural Research Center (USMARC), inbred lines developed by USM maize breeders are increasing in number these recent years. However, no local-version of QPM lines are available. To utilize the nutritional potential of QPM in Southern Philippines, efforts are being made by USMARC to introgress opaque mutation and its associated modifier into elite breeding lines. Therefore, this study aimed to transfer the QPM allele (*opaque2*) to an elite USM inbred through marker assisted backcrossing.

Methodology

Survey of parental polymorphism To determine which parental lines should be used for marker-aided selection, survey of parental polymorphism were conducted using three SSR markers for *opaque 2 gene*. These markers were *phi 057, phi 112,* and *umc 1066*. QPM lines and non QPM lines were used to survey parental polymorphism on these three SSR markers. Primer sequences (F=forward and R=reverse) used were as follows (www.maizegdb.org):

phi057:
F, 5'-CTCATCAGTGCCGTCGTCCAT-3';
R, 5'-CAGTCGCAAGAAACCGTTGCC-3':
umc1066:
F, 5'-ATGGAGCACGTCATCTCAATGG-3';
R, 5'-AGCAGCAGCAACGTCTATGACACT-3':
phi112:
F, 5'-TGCCCTGCAGGTTCACATTGAGT3';
R, 5'-AGGAGTACGCTTGGATGCTCTTC- 3':

DNA isolation, PCR amplification, and analysis Template DNA was extracted from the seedlings at the 3-4 leaf stage following procedures of CIMMYT ABC LAB protocol. Twenty (10 µl) PCR mix containing the DNA template, dNTPs, primer, magnesium chloride, and *taq* polymerase were added in each well. PCR amplification was carried using the profile of one cycle initial denaturation for 2 min at 94°C, and 30 cycles of denaturation for 1 min at 94°C, annealing temperature of 60°C for 2 min, extension for 1 min at 72°C and a final extension of 5 min at 72°C. The amplified fragments were separated on 6% polyacrylamide gels in a Bio-Rad vertical electrophoresis gel apparatus. Thereafter, the gel was silver-stained as described by manufacturer's instruction with little modification.

Development of BC populations Based on the results of parental polymorphism survey, four inbred lines were selected to develop BC populations for the marker assisted backcrossing. These lines were SMWL 10-8, SMYL 104, CML 144, and CML 168. The former lines were used as recurrent parents and the later lines were *opaque 2 gene* donor. To form white BC population, line SMWL 10-8 was crossed with CML 144 using CML 144 as male parents; the resulting F_1 plants were backcrossed to SMWL 10-8 (recurrent parent) as pollinator to obtain BC_1F_1 plants and followed by selfing to generate BC_1F_2 plants. These procedures were repeated until BC_3F_3 population. For the yellow BC population, SMYL 104 and CML 168 were used to develop the desired population using similar approach in white BC population as described above. The schematic diagram in producing BC populations is illustrated in Figure 1.

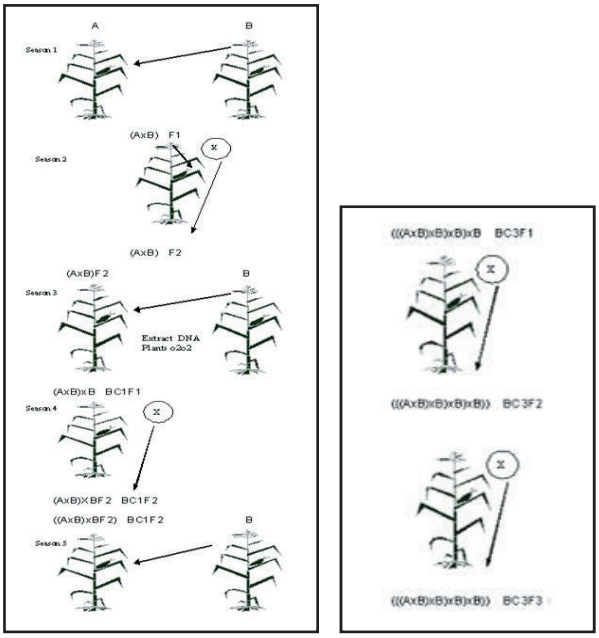


Figure 1. Breeding scheme used in the development of backcross population (A=QPM donor parent, B=normal parent).

