UDC 575. 633.15
https://doi.org/10.2298/GENSR220947K
Original scientific article

MARKER ASSISTED CONVERSION OF MAIZE INBRED LINES TO QUALITY PROTEIN MAIZE (QPM) ADAPTED TO TEMPERATE CLIMATE

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Kostadinović M., D. Ristić, S. Božinović, O. Đorđević Melnik, D. Ignjatović-Micić, J. Vančetović (2022*). Marker assisted conversion of maize inbred lines to quality protein maize (QPM) adapted to temperate climate*. - Genetika, Vol 54, No.2, 947-957.

Maize is a poor source of protein both for humans and monogastric animals due to the lack of essential amino acids, such as lysine and tryptophan. Naturally occurring *opaque2* (*o2*) mutation increases content of these amino acids, but also confers an undesirable phenotype leading to low yields. Quality protein maize (QPM) is agronomically acceptable and nutritionally improved *opaque2* maize obtained through conventional breeding. Marker assisted breeding program aimed at developing QPM genotypes for growing in temperate regions is being conducted at Maize Research Institute Zemun Polje (MRIZP). The results presented in this paper relate to foreground selection aimed to identify plants that attained homozygosity at *o2* locus in BC_2F_2 generation in conversion of four MRIZP commercial inbred lines. Maize inbred line ZPL5 converted to its QPM counterpart and adapted to temperate climate was used as *o2* donor to the four recurrent parents (RP). Foreground selection was carried out with gene-specific markers phi057 and umc1066, both segregating as per the expectation. The percentage of recessive homozygotes in BC_2F_2 generation was approximately 25% (24.6% in RP_1, 23.3% in RP_2, 25% in RP_3 and 24.4% in RP_4). After the self-pollination of selected recessive homozygotes, BC_2F_3 progenies were screened for phenotypic and biochemical characteristics to confirm their nutritional and agronomical superiority. The results of scoring endosperm modifications revealed over 95% of hard endosperm kernels. The average tryptophan content ranged from 0.070% in RP_1 to 0.087% in RP_3. Out of 39 derivations from four lines, 19 had tryptophan content above the QPM threshold (0.075%). A total of 16 derivations were chosen for their highest tryptophan content. Their quality index was increased by 2-46% relative to the recurrent parent. These lines

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will serve as an important breeding material for developing QPM maize hybrids adapted to temperate regions.

Key words: maize, marker assisted selection, *opaque2*, quality protein maize, SSR

INTRODUCTION

Maize is one of the most important cereal crops across the world. In addition to being staple food for human consumption, maize is also important for livestock and poultry industry (TANUMIHARDJO *et al*., 2019). However, maize is naturally lacking in several essential amino acids such as lysine, tryptophan and methionine (KRIVANEK *et al*., 2007). Out of several mutations responsible for increase of essential amino acids (*opaque2* - *o2*, *floury2* - *fl2*, *opaque7* - *o7*, *opaque6* - *o6* and *floury3* - *fl3*) discovered in the 1960s (MA and NELSON, 1975; MCWHIRTER, 1971; MERTZ *et al.,* 1964; NELSON *et al*., 1965), recessive mutation *o2* has been the most widely studied and used as a source for genetic improvement of the nutritional value of maize proteins. However, the *o2* mutation also confers an undesirable phenotype leading to low yields, soft and chalky kernels that renders seeds susceptible to storage pests and ear rots.

Development of quality protein maize (QPM), an ideotype of maize having high yield potential, enriched with essential amino acids and without negative pleiotropic effects on the agronomic traits, is comprised of a series of efforts across many decades (MAQBOOL *et al.,* 2021). The QPM is reported to have increased levels of lysine and tryptophan in the endosperm protein, which enhances the biological value of protein close to the milk protein (MOTUKURI, 2019). Only 37% of protein intake from non-QPM is utilized compared to 74% of the same amount of QPM (BIDI *et al*., 2019). Results of a meta-analysis by GUNARATNA *et al.* (2010) indicated that consumption of QPM leads to a 12% increase in the rate of growth in weight and a 9% increase in the rate of growth in height in infants and young children with mild to moderate undernutrition from populations in which maize is a significant part of the diet. On the other hand, many studies showed that QPM had a positive overall impact on the weight gain of both poultry and pigs (MBUYA *et al*., 2011; PANDA *et al.,* 2010; SOFI *et al.,* 2009).

