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Original scientific paper

GENETIC AND BIOCHEMICAL CHARACTERISATION OF PARENTAL INBRED LINES IN MARKER ASSISTED SELECTION FOR QUALITY PROTEIN MAIZE

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Maize protein is deficient in two essential amino acids, lysine and tryptophan. Naturally occurring *opaque2* (*o2*) mutation which increases the levels of tryptophan and lysine in the grain also confers an undesirable phenotype leading to low yields and more susceptible, soft and chalky kernels. Quality Protein Maize (QPM) is agronomically acceptable and nutritionally improved *opaque2* maize. Marker assisted selection increases reliability and efficiency, reduces time and costs taken to obtain QPM. The results presented in this paper are a part of the Maize Research Insitute project on conversion of normal maize lines to QPM lines adapted for growing in temperate regions trough marker assisted backcross (MAB). Genetic and biochemical variability was analyzed between normal (ZPL 5) and QPM (CML 144) parental inbred lines and the efficiency of the three *opaque2* specific SSR markers was examined. Markers phi057 and umc1066 can discriminate homozygous and heterozygous backcross progeny, thus can be used as foreground selection markers for the *opaque2* gene. The genetic similarity between analyzed lines was 0.05, which confirmed good selection of parental lines for the creation of hybrids. Tryptophan content was 0.093 and QI was 0.85 in CML 144, approximately twice as high compared to 0.054 and 0.48 in ZPL 5. Both tryptophan and QI values for CML 144 were above the QPM treshold, i.e. tryptophan for 24% and QI for 0.625%, making it a suitable donor line of *o2*. These results represent the framework for marker assisted introgression of the quality protein trait into local maize genotype.

Key words: maize, marker assisted selection, *opaque2*, Quality Protein Maize, SSR

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INTRODUCTION

Maize (*Zea mays* L.) is one of the three most important crops in human and animal nutrition. However, maize protein (as most cereal protein) is deficient in two essential amino acids, lysine and tryptophan. Therefore, maize is a poor source of protein for both humans and monogastric animals. The enhanced nutritional quality was discovered in the *opaque2* maize (EGGUM *et al*., 1983, 1985, MERTZ *et al*., 1964). The *opaque2* (*o2*) 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, created the new, agronomically acceptable and nutritionally improved *opaque2* types by the name of Quality Protein Maize or QPM (VIVEK *et al*., 2008). They 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.

QPM is primarely developed for tropical and sub-tropical regions, but it could also have many advantages in human nutrition and animal feed in other parts of the world. Numerous studies reported the nutritional and biological superiority of QPM. AKUAMOA-BOATENG (2002) found that children in Ghana fed with high lysine/tryptophan maize were healthier, had reduced stunting and better growth capabilities compared with children fed with normal maize porridge. Significantly higher nitrogen retention was found with people eating QPM than those who ate normal maize, indicating that QPM protein is more „bioavailable“ (BRESSANI, 1991). QPM can significantly improve 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. The impact of QPM on the increase in body growth of poultry and pigs was proved in a number of studies (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, reducing its cost (SCOTT *et al*., 2009).

The use of molecular markers in conventional breeding has improved the accuracy of crosses and allowed breeders to produce strains with combined traits that were impossible before the advent of DNA technology (STUBER *et al*., 1999). For the reason of cost and simplicity, single sequence repeat (SSR) markers are widely applied in maize breeding. SSRs are short nucleotide sequences, usually from two to six bases in length that are repeated in tandem arrays. Amplifiable polymorphisms are revealed because of the diferences in the number of tandem repeats, which lie between sequences that are otherwise conserved for each locus. They are based on naturally occurring polymorphisms in DNA sequences, such as base pair deletions, substitutions or additions (GUPTA *et al*., 1999).

Three simple sequence repeats (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). In any particular cross, it is critical that the markers be run first on the two parents, to confirm the polymorphisms (size differences) for the marker alleles. Occasionally the two parents may share the same marker alleles and, in such cases, one of the two remaining markers must be tried (VIVEK *et al*., 2008).

Marker assisted selection (MAS) increases reliability and efficiency, reduces time and costs taken to obtain QPM (BABU *et al*., 2004). 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. GUPTA *et al*. (2013) used MAS for development of QPM parental lines of Vivek-9 hybrid and could develope the 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.

