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THE TWO-LEVEL MARKER ASSISTED SELECTION IN BC_2 GENERATION OF THE CONVERSION OF STANDARD MAIZE LINES TO THEIR QPM VERSION

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ABSTRACT

Quality Protein Maize (QPM) is nutritionally enhanced maize. To shorten the period required for development of QPM hybrids through the conventional method of backcrossing, marker assisted selection (MAS) is being used. After a successful conversion of one commercial maize inbred line to its QPM counterpart for growing in temperate climate, four commercial Maize Research Institute (MRI) inbreds, chosen for marker assisted introgression of the quality protein trait, and their BC_2 progenies were subjected to two-level selection procedure. First, BC_2 plants were analyzed with *opaque2* (*o2)* specific molecular markers to identify heterozygotes. Second, the selected heterozygotes were screened with SSR markers to identify genotypes with the highest recovery of recurrent parent's genome (RPG). The specific markers identified 100 out of 192 plants (52%) as heterozygous. Genetic similarity values between parental lines and their BC_2 heterozygous progenies were in the range from 0.77 to 0.99 (77-99% RPG). The highest proportion of RPG was found in L_1 (93-99%) and the lowest in L_3 progenies (77-89%). Average values for the RPG content ranged from 83.9 to 95.8%. Progenies with RPG above 95% were selfed to produce BC_2F_2 plants which will be subjected to foreground selection. This time selection will be focused on homozygous recessive individuals, given that the presence of *opaque2* gene in the homozygous recessive state is the aim of the QPM selection. Finally, those *o2o2* genotypes will be screened for biochemical and phenotypic traits to confirm their nutritional and agronomical superiority.

Keywords: Maize, Marker assisted selection (MAS), *opaque2*, Quality Protein Maize (QPM)

INTRODUCTION

The discovery of *opaque2 (o2)* maize mutants (MERTZ *et al*., 1964) initiated the beginning of the breeding for improved protein quality in maize. This mutation has been

used as a source for genetic improvement of the nutritional value of maize proteins. Agronomically acceptable and nutritionally improved *opaque2* types (Quality Protein Maize -QPM) were created through conventional breeding programs by interdisciplinary research team in the International Maize and Wheat Improvement Center (CIMMYT), Mexico (VILLEGAS *et al*., 1992). In comparison to normal maize, QPM differs in protein quality because it contains double the amount of Lys and Try, the two amino acids deficient in maize proteins (PRASANNA *et al*., 2001; PANDA *et al*., 2010).

Marker assisted selection (MAS) has been increasingly used in maize protein quality improvement programs (BABU *et al*., 2005, DANSON *et al*., 2006; GUPTA *et al*., 2013). Foreground selection enables maintenance of recessive genes without the need for progeny testing in each generation of selection, as homozygous and heterozygous plants can be distinguished using gene-specific SSR markers. Also, DNA markers in background selection accelerate recurrent parent's genome (RPG) recovery.

Marker assisted selection contributes immensely to the conventional breeding. The tremendous benefits of the combined approach, as pointed out in MIAH *et al*., (2015), are: competent foreground selection for the locus of interest, effective background selection for the recovery of recurrent parent's genome, reduced linkage drag adjacent to the introgressed locus, prompt breeding for the development of new genotypes with favorable traits.

As a result of a breeding program at MRI, one commercial maize inbred line was converted to its QPM counterpart for growing in temperate climate (KOSTADINOVIC *et al*., 2014; KOSTADINOVIC *et al*., 2016). The aim of this research was to examine the efficiency of molecular markers for direct selection of the target gene (foreground selection), as well as for fast recovery of recurrent parent's genome (background selection) in BC_2 generation of this conversion process.