Although conventional breeding has been successfully used to create QPM, marker assisted selection (MAS) gained considerable importance as it increases efficiency, reduces time and costs taken to obtain QPM (BABU *et al.,* 2004). Marker assisted selection can aid selection of all target alleles that are difficult to assay phenotypically, in less time and with minimum linkage drag (PITAMBARA and SINGH, 2017). BENCHIMOL *et al.* (2005) demonstrated that microsatellites could be used efficiently for introgressing a target allele, simulating a monogenic trait, without any intermediate field selection. The idea of using a marker was to control all steps for introgression of a target locus from a donor to a recipient line. Three simple sequence repeats (SSR) markers are located within the *opaque2* gene, i.e., there is a very high correlation between marker data and phenotypic expression (VIVEK *et al.,* 2008).

Several successful examples of MAS in QPM breeding were published, but mostly for growing in tropical and sub-tropical regions (BABU *et al.,* 2005; GUPTA *et al*., 2013; PRASANNA *et al.,* 2010). In MRIZP, breeding program on QPM is aimed at development of QPM genotypes for growing in temperate regions (DENIC *et al.,* 2012; IGNJATOVIC-MICIC *et al.,* 2009, 2010, 2013). After the first successful marker assisted conversion of one commercial maize inbred line to its QPM counterpart for growing in temperate climate (KOSTADINOVIC *et al.,* 2014, 2016), this

program was continued with a larger number of maize inbred lines. The results presented in this paper relate to: 1) foreground selection in BC_2F_2 generation in conversion of four MRIZP commercial inbred lines aimed to identify plants that attained homozygosity at *o2* locus and 2) phenotypic and biochemical evaluation of BC_2F_3 progenies to confirm their nutritional and agronomical superiority.

MATERIAL AND METHODS

Plant material

One commercial MRIZP inbred line ZPL5 was converted to its QPM counterpart as presented in KOSTADINOVIC *et al.* (2016). Backcross progenies of ZPL5QPM with the highest genetic distance with original line during its conversion were used as *o2* donors for four commercial inbred lines, components of the leading MRIZP hybrids that were used as the recurrent parents. These lines are of the opposite heterotic pattern to ZPL5. In conversion program, F_1, BC_1F_1, BC_2F_1, BC_2F_2 and BC_2F_3 generation were developed. BC_1 plants heterozygous for the gene-specific phi057 and umc1066 loci were selected for backcrossing. A two-level selection procedure was carried out in BC_2 generation (KOSTADINOVIC *et al.*, 2020) and heterozygotes with the highest recovery of recurrent parent's genome were selfed to produce BC_2F_2 generation. These plants were subjected to foreground selection to identify homozygous recessive individuals. The conversion process is given in Figure 1.

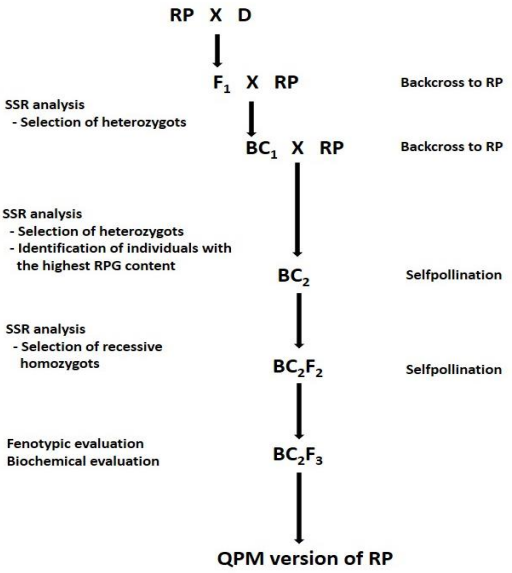

Figure 1. Schematic presentation of MAS for conversion of a standard maize inbred line (recurrent parent-RP) to its QPM version employing a QPM donor line (D).

Molecular analyses

Genomic DNA was isolated according to DOROKHOV and KLOCKE (1997) from four-weeks-old plants. DNA concentration was quantified using biospectrometer (BioSpetrometer kinetic, Eppendorf) and diluted to a working concentration of 20 ng/μL. Simple sequence repeat analysis was done with two *opaque2*-specific primers (phi057 and umc1066). Polymerase chain reaction was carried out in 20 μl reaction volume containing: DreamTaq™ Green PCR Master Mix (2X), 0.25 μM primers and 20 ng of DNA. Amplifications were performed in thermocycler Biometra TProfessional Standard 96 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 extension at 72ºC/10min. The amplified fragments were resolved by electrophoresis on 8% polyacrylamide gel with 50bp ladder as a marker. After staining with ethidium bromide, gels were visualized under UV transilluminator and documented using gel documentation system (BioDocAnalyze, Biometra). The amplification products were determined based on the positions of the bands relative to the standard (dominant allele) and QPM (recessive allele) lines used as controls.

Endosperm modifications

Kernel endosperm modifications were visually assessed using light table, according to the scoring scale from 1 (completely translucent, with no opaqueness) to 5 (completely opaque), as presented in VIVEK *et al.* (2008). Kernels with scores 1, 2 and 3 (≤50% opaque) were selected for tryptophan content determination.

Biochemical analyses

Two sub-samples per genotype, consisting of 30 kernels each, were dried in a controlled oven at 65°C/16-18 hours, milled and flour was defatted by hexane treatment in Soxhlet extractor. Tryptophan content was determined by the colorimetric method given in NURIT *et al.* (2009). Shortly, the color was developed in the reaction of flour hydrolysate, obtained by overnight digestion with papain solution at 65°C, with a reagent containing glyoxylic acid and ferric chloride dissolved in sulfuric acid. After incubation at 65°C/30 min, absorbance was read at 560 nm. Tryptophan content was calculated using a standard calibration curve, developed with known amounts of tryptophan, ranging from zero to 30 μg/μl. Protein content was determined by the standard Kjeldahl method based on nitrogen determination as explained in VIVEK *et al.* (2008). The protein was estimated from the nitrogen value as: % protein = % nitrogen × 6.25 (conversion factor for maize). Quality index (QI), defined as tryptophan to protein ratio in the sample, was calculated as: QI = 100 × (tryptophan content in the sample / protein content in the sample).

RESULTS AND DISCUSSION

The use of molecular markers in QPM breeding programs shortens the selection process during development of improved genotypes, making it more efficient across environments (TANDZI *et al.,* 2017). Two co-dominant SSRs (phi057 and umc1066), located as internal repetitive sequences within the *opaque2* gene, were utilized as foreground selection markers in

MAS for developing QPM lines. As presented in KOSTADINOVIC *et al.* (2020), the efficiency of molecular markers was confirmed for identification of heterozygous plants in BC_1 and BC_2 generations, as well as in the recovery of the recurrent parent's genome (RPG) (background selection). Individuals with the highest RPG values were self-pollinated to produce BC_2F_2 plants.

Out of 366 BC_2F_2 plants, 89 (24.3%) were identified as homozygous recessive, which is in accordance with the expected Mendelian ratio of 1*O2O2* : 2*O2o2* : 1*o2o2* (25:50:25%). This also applies for each line individually, as the percentage of recessive homozygotes was approximately 25% (24.6% in RP_1, 23.3% in RP_2, 25% in RP_3 and 24.4% in RP_4). These results confirmed the expectations stated in KOSTADINOVIC *et al.* (2016) that the use of donors adapted to temperate climate and bearing high percentage of domestic germplasm will outbalance the impediments met in stated research. Small number of recessive homozygotes (7.6%) was emphasized as the major impediment in backcross breeding for QPM. As probable causes, the authors suggested the exotic origin of the donor germplasm, as well as the incompatibility between pollen and style. However, this was not the case when QPM version of commercial MRI inbred line adapted to temperate climate was used as the donor parent.