Research on *opaque2* mutation and high tryptophan was initiated in the 1970-ies in Maize Research Institute Zemun Polje - MRI (DENIC *et al*., 1979) and today's breeding program is aimed at conversion of standard maize inbred lines to QPM genotypes for growing in temperate regions (DENIC *et al.,* 2012; IGNJATOVIC-MICIC *et al*., 2008, 2009, 2010, 2013). The results presented in this paper are part of this program and they relate to: 1) genetic and biochemical variability analysis between one commercial normal maize inbred line and one QPM inbred line in order to be used as parents in marker assisted selection for quality protein maize and 2) efficiency of the three *opaque2* specific SSR markers and the choice of appropriate markers for marker assisted introgression of the *opaque2* gene into the commercial normal inbred line.

MATERIALS AND METHODS

Plant material

MRI commercial inbred line ZPL 5 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. ZPL 5 has good agronomic properties, excellent combining abilities and it is the component of the leading MRI hybrids. CML 144, a QPM inbred line obtained from the CIMMYT, was selected as the donor line of *o2*.

Molecular analysis

DNA isolation

Genomic DNA was isolated 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. DNA was quantified using spectrophotometer (UV-1601, Shimadzu) and a working stock of 20 ng/µl was prepared to perform PCR.

Parental polymorphism analysis using opaque2 specific SSR marker

Simple sequence repeat analysis was done with three primers specific for *opaque2* gene (phi057, umc1066 and phi112). 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 in 1 x TBE buffer, with 100bp ladder as a marker. Gels were run on small

format (7.3 x 10 cm) 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). Allele designations were made and approximate size range for the amplification products for each SSR locus was determined based on the positions of the bands relative to the 100 bp molecular weight ladder.

Genetic similarity analysis using SSR markers

Simple sequence repeat analysis was done with 40 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 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 were performed. Final elongation was at 72°C for 4 min. The amplified fragments were resolved by electrophoresis on 8% polyacrylamide gel in 1 x TBE buffer, with 100bp ladder as a marker. Gels were run on small format (7.3 x 10 cm) 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). Marker data analyses were performed using statistical NTSYSpc2 program package (ROHLF, 2000).

Biochemical analysis

Sample preparation

Each genotype was represented by 60 randomly chosen kernels, divided into two sub-samples consisting of 30 kernels each. Kernels were dried in a controlled oven at 65°C over night (16-18 hours), and milled in a Cyclone sample mill - Simmons Fastener, USA. The flour was defatted by hexane treatment for 4 hours in Soxhlet extractor.

Tryptophan content

Tryptophan content was determined using the colorimetric method of NURIT *et al.* (2009). 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.

Total protein content and Quality index

The protein content was determined by the standard Kjedahl method based on nitrogen determination as explained in VIVEK *et al* (2008). The protein was estimated from the nitrogen value as: % protein = % nitrogen x 6.25 (conversion factor for maize).

Quality index (QI), defined as tryptophan to protein ratio in the sample, was calculated as: QI = 100 x (tryptophan content in the sample / protein content in the sample).

RESULTS

An illustration of the amplification with all three *opaque2* specific SSR markers is given in Figure 1. The markers phi057 and umc1066 exhibited co-dominant polymorphism between the normal and QPM inbred lines. The phi057 amplified ~170 bp fragment in CML 144 and ~160 bp fragment in ZPL 5. The umc1066 amplified ~150 bp fragment in CML 144 and ~160–170 bp fragment in ZPL 5. The SSR marker phi112 exhibited dominant polymorphism between inbreds. The null allele was present in CML 144 and ~170 bp DNA fragment in ZPL 5.