Marker-assisted selection for Opaque 2 gene and endosperm hardness The plants carrying *σ2* gene from the derived BCF_2 populations were selected using three SSR markers. Of these markers, we used only phi 057 marker since it is a codominant marker and showed good polymorphism in the parental survey. The plants from the segregating BCF_2 populations were genotyped using *phi57 marker* to identify plants carrying the *σ2* gene. To select hard endosperm with *σ2* gene, only those lines and plants with *σ2* gene were subjected to light box. Only those kernels with endosperm harness (EH) of 2-3 were selected using light box analysis (Figure 2). The rating scale used was 1-5, wherein 1-hard endosperm and 5-soft endorperm is shown below. The percentage of opaqueness (EH) is visually assessed and best illustrated in Figure 3.

Type (Modification score) 1: Not opaque.
Type (Modification score) 2: 25% opaque.
Type (Modification score) 3: 50% opaque.
Type (Modification score) 4: 75% opaque.
Type (Modification score) 5: 100% opaque.

Figure 2. Locally made light box used in selecting opaqueness of endosperm.

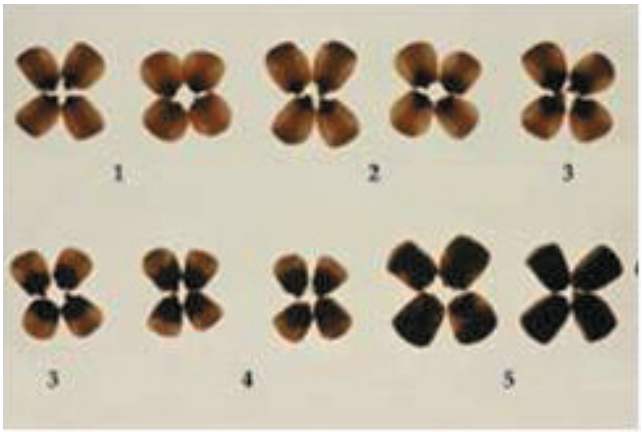


Figure 3. Kernels on a light table sorted into modification classes (adapted from Vivek et al 2006).

Protein quality analysis The percentage protein and tryptophan content were determined in the final selected BC3F3 lines. Kernel samples of the selected lines were submitted to the Biochemistry Laboratory of the Institute of Plant Breeding for protein and tryptophan analyses. Since the cost of lysine content analysis was very expensive, we only estimated it based on the tryptophan content following CIMMYT procedure (Vevik et al 2008). The percent lysine content was estimated as % lysine = percent tryptophan in protein times 4. The quality index was calculated as (% tryptophan sample/ %protein) x 100.

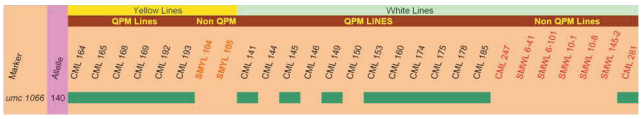
Results and discussion

Maize germplasm About 55 QPM (20 yellow and 35 white) inbred lines were obtained from CIMMYT. They were multiplied to obtain adequate seeds for evaluation. However, some of the inbred lines had very poor seed germination. During the seed increase, six inbred lines (2 yellow and 4 white) were initially selected based on visual acceptable phenotypic appearance. At harvest, only 7 and 18 inbred lines were finally seed increased (Table 1). When enough seeds of these inbred lines were obtained, the lines were evaluated for their agronomic traits. The selected lines were used as donor parents of ***opaque2*** gene for marker-assisted backcrossing.

Table 1. Survey on parental polymorphism using SSR marker *phi 057*.