The co-dominant nature of marker umc1066 is presented in **Figure 2**. The homozygous dominant individuals (lanes 1, 3 and 12) were clearly distinguished from heterozygous (lanes 2, 4, 5, 6, 9 and 11) and homozygous recessive genotypes (lanes 7, 8 and 10). Homozygous recessive plants were self-pollinated to produce BC_2F_3 kernels for endosperm modifications score and tryptophan content analyses.

Figure 2. SSR profile of BC_2F_2 individual plants detected with *opaque2*-specific marker umc1066. M: 50 bp DNA ladder, P1: standard line (recurrent parent), P2: QPM line (donor parent), 1-12: BC_2F_2 individuals (7, 8 and 10 - recessive homozygotes).

Selection for hard endosperm modifications was rapidly incorporated into *opaque2* breeding, since the importance of vitreous endosperm in reducing the negative pleiotropic effects of the *o2* mutation was known since 1969 (PAEZ *et al.*, 1969). Visual phenotypic selection using the light table helps to separate *o2o2* genotypes with modified endosperm (TWUMASI-AFRIYIE *et al.*, 2016). Percentages of average endosperm modifications for each derivation of the four recurrent parents are given in Figure 3. The results of scoring kernel types revealed over 95% of good (types 1 and 2) and medium (type 3) endosperm modifications, which corresponds to

standard maize kernels (VIVEK *et al.*, 2008). Vitreous kernels (types 1 and 2) were around 50% in all lines (from 48.95% for RP_1 to 58.95% for RP_2). While percentage of poor (type 4) or no modifications (type 5) was generally small, RP_3 was devoid of soft endosperm kernels. Therefore, it could be assumed that sufficient degree of endosperm modifications was accomplished, i.e. undesirable characteristics such as kernel cracking and susceptibility to ear rots and pests are not to be expected. Based on these results, derivations with soft kernels, as well as the derivations with the insufficient number of kernels, were discarded out of further biochemical analyses.

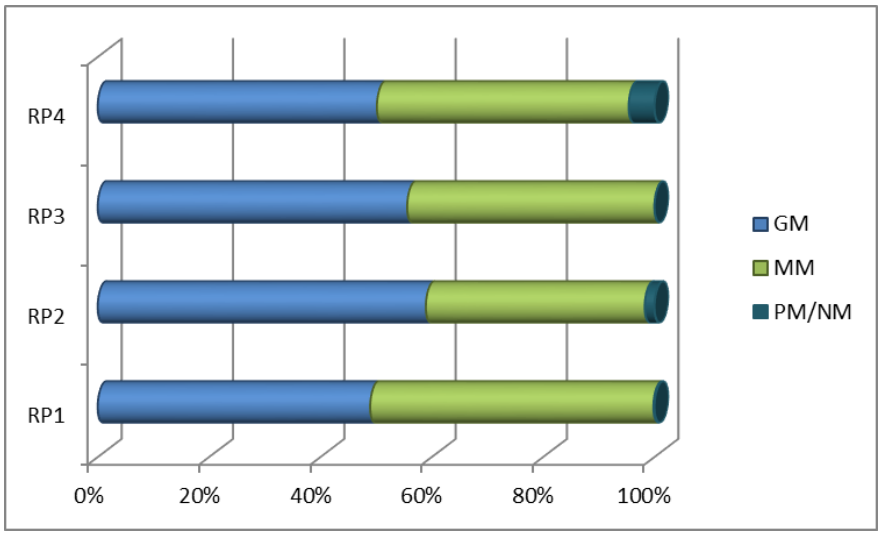

Figure 3. Percentage of good (GM) medium (MM) and poor/no endosperm modifications (PM/NM) in four recurrent parents (RP_1, RP_2, RP_3 and RP_4). GM: Kernel types 1 and 2; MM: kernel type 3; PM/NM: kernel types 4 and 5.

The expression of the QPM trait is confirmed through elevated kernel tryptophan content (TWUMASI-AFRIYIE *et al.*, 2016) and laboratory analyses are used to quantify and select for acceptable tryptophan concentrations - above 0.075% (VIVEK *et al.*, 2008). Out of 39 derivations from four lines, 19 had tryptophan content (TC) above the QPM (Figure 4). Overall, RP_3 derivations had the highest TC (0.084-0.093%), while the lowest TC was found in RP_1 (0.066-0.088%). High TC was also found in RP_4 derivations (0.072-0.088), with only one derivation below the QPM threshold. Similar results were found in BABU *et al*. (2005) where biochemical analysis showed TC in the range from 0.078 to 0.094%, as well as in JOMPUK *et al*. (2011) where TC ranged from 0.070 to 0.084%.