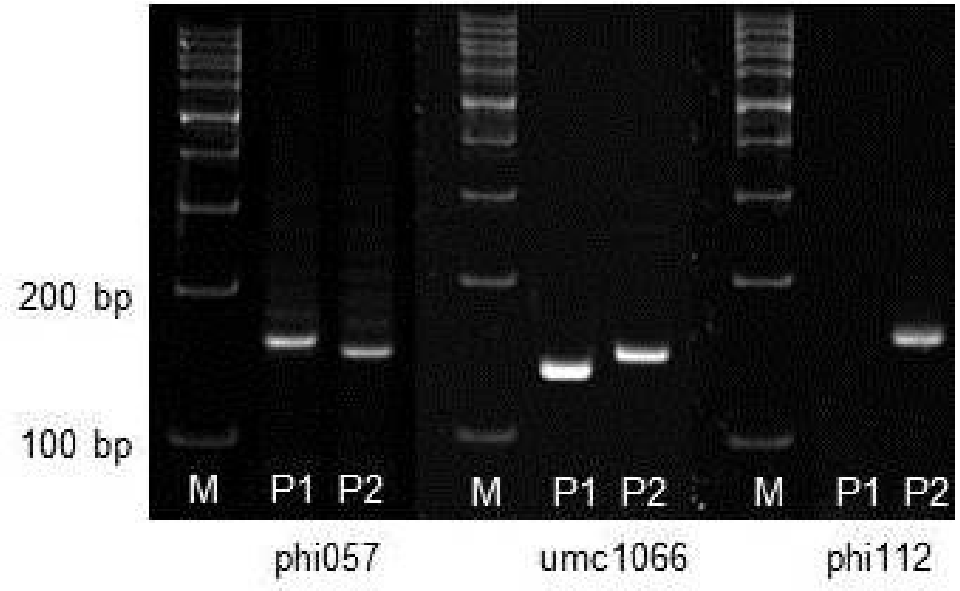

Figure 1. SSR profile of *opaque2* markers phi057, umc1066 and phi112. M: 100bp DNA ladder, P1: CML 144, P2: ZPL 5.

Out of the 40 SSR markers used in the screening, 24 markers showed polymorphism between ZPL 5 and CML 144 (Table 1). There was wide variation in the number and size of alleles amplified. Total number of alleles detected with informative SSR markers was 72 and the average value was 3. Genetic similarity value between the ZPL 5 and CML 144 calculated on Dice coefficient was 0.05.

Table 1. List of SSR markers (name, bin, repeat and number of alleles) used in genetic similarity (parental polymorphism) analysis of ZPL 5 and CML 144

Name	Bin	Repeat	Number of alleles	Name	Bin	Repeat	Number of alleles
umc1282	1.01	(AT)6	2	bnlg1443	6.05	(AG)25	4
umc1013	1.08	(GA)9	2	umc1695	7.00	(CA)8	4
bnlg1643	1.08	(AG)24	4	umc1393	7.02	(GTC)4	3
umc2047	1.09	(GACT)4	2	umc1944	7.04	-	4
bnlg198	2.08	-	4	phi116	7.06	ACTG/ACG	2
bnlg1520	2.09	(AG)22	4	umc1858	8.04	(TA)8	2
phi036	3.04	AG	2	phi080	8.08	AGGAG	2
bnlg1350	3.08	(AG)13	3	umc1492	9.04	(GCT)4	2
umc1594	3.09	(TA)10	2	umc1827	10.04	(GAC)6	4
umc2039	4.03	(CAG)5	4	bnlg1526	10.04	(AG)15	4
bnlg557	5.03	-	2	umc1506	10.05	(AACA)4	2
umc1274	5.03	(TGC)5	4				
umc1006	6.02	(GA)19	4	total			72
				mean			3

- data not available

Protein and tryptophan contents and quality index of parental lines together with QPM treshold values for tryptophan and QI are presented in Table 2. CML 144 had approximately twice the amount of tryptophan compared to ZPL 5. QI was also higher for CML 144. Protein content was similar for both lines. Difference (%) in biochemical components between the standard and QPM inbred line is given in Figure 2. Corresponding treshold values for QPM are given as 1 (100%). Compared to the treshold values, ZPL 5 had lower tryptophan content for 28% and QI for 40%. Both tryptophan and QI values for CML 144 were above the treshold, tryptophan was higher for 24% and QI for 0.625%.