MATERIAL AND METHODS

Plant material

The QPM version of one commercial MRI inbred line was used as the donor parent (DP) of the favourable allele of the *opaque2* gene. Four MRI commercial inbred lines, components of the leading MRI hybrids, were used as the recurrent parents (RP_1, RP_2, RP_3 and RP_4). The conversion process is given in Figure 1. F_1 plants were pollinated with reccurent parent line to generate BC_1 progeny. The BC_1 plants heterozygous for the gene specific phi057 and umc1066 locus were selected for backcrossing. A two-level selection procedure was carried out in BC_2 generation. First, the individuals were screened with *opaque2* specific molecular markers to identify the heterozygotes. Second, these selected heterozygotes were screened with SSR markers distributed throughout the maize genome. Genotypes with the highest recovery of recurrent parent's genome were selfed to produce BC_2F_2 seeds.

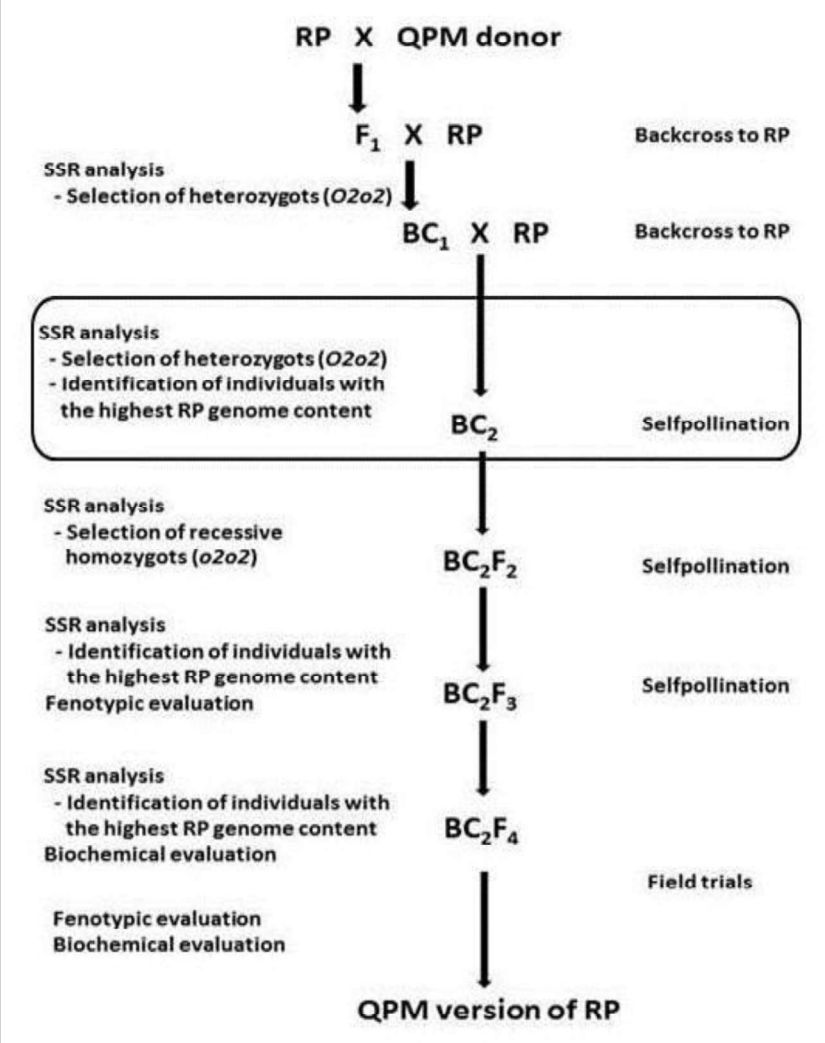

Figure 1. Scheme of MAS for conversion of standard maize inbred line to its QPM version.

DNA extraction

Genomic DNA was isolated from the kernel bulk according to DOYLE and DOYLE (1987) from the four-weeks-old plants. Bulks were prepared by pooling an equal amount of leaf tissue taken from 20 leaves per line. The concentration and the quality of the DNA was determined using biospectrometer BioSpectrometer kinetic (Eppendorf, Germany).