Three derivations with the highest TC from RP_1 and RP_2 each, as well as five from RP_3 and RP_4 each, were chosen for development of QPM hybrids. Unlike tryptophan content which

was above QPM threshold in all 16 chosen lines, quality index was above it in only six of them. However, increase in percentage of QI relative to the recurrent parent ranged from 2 to 46% (Figure 5). The highest increase of QI (23-46%) was achieved in four RP_4 derivations. Biochemical analysis in some other researches showed similar increase of tryptophan content and tryptophan concentration in protein. In two improved versions of parental lines obtained by GUPTA *et al.* (2013), TC increase over original parental lines was about 20% and less than 5%. Tryptophan concentration in protein, the major indicator of protein quality, was enhanced more than twice compared to original recipient lines in BABU *et al*. (2005), as well as in JOMPUK *et al*. (2011).

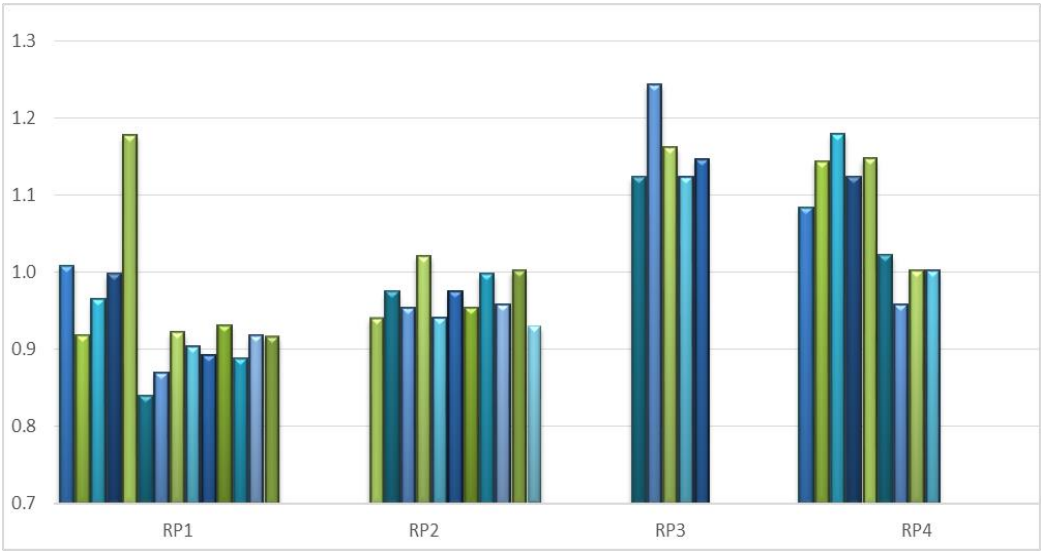

Figure 4. Tryptophan content of the four recurrent parents′ derivations given as % of the QPM threshold (given as 1).

As already stated, inbred lines chosen for conversion into their high protein quality counterparts are components of the leading MRIZP hybrids. These four improved lines can be considered the candidate parents for developing QPM hybrids adapted to temperate regions. QPM hybrids obtained in such a way will be further tested in the field for agronomic, as well as biochemical characteristics. Hybrids that express characteristics of a QPM genotype - good grain yield, hard endosperm, high tryptophan and quality index, as well as stability of tryptophan content in diverse environmental conditions, will be used in broiler feeds. Due to superior feeding value compared to the normal maize, QPM could substitute costly synthetic lysine in their diets, i.e. reduce the feed costs making animal products more affordable.