Table 2. Protein and tryptophan contents (%) and quality index (QI) of parental lines and QPM treshold values for tryptophan and QI

	Protein	Tryptophan	QI
ZPL 5	11.07	0.054	0.48
CML144	10.95	0.093	0.85
QPM treshold		>0.075	>0.8

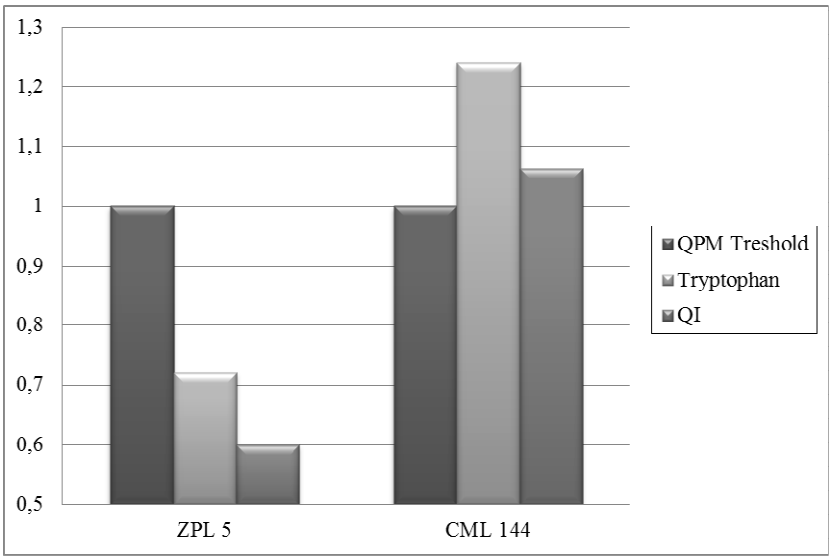

Figure 2. Tryptophan content and quality index (QI) of the ZPL 5 an CML 144, given as % of the treshold set for QPM. Corresponding treshold values are given as 1 (100%).

DISCUSSION

The results presented in this paper are a part of a MRI project on conversion of normal maize lines to QPM lines adapted for growing in temperate regions trough marker assisted backcross breeding. The main goal is to achieve high levels of essential amino acids and high grain yield at the same time. The donor line was one of the CIMMYT Maize Lines (CMLs). CMLs are carefully selected inbred lines with good general combining ability and a significant number of value-adding traits such as drought tolerance, enhanced levels of the essential amino acids lysine and tryptophan, N use efficiency, acid soil tolerance, resistance to diseases, insects and parasitic weeds. In many instances, they are parental lines of hybrids which have proven successful in one or several maize mega-environments. CML144, one of the CIMMYT's most successful CMLs, is a white flint, QPM tropical line with excellent combining ability (http://www.cimmyt.org/en/order-seed/maize-or-related-species-purpose/maize-germplasm-bank-menu). ZPL 5, one of the commercial MRI inbred lines 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. ZPL 5 is a orange, dent-like line adapted to the local environmental conditions. Due to its excellent combining abilities, it is the component of the leading MRI hybrids.

Recipient parent and donor lines were clearly distinguishable with three *opaque2* specific SSR markers. Similar to BABU *et al.* (2005) and JOMPUK *et al.* (2011), the phi057 marker detected amplified products of 160 bp in non QPM and 170 bp fragments in QPM maize. The

umc1066 marker amplified 150 bp fragment in QPM and 160–170 bp fragment in normal inbred line. In the work of DANSON *et al* (2006) primer umc1066 was monomorphic between QPM and non-QPM lines, giving a band of 134bp. However, BABU *et al.* (2005) got a 150bp band for QPM and 160 – 170bp band for non-QPM lines. Being the co-dominant markers, phi057 and umc1066 can detect homozygous dominant (*O2O2*), heterozygous (*O2o2*) and homozygous recessive (*o2o2*) plants separately. Therefore, they will be used for marker assisted selection for the *opaque2* gene. Marker analysis will be done using DNA samples extracted from leaf tissue of very young plants. This enables that plants without *o2* allels be discarded prior to pollination, reducing the size of the breeding population and saving both tome and money.