Foreground selection

Two gene specific SSR markers (phi057 and umc1066) were employed in foreground selection for the incorporation of *opaque2* into recepient maize lines (Table 1). Polymerase chain reaction was carried out in 20 μL reaction volume containing: 1×DreamTaq Green Buffer (Thermo Scientific, USA), 200 μM dNTP (Thermo Scientific, USA), 0.25 μM primers, 1U DreamTaq DNA Polymerase (Thermo Scientific, USA) and 20 ng DNA template. Amplifications were performed in thermocycler

Biometra TProfessional Standard 96 (Biometra, Germany) with the following program: an initial denaturation at 94ºC/2min, followed by 40 cycles each of denaturation at 94ºC/1min, annealing at 60ºC/2min and extension at 72ºC/2min with final elongation at 72° C for 10 min. The amplified fragments were resolved by 8% polyacrylamide gel electrophoresis on small format vertical gel system (Mini Protean Tetra-Cell, BioRad, USA). After staining with ethidium bromide, they were visualized under UV transilluminator and documented in gel documentation system BioDocAnalyze (Biometra, Germany). The size of the amplification products was determined comparing to the 50 bp molecular weight ladder (Thermo Scientific, USA).

Table 1. The set of SSR markers used in foreground selection for the opaque2 gene

Primer	Sequence
phi057 F	5'-CTCATCAGTGCCGTCGTCCAT-3'
phi057 R	5'-CAGTCGCAAGAAACCGTTGCC-3'
umc1066 F	5'-ATGGAGCACGTCATCTCAATGG-3'
umc1066 R	5'-AGCAGCAGCAACGTCTATGACACT-3'

Background selection

For the background selection, SSR analysis was done with 30 polymorphic markers spanning over the whole genome, selected from the maize database (www.maizegdb.org). Polymerase chain reaction (PCR) was carried out in 25 µL reaction volume containing: 1×DreamTaq Green Buffer (Thermo Scientific, USA), 200 µM dNTP (Thermo Scientific, USA), 0.5 µM of each primer, 1U DreamTaq DNA Polymerase (Thermo Scientific, USA) and 20 ng DNA template. The following touch-down program in the thermocycler Biometra TProfessional Standard 96 (Biometra, Germany) was performed: an initial denaturation at 95ºC/5min, followed by 15 cycles each of denaturation at 95ºC/30 s, annealing at 63.5ºC/1min (-0.5ºC/cycle) and extension at 72ºC/1min; another 22 cycles of 95ºC/30 s, 56ºC/1min and 72ºC/1min with final elongation at 72° C for four min. The PCR products were separated by electrophoresis on 8% polyacrylamide gel, with 20 bp molecular weight ladder (Thermo Scientific, USA) as a marker. After staining with ethidium bromide, they were photographed under UV light using Biometra BioDocAnalyze gel documentation system (Biometra, Germany). SSR profiles were converted into a binary matrix based on the presence (1) or the absence (0) of a specific allele. Genetic similarity (GS) was calculated in accordance with DICE (1945): GSij = 2a/2a+b+c; where: a is the number of fragments present in both variety i and j (1,1), b is the number of fragments present in i and absent in j (1,0), c is the number of fragments absent in i and present in j (0,1). Marker data analyses were performed using statistical NTSYSpc2 program package (ROHLF, 2000).

RESULTS AND DISCUSSION

The first level of selection procedure in BC_2 generation was identification of heterozygous plants with *opaque2* specific molecular markers phi057 and umc1066. Out of 192 plants, 100 (52%) were identified as heterozygous, which is in accordance with the expected Mendelian ratio of 1 *O2O2*: 1 *O2o2* in backcross generations. Out of these 100 heterozygous individuals, 30 originated from RP_1, 24 from RP_2, 20 from RP_3 and 26 from RP_4. Figure 2 shows the co-dominant nature of marker umc1066 that can distinguish homozygous and heterozygous genotypes. Lanes 3, 5, 8, 10, 11, 12 and 13 were heterozygous (*O2o2*) and lanes 4, 6, 7 and 9 were dominant homozygous individuals (*O2O2*).

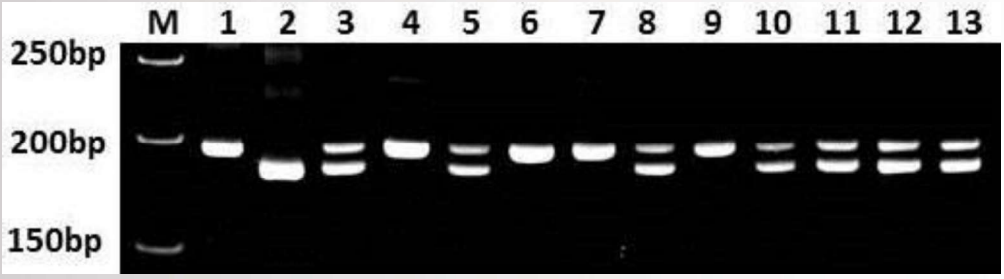

Figure 2. SSR profile of BC_2 individual plants detected with opaque2 specific marker umc1066. M: 50 bp DNA ladder, 1: standard line (recurrent parent), 2: QPM line (donor parent), 3-13: BC_2 individuals.

The second level of selection procedure in BC_2 generation was background selection performed on previously identified heterozygous plants (*O2o2*). Genetic variability between these individuals and their recurrent parents was analyzed with SSR markers distributed over the maize genom. As stated in MIAH *et al*., (2015), monomorphic markers bear no value in selection work since this type of marker is not able to distinguish the two parental genotypes. Markers that showed polymorphism were used in backcross generation. Total number of alleles detected with 30 informative markers was 39 for RP_1, 48 for RP_2, 68 for RP_3 and 61 for RP_4, average being 1.3, 1.6, 2.27 and 2.03, respectively. These values are somewhat lower than those previously reported in maize inbreds (BANTE and PRASANNA, 2003; LEGESSE *et al*., 2007; KOSTADINOVIC *et al*., 2018). The higher number of alleles per locus in other studies can be explained by the fact that analyses were performed on a larger number of different maize genotypes or by the use of a larger number of SSR markers in the analysis (MEHTA *et al*., 2017).

Genetic similarity values between the recurrent parents and their corresponding BC_2 progenies, calculated using Dice coefficient, ranged from 0.77 to 0.99 (77-99% RPG). The highest proportion of RPG was found in RP_1 (93-99%) and the lowest in RP_3 progenies (77-89%). Average values for the RPG content ranged from 83.9 to 95.8%. Similar percentages of parental genome renewal in the BC_2 generation have been reported in other studies (BABU *et al*., 2005; GUPTA *et al*., 2013; SINGH and RAM, 2014; THAKUR *et al*., 2014).

There was a great acceleration of recipient genome recovery in the present study. Theoretically, the proportion of the RPG after *n* generations of backcrossing is given by $(2^{n+1} - 1)/2^{n+1}$ (COLLARD *et al*., 2005). In our case, 66% of progenies had RPG above this theoretical value. Also, the RPG value of 99% was achieved in a few individuals, what is the value theoretically achieved in BC_6 generation. Fast recovery of RPG was attained probably due to the genetic similarity between donor and recipient lines, as well as the absence of linkage between the target gene and nearby genes from the donor parent and/or random genetic recombination.

Our study showed that molecular markers are efficient in reducing the time and resources involved in selection process. Selected heterozygous individuals with the highest RPG values were self-pollinated to produce BC_2F_2 plants. DNA samples from BC_2F_2 progenies will be collected and subjected to the foreground selection before flowering to identify the progenies that attained homozygosity at *o2* locus. Finally, those *o2o2* genotypes will be screened for biochemical and phenotypic traits to verify their nutritional and agronomical superiority.

CONCLUSIONS

Co-dominant nature of the polymorphism exibited by the phi057 and umc1066 primers enables their utility in MAS program to successfully discriminate between homozygotes and heterozygotes. Using polymorphic SSR markers in background selection, heterozygotes with the highest percentage of recurrent parent's genome were successfully identified. Our study confirmed the efficiency of molecular markers in determination of the success rate of genomic regions transfer (foreground selection), as well as the recovery rate of the recurrent parent’s genome (background selection).

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