Survey for parental polymorphism Three SSR markers (*phi 057, phi 112, and ucm 1066*) were used to survey the parental polymorphism for the *opaque 2 gene*. However, only phi057 and umc 1066 showed polymorphism among the lines surveyed. Marker *phi 112* did not show any polymorphism among the lines surveyed. Based on these results we selected marker *phi 057* for the marker-assisted selection since it is a codominant marker. This primer showed a very good polymorphism with QPM donor parents, showing a band size at 169 bp (base pairs) and non QPM parent with a peak size 159 bp (Table 2). This *phi 057* SSR marker was also reported earlier as a good co-dominant marker by several workers in their marker assisted backcrossing studies (Manna et al 2005; Danson et al 2006). The polymorphism was well applicable for discriminating between the QPM and non-QPM inbred lines and between homozygous and heterozygous opaque2 progeny (Figure 4). Based on these results, we chose CML 144 and SMWL 10-8 and CML 168 and SMYL 104 to serve as the parent lines for the development of BC populations.

Table 2. Survey on parental polymorphism using SSR marker umc 1066.



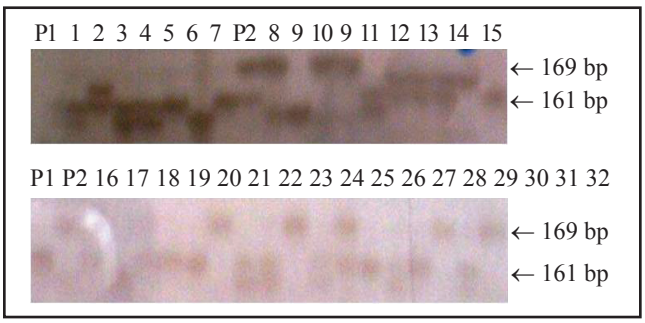


Figure 4. Profile of BC3F2 individual plants detected with phi075 SSR marker [P1-normal parent (161 base pairs {bp}), P2-QPM parent (169 bp), 1-32-BCF2 plants].

Development of BC populations and Marker-aided selection for opaque 2 gene Two BC populations were developed to employ marker-assisted selection for *opaque 2 gene*. One white population was formed by crossing CML 144 (donor parent) x SMWL 10-8 (recurrent parent). A total of 122 BC_0F_2 plants was formed. Of these 122 BC_0F_2 plants, 11 plants were genotyped for *opaque 2 gene* using *SSR phi 057* marker coupled with the selection of endosperm hardness (EH) of 2-3 rating using locally made light box. Figure 4 shows the DNA profile of BCF_2 plants detected by *phi057* SSR marker. The 11 lines were selected and used to form BC_3F_2 population. From this BC_3F_2 population, 25 plants were selected based on presence of *opaque 2 gene* and EH of 2-3 rating. At BC3F3 population, 16 lines were selected based on acceptable phenotypic appearance, presence of opaque 2 gene, and endosperm hardness. The protein quality contents of the selected 16 lines are shown in Table 3. These selected lines had percent protein content ranging from 8.63 to 9.68%. The percent tryptophan sample ranged from 0.067 to 0.094%, and the quality index of the same lines ranged from 0.72 to 1.09. The estimated percent lysine content ranged from 2.89 to 4.22%. The endosperm hardness ratings of all the lines were 2. Among the 11 lines, four lines (L-001, L-002, L-005, and L-016) were finally selected based on high quality index and lysine content. We only analyzed the tryptophan content because tryptophan is highly correlated (r=0.99) with lysine (Cordova 2001, personal communication of Vivek, 2006). CIMMYT scientists have devised selection and standard criteria for *quality protein maize* (*QPM*). It can be called QPM when it has a minimum level of 8% protein, 0.075% tryptophan in sample, 4.0% lysine, and quality index (QI) of 0.8. Using these criteria, four lines were identified to be considered as QPM. Lysine levels in normal and o2 maize averaged respectively at 2.0% and 4.0% of total protein in whole grain flour. However, across diverse genetic backgrounds, these levels range from 1.5-2.8% in normal maize to 2.6-5.0% in their O2 converted counterparts (Moro et al 1996).