Phi112 primer showed a null fragment with QPM line and a band size around 170bp with normal maize line. These results are in agreement with results given in DANSON *et al.* (2006) and BABU *et al*. (2005), where a null allele was present in all QPM lines and a band size of 136bp and 160bp, and 150bp respectively, for non-QPM lines analyzed. The phi112 exhibited dominant (presence-absence) polymorphism, thus it could not be used for discriminating homozygous and heterozygous backcross progeny. Therefore, its utility is limited to the initial parental genotype discrimination, but it could also be of use in checking the seed purity during routine field maintenance of QPM inbred lines (BABU *et al,* 2005).

The use of DNA markers makes the process of selecting parental lines more efficient. Assessment of genetic similarity/distance based on DNA markers can be useful for predicting the yields of crosses between lines. This information can be extremely helpful for identifying the most appropriate parental lines to be crossed (RIBAUT and HOISINGTON, 1998). Field trials for the identification of promising heterotic patterns can be planned more efficiently based on the prior information obtained by markers, and that would make a great contribution to the efficiency of maize breeding (DRINIC MLADENOVIC *et al*., 2012). In order to estimate genetic similarity between parental lines, 40 SSR markers were used. The average number of alleles for the 24 informative markers was 3, which is similar to those previously reported in maize inbreds. MAPHOSA *et al.* (2011) reported 4.15 alleles using 24 SSR loci, BANTE and PRASANNA (2003) reported 3.25 alleles using 36 SSR loci, LEGESSE *et al*. (2007) 3.85 with 27 SSR loci, while WARBURTON *et al*. (2002) observed 4.9 alleles from 88 SSR loci. Somewhat higher average number of alleles can be explained by the larger number of lines than analyzed in our study. Variability in the nature and number of SSR alleles can be explained by the factors such as number of SSR loci and repeat types or methodologies used for detection of polymorphic markers as well as mutations at specific loci (LIU *et al.*, 2003; LEGESSE *et al*., 2007). Analyzed lines revealed more than one band during amplifications, which may have resulted from the co-dominant nature of the SSR markers (BANTE and PRASANNA, 2003). Similar results have been reported in maize inbred lines (LEGESSE *et al*., 2007, LIU *et al*., 2003). As probable causes, these authors suggested residual heterozygosity, pollen or seed contamination, mutation at specific SSR loci, or amplification of similar sequences in different genomic regions due to duplication. Genetic similarity value between the ZPL 5 and CML 144 calculated on Dice coefficient was 0.05, which comfirmed good choice of parental lines for the creation of high grain yield hybrids.

Due to the well established relationship between lysine and tryptophan in the maize protein (4:1 ratio), tryptophan can be used as a single parameter for evaluating the nutritional quality of the protein. When interpreting the results of laboratory analysis for making selections, tryptophan content and quality index have to be above the acceptable limits (VIVEK *et al*, 2008). CML 144 had tryptophan content and QI higher than ZPL 5 and both above treshold set for

QPM. The results agreed with JOMPUK *et al*. (2006) 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.

CONCLUSIONS

All three *opaque2* specific SSR markers showed polymorphism between the normal and QPM parent but only phi057 and umc1066 can be used for discriminating homozygous and heterozygous backcross progeny, i.e. as foreground selection markers for the *opaque2* gene. The results of the genetic similarity obtained by polymorphism of SSR markers confirmed good selection of parental lines in the creation of hybrids. Biochemical analysis showed high protein quality of CML 144 making it suitable donor line of *o2*. The results of our analysis constitute the framework for marker assisted introgression of the *opaque2* (quality protein trait) into the local maize genotype.

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GENETIČKA I BIOHEMIJSKA KARAKTERIZACIJA RODITELJSKIH INBRED LINIJA U MARKER ASISTIRANOJ SELEKCIJI KUKURUZA VISOKOG SADRŽAJA PROTEINA

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Izvod

Proteini kukuruza su siromašni u sadržaju dve esencijalne aminokiseline – lizina i triptofana. Prirodna *opaque2* (*o2*) mutacija povećava sadržaj ovih aminokiselina, ali istovremeno smanjuje prinos i čini zrno kukuruza mekim, brašnjavim i osetljivijim. Kukuruz visokog kvaliteta proteina (QPM) predstavlja *opaque2* kukuruz poboljšanih agronomskih i nutritivnih karakteristika. Selekcija pomoću molekularnih markera (MAS) povećava pouzdanost i efikasnost, kao i uštedu vremena i novca potrebnih za stