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MARKER ASISSTED BACKCROSSING FOR INCORPORATION OF THE *OPAQUE2* GENE INTO A STANDARD MAIZE INBRED LINE

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Abstract

Quality protein maize (QPM) is the maize with increased levels of two essential amino acids, hard endosperm and good agronomic performances. The recessive opaque2 (o2) mutation increases lysine and tryptophan content in maize grains. It has recently become possible to use marker assisted selection (MAS) to accelerate selection for the o2 allele in QPM breeding process. Maize Research Institute Zemun Polje has a program on developing QPM genotypes for growing in temperate regions. The results presented herein are the part of marker assisted backcross breeding program for incorporation of the o2 into maize inbred line. Marker selected o2 recessive homozygous BC_2F_2 individuals were subjected to the whole genome background selection, which identified five progenies with 83 to 94% recurrent parent genome content. BC_2F_3 families are being evaluated for desirable agronomic and biochemical traits in replicated trials and the best lines will represent the QPM version of the standard line.

Key words*: maize, marker assisted selection (MAS), opaque2 (o2), quality protein maize (QPM)*

1. INTRODUCTION

Maize (*Zea mays* L.) is one of the three major cereal crops. It represents an important source of food and feed, but due to the low level of two essential aminoacids – lysine and tryptophan, it does not meet the daily balanced protein requirement. The enhanced nutritional quality of the maize mutant *opaque2* (*o2*) was discovered in the 1960s (Eggum et al., 1983, 1985, Mertz et al., 1964). The *opaque2* mutation which increases lysine and tryptophan content also confers an undesirable phenotype leading to low yields, soft and chalky kernels that renders seeds susceptible to storage pest and ear rots. Using conventional breeding methodologies, interdisciplinary research team in the International Maize and Wheat Improvement Center (CIMMYT), Mexico converted the floury soft endosperm kernels into harder types, increased grain yield potential to the level of the best normal maize types, endowed the *opaque2* maize with disease and insect resistance and with utilization and storage qualities similar to those of superior normal maize materials. The new, agronomically acceptable and nutritionally improved *opaque2* types were renamed "Quality Protein Maize" or QPM (Vivek et al., 2008).

QPM varieties have been released in more than 23 developing countries for large scale cultivation on the area over 2.5 million ha (Akande & Lamidi, 2006; Sofi et al., 2009). QPM is primarely developed for tropical and sub-tropical regions, but it could also have many advantages in other parts of the world for animal feed and also in human nutrition. The nutritional and biological superiority of QPM has been reported in numerous studies. Akuamoa-Boateng (2002) in Ghana found that children fed with high lysine/tryptophan maize were healthier, had reduced stunting and better growth capabilities, compared with children fed with normal maize porridge. People eating QPM had significantly higher nitrogen retention than those who ate normal maize, indicating that QPM protein is more „bioavailable“ (Bressani, 1991). QPM holds the promise of improving the nutritional status of groups whose main staple is maize and who cannot afford protein-rich foods to supplement their diet. The utility of QPM as animal feed is greater in the parts of the world which have high per capita meat supply. A number of studies have proved the impact of QPM on the increase in body growth of poultry and pigs (Krivanek et al., 2007, Mbuya et al., 2011, Sofi et al., 2009). Utilization of QPM could substitute protein additives which are used in animal feed composites.

It has recently become possible to use marker assisted selection (MAS) to accelerate selection for the *o2* allele in QPM breeding. Molecular markers are superior to other forms of MAS (performed with morphological or biochemical markers) because they are relatively simple to detect, abundant throughout the genome even in highly bred cultivars, completely independent of environmental conditions and can be detected at any stage of plant development. They are based on naturally occurring polymorphisms in DNA sequences (base pair deletions, substitutions, additions or patterns) (Gupta et al., 1999). Properties that characterize a suitable

molecular marker are high polymorphism, co-dominant inheritance, random and frequent distribution throughout the genome, easy and cheap detection, high reproducibility (Semagn et al., 2006) Molecular markers can be used for several different applications including: germplasm characterization, genetic diagnostics, characterization of transformants, study of genome organization and phylogenic analysis.

Three Simple Sequence Repeats (SSR) (phi057, phi112 and umc1066) located as internal repetitive sequences within the *opaque2* gene are being utilized as foreground selection markers for the *opaque2* gene (Danson et al., 2006). The phi112 marker is dominant and therefore identifies normal (*O2O2*) and heterozygous (*O2o2*) genotypes and it is useful in the contamination of QPM seeds with non-QPM. The other two markers are co-dominant and can identify all three genotypes. Recessive genes can be maintained without the need for progeny tests in each generation, as homozygous and heterozygous plants can be distinguished with the aid of (co-dominant) markers. In backcrossing, DNA markers can help to reduce the generations required to recover a recurrent parent's genetic background.

Marker assisted selection increases reliability and efficiency, reduces time and costs taken to obtain QPM (Babu et al., 2004, 2005). Marker analysis can be done using DNA samples extracted from leaf tissue of very young plants, thus enable identification of QPM plants early in the breeding cycle. This allows breeders to discard plants without *o2* allels prior to pollination, reducing the size of the breeding population and saving both tome and money. Also, with molecular markers breeders can identify heterozygous plants in order to get homozygous recessive plants after selfing, given that the presence of *o2* in the homozygous recessive state (*o2o2*) is the aim of the selection of QPM genotype (Vivek et al., 2008).

Maize Research Institute Zemun Polje (MRI) has a breeding program with the aim to convert standard maize inbred lines to QPM genotypes for growing in temperate regions (Ignjatovic-Micic et al., 2008, 2009, 2010, 2013). The objective of analysis presented in this paper was to identify the BC_2F_3 progeny with the highest proportion of the recurrent parent genome using SSR markers evenly distributed throughout the genome. The results also contributed in reducing the number of lines that will be subjected to biochemical and phenotypic selection.

2. MATERIALS AND METHODS

2.1. Plant material

One MRI commercial inbred line was selected as the recurrent parent for marker assisted backcross programme to be the recipient of the *o2* allele and to improve the tryptophan content. CML 144, a QPM inbred line obtained from CIMMYT, was selected as the donor line of o2. F_1 plants were backcrossed onto reccurent parent line to generate BC_1F_1 progeny. The genotypes that were heterozygous for the gene-specific phi057 and umc1066 locus were selected for backcrossing. A two-level selection procedure was carried out in BC_2F_1. First, the genotypes were screened for phi057 and umc1066 locus to identify the heterozygotes. Second, the selected heterozygotes for *o2* allele were screened by a large number of SSR markers distributed over the maize genome. Genotypes with the highest recovery of recurrent parent genome were selected among the heterozygotes. Selected progenies were selfed to produce BC_2F_2 seeds. The harvested BC_2F_2 seeds of all selected plants were raised and the DNA samples from BC_2F_2 progenies were collected and subjected to foreground selection before flowering to identify the progenies that attained homozygosity at *o2* locus. The genotypes having homozygous recessive for *opaque2* locus were selfed to produce BC_2F_3. Ten BC_2F_3 families were subjected to the whole genome background selection.

2.2 DNA isolation

Genomic DNA was isolated from the four-weeks-old seedlings of the recurrent parent and ten progenies from backcrossed and selfed generations. Bulks were prepared by pooling an equal amount of leaf tissue taken from 10 leaves per progeny. Young and healthy leaf tissue was ground into a fine powder in liquid nitrogen using a pre-chilled mortar-pestle. Approximately 100 - 150 mg of ground material was transferred into a 2 ml microcentrifuge tube containing 900 µl extraction buffer (CTAB 2%, Tris -HCL 100 mM, pH 8.0, EDTA 25 mM, pH 8.0, NaCl 1.4 M, β-mercaptoethanol 0.2%). The contents were mixed gently and incubated at 65 ºC for one hour. After the samples were shortly stored at the room temperature (RT), 900 µl of ice-cold chloroform:iso-amylalcohol (24:1) mixture was added, mixed well with the suspended tissue and spun in a microcentrifuge at 12,000 rpm for 5 min at RT. Without disturbing the interface, the supernatant was transferred to another 2 ml microcentrifuge tube, 0.7 V of isopropyl alcohol was added and stored at -20 ºC over night for DNA to precipitate. The next day samples were spun at 12,000 rpm for 5 min at RT and the supernatant was decanted.

Then 1 ml of 75% ethyl alcohol was added, mixed gently and stored at room temperature for at least an hour. The tubes were spun at 12,000 rpm for 10 min at RT and the supernatant was decanted. Once more, 1 ml of 75% ethyl alcohol was added, mixed gently, spun at 12,000 rpm for 3 min and the supernatant was decanted. DNA was suspended in 50 µl of 0.1xTE (Tris 1 mM, pH 8.0, EDTA 0.1 mM, pH 8.0) buffer and then stored at -20 ºC for long term storage or used directly for PCR amplification reactions. DNA was quantified using spectrophotometer (UV-1601, Shimadzu) and a working stock of 20 ng/µl was prepared to perform PCR.

2.3. SSR Analysis

Simple sequence repeat analysis was done with 32 primer pairs distributed over the maize genom. Polymerase chain reaction was carried out in 25 µl reaction volume containing: DreamTaq™ Green PCR Master Mix (2X), 0.5 µM primers and 20 ng of DNA. Amplifications were performed in thermocycler Biometra TProfessional Standard 96 with the following touch-down program: an initial denaturation at 95ºC/5min, followed by 15 cycles each of denaturation at 95 ºC /30 s, annealing at 63.5 ºC/1min (-0.5 ºC/cycle) and extension at 72 ºC/1min; another 22 cycles of 95 ºC /30 s, 56ºC/1min and 72ºC/1min were performed. Final elongation was at 72°C for 4 min. The amplified fragments were resolved by electrophoresis on 8% polyacrylamide gel, with 20bp ladder as a marker. Gels were run on small format (7,3x10cm) vertical gel system (Mini Protean Tetra-Cell, BioRad), at 80 V for 1,5 h. After staining with 0.5 µg/µl ethidium bromide they were visualized under UV transilluminator and documented in gel documentation system (BioDocAnalyze, Biometra).

SSR profiles were scored as presence/absence of fragments in each sample and the data were assembled into a binary matrix. Genetic similarity (GS) was calculated in accordance with Dice (1945): GS_{ij} = 2a/2a+b+c; where:

a - the number of fragments present in both variety *i* and *j* (1.1)

b - the number of fragments present in iand absent in *j* (1.0)

c - the number of fragments absent in *i* and present in *j* (0.1)

Unweighted Pair Group Method with Arithmetic mean (UPGMA) method was applied for cluster analysis. The co-phenetic coefficient value were computed and the significance of the co-phenetic correlation observed was tested using the Mantel matrix correspondence test (Mantel, 1967). All marker data analyses were performed using statistical NTSYSpc2 program package (Rohlf, 2000).

3. RESULTS AND DISCUSSION

The backcross procedure is used in plant breeding to introgress favorable alleles from a donor plant into a recipient elite genotype (recurrent parent). Markers can be used in the context of marker assisted backcrossing (MABC) to either control the target gene (foreground selection) or to accelerate the reconstruction of the recurrent parent genotype (background selection). In traditional backcross breeding the reconstruction of the recurrent parent genotype requires more than six generations, while this may be reduced to only three generations in MABC (Frisch et al., 1999, Gupta et al., 2013, Tanksley et al., 1989). However, the authors also state that large numbers of marker data points are required to achieve such results.

With the development and access to reliable PCR-based allele specific markers such as SSRs, marker assisted selection is becoming an attractive option for simply inherited traits (Babu et al. 2004). There is a large amount of research that addresses MAS in some form. However, there are few data on MAS experiments - the majority of work is aimed at identifying genomic regions of interest, from which MAS experiments are an attractive next step (Ribaut & Hoisington, 1998). Babu et al. (2005) reported conversion of normal maize inbred line V25 to its QPM version employing the QPM donor CML176. Their rapid line conversion strategy included a two-generation backcross program that employs foreground selection for the *opaque2* gene in two backcross generations, background selection at non-target loci in the BC_2 generation, and phenotypic selection for kernel modification and other desirable agronomic traits in two subsequent selfed generations.

In this analysis, three out of 32 SSR primers used for background selection could not be applied for analysis due to absence of amplification product (two primers) or poor amplification (one primer). Total number of alleles detected with 29 informative SSR markers was 163. The number of alleles varied from 2 (phi116, bnlg557 and umc1858) to 10 (bnlg1643), with the average value of 5.6.

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Genetic similarity values between the recurrent parent (RP) and progenies (P-1 to P-10) calculated on Dice coefficient were in the range from 0.71 to 0.94, the average being 0,81. A total of five progenies had more than average recurrent parent genome (RPG) content (from 82 to 94%) (Table 1). P-5 had the highest proportion of RPG of 94%. P-6 had 91%, while P-4, P-7 and P-1 had 84, 83 and 82%, making them all candidates for developing further selfed generation. Similar to Babu et al. (2005), we selected the first five progenies with the highest proportion of recurrent parent genome, considering the unpredictable field problems especially with QPM germplasm (which is prone to poor germination and insufficient kernel modification). Other five progenies had 71- 78%, which is less than average RPG content and they were not used in further analysis.

Table 1. Recurrent parent genome content of progenies revealed by background marker analysis

Progenies	Recurrent parent genome content
P-1	**82%**
P-2	78%
P-3	75%
P-4	**84%**
P-5	**94%**
P-6	**91%**
P-7	**83%**
P-8	71%
P-9	71%
P-10	76%

(genotypes with more than average recurrent parent genome content (81%) are emphasized by Bold letters)

Dendrogram generated on Dice genetic similarities showed that progenies 5 and 6 have the highest procent of the RPG (Figure 1). The co-phenetic correlation coefficient (which indicates the extent to which the clustering of genotypes depicted in the trees accurately represents the estimates of genetic distances between populations obtained with a particular marker system) was 0.94, indicating a very good concurrence (fit).

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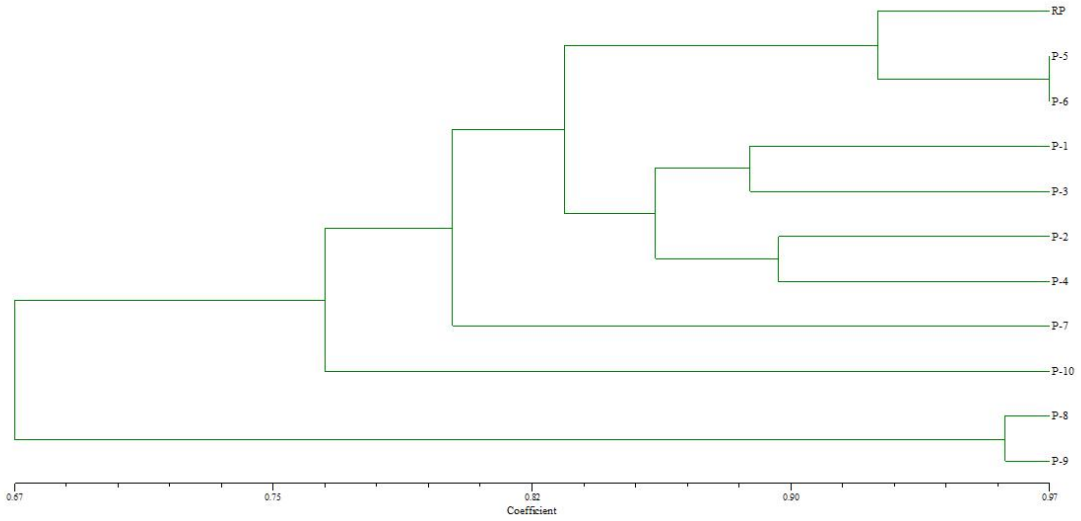

Figure 1. Dendrogram of the recurrent parent (RP) and BC_2F_3 progenies (P1-P10) constructed using UPGMA cluster analysis of *Dice* similarity values obtained by SSR binary analysis

Marker assisted selection in combination with conventional breeding can greatly accelerate the introgression of QPM genotype into normal maize. Gupta et al. (2013) used MAS for development of QPM parental lines of Vivek-9 hybrid and could developed QPM hybrid in less than half the time required through conventional breeding. SSR markers were effectively employed both for selecting *o2* homozygous recessive genotypes and genotypes with the highest proportion of recurrent parent genome. The recovery of the recipient genome in their best lines varied from 83.7% to 94.4% for one type of cross and from 80% to 93.7% for another. The results obtained in our study are in accordance with their results.

Considering that quality traits and yield depend on environmental conditions and stress environments produce high genotype by environment interaction (Banziger et al, 2000; Banziger & Diallo, 2004), field trials on several locations and in different years will be performed for the five BC_2F_3 families (P-1, P-4, P-5, P-6 and P-7) to test their stability. The following agronomic traits were recorded in the first year ot the trial: plant height, ear height, leaf number, leaf number up to the ear insertion, days to 50% silking, days to 50% pollen shed, ear lenght, ear diameter, kernel row number, number of kernels per row, kernel lenght and grain yield. Grain samples were also collected for estimation of kernel modification, total protein content, tryptophan concentration in protein and the recurrent parent genome content. After processing all these results, we will be able to select lines with the highest proportion of RPG coupled with higher level of tryptophan content without losing good agronomic performances of the original line.

4. CONCLUSIONS

The use of molecular markers for screening and selecting of plants in a breeding program provides several advantages and is therefore very attractive to plant breeders. Marker assisted selection has proven to be efficient both for transfer of *o2* into a different genetic background in order to improve an existing variety for a specific trait and for RPG content determination. We recovered inbred lines homozygous for *o2* allele with 94% RPG recovery. Improvement of protein quality along with the good agronomic performances maintenance is yet to be confirmed. By selecting the progenies with the highest proportion of recurrent parent genome, number of lines that need to be tested was reduced.

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