Table 3. Percent protein, tryptophan, and lysine contents of USM white quality protein maize lines.

Lines	Pedigree (Sample)	% Protein	% Try	Estimated %Lys	Quality Index	Endospern Hardness
L-001	BC3F3-45-6	8.78	0.093	4.22	1.06	2
L-002	BC3F3-45-10	8.64	0.094	4.37	1.09	2
L-003	BC3F3-45-13	9.22	0.088	3.84	0.96	2
L-004	BC3F3-48-11	8.92	0.084	3.77	0.94	2
L-005	BC3F3-48-17	8.83	0.092	4.16	1.04	2
L-006	BC3F3-58-1	9.68	0.089	3.67	0.92	2
L-007	BC3F3-58-2	9.18	0.083	3.62	0.91	2
L-008	BC3F3-58-3	9.30	0.067	2.89	0.72	2
L-009	BC3F3-58-4	9.47	0.080	3.37	0.84	2
L-010	BC3F3-58-5	9.53	0.074	3.13	0.78	2
L-011	BC3F3-58-6	9.54	0.083	3.47	0.87	2
L-012	BC3F3-58-7	9.16	0.084	3.67	0.92	2
L-013	BC3F3-58-9	9.56	0.092	3.86	0.96	2
L-014	BC3F3-58-10	9.13	0.084	3.68	0.92	2
L-015	BC3F3-58-11	8.71	0.075	3.44	0.86	2
L-016	BC3F3-58-12	8.63	0.089	4.13	1.03	2
CML 144	Donor Parent	10.1	0.100	4.00	0.99	2
SMWL 10-8	Recipient Parent	9.58	0.054	2.16	0.56	1

1-hard endosperm, 5-soft endosperm

For the yellow BC population, it was formed by crossing CML 168 (donor parent) x SMYL 104 (recurrent parent). At BC_0F_2, 152 plants were obtained and 11 plants were selected based on presence of *opaque 2 gene*. These 11 lines were used to form, BC_2F_2 population wherein 690 plants were produced. Ten lines were selected based on presence of opaque 2 gene, endosperm hardness, and desirable phenotypes. The protein quality of the 10 selected yellow lines is presented in Table 4. Using the same CIMMYT standard for QPM, three lines (L-019, L-022, and L-023) may be considered as quality protein maize.

Table 4. Percent protein, tryptophan, and lysine contents of USM yellow quality protein maize lines.

Lines	Pedigree (Sample)	% Protein	% Try	Estimated %Lys	Quality Index	Endospern Hardness
L-017	BC3F2-16-47-7	9.79	0.089	3.63	0.91	2
L-018	BC3F2-6-51-28	8.70	0.087	3.98	0.99	2
L-019	BC3F2-6-51-10	8.99	0.091	4.07	1.02	2
L-020	BC3F2-6-51-27	9.59	0.084	3.48	0.87	2
L-021	BC3F2-16-128-51	9.55	0.079	3.33	0.83	2
L-022	BC3F2-6-51-21	8.80	0.090	4.12	1.03	2
L-023	BC3F2-6-51-18	8.61	0.089	4.14	1.04	2
L-024	BC3F2-6-51-12	8.71	0.080	3.66	0.91	2
L-025	BC3F2-6-51-24	9.59	0.077	3.21	0.80	2
L-026	BC3F2-6-51-15	9.37	0.076	3.23	0.81	2
CML 168	Donor Parent	9.45	0.099	4.00	1.05	2
UPYL 101	Recipient Parent	9.71	0.080	3.30	0.82	1

1-hard endosperm, 5-soft endosperm

Summary and conclusion

SSR phi 057 marker was used in marker-assisted backcrossing in the two BC maize populations. At BC_3F_2 populations, seven lines were converted into QPM versions which contained more than 1.0% tryptophan and 4% lysine, and more than one g/unit protein quality contents. These newly converted QPM lines can be utilized for QPM variety development. These results demonstrate the utility of marker-assisted selection in expediting selections in plant breeding.

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