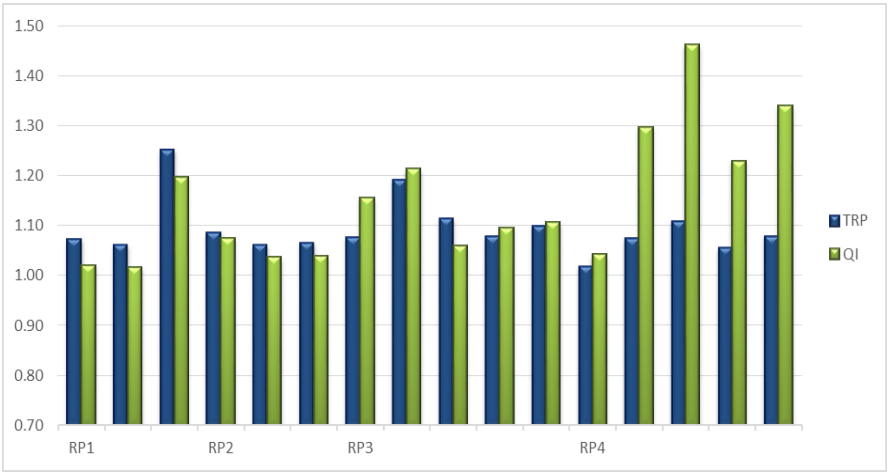

Figure 5. Tryptophan content (TRP) and quality index (QI) of the four recurrent parents′ derivations chosen for QPM hybrids development, given as % of the RP values (given as 1).

CONCLUSIONS

Co-dominant nature of the polymorphism exhibited by the phi057 and umc1066 markers enabled their utility in MAS program to successfully discriminate between homozygotes and heterozygotes. Self-pollinated recessive homozygous plants showed high percentage of kernels with hard endosperm, while half of them also had high tryptophan content. The ultimate goal is selection of the recessive genotypes with the highest proportion of recurrent parent′s genome along with high tryptophan content without losing good agronomic performances of the original line. These lines will serve as an important breeding material for developing QPM maize hybrids adapted to temperate regions.

ACKNOWLEDGEMENT

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia under Grant no. 451-03-68/2022-14/200040.

Received, November 14th, 2021
Accepted May 28th, 2022

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PREVOĐENJE INBRED LINIJA KUKURUZA U KUKURUZ VISOKOG KVALITETA PROTEINA ZA UMERENO KLIMATSKO PODRUČJE PRIMENOM MOLEKULARNIH MARKERA

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Izvod

Hranljiva vrednost kukuruza je niska zbog niskog sadržaja dve esencijalne aminokiseline - lizina i triptofana. Prirodna *opaque2* (*o2*) mutacija povećava sadržaj ovih aminokiselina, ali istovremeno smanjuje prinos čineći zrno kukuruza mekim i osetljivijim na štetočine. Kukuruz visokog kvaliteta proteina (*Quality protein maize - QPM*) predstavlja *opaque2* kukuruz poboljšanih agronomskih i nutritivnih karakteristika nastao konvencionalnim oplemenjivanjem. Rezultati ovog rada su deo projekta Instituta za kukuruz koji za cilj ima prevođenje linija standardnog kvaliteta zrna u QPM linije adaptirane na umerene klimatske uslove pomoću molekularnih markera. Kao *o2* donor korišćena je QPM verzija linije ZPL5, a kao rekurentni roditelji četiri komercijalne linje, roditeljske komponente vodećih ZP hibrida. Procenat recesivnih homozigota identifikovan *o2*-specifičnim markerima phi057 i umc1066 u BC_2F_2 generaciji bio je oko 25%, što je u skladu sa očekivanim odnosom prema pravilima Mendelovog nasleđivanja. Nakon njihove samooplodnje, uradjena je fenotipska i biohemijska potvrda kvaliteta. Preko 95% zrna imalo je tvrdi endosperm, a prosečne vrednosti sadržaja triptofana bile su od 0,070-0,087%. Kod 16 odabranih linija indeks kvaliteta (procenat triptofana u proteinu) bio je uvećan za 2-46% u odnosu na roditeljsku liniju. Ove linije će se koristiti za stvaranje QPM hibrida adaptiranih na uslove umerenog klimatskog područja.

Primljeno 14.XI.2021.
Odobreno 28. V. 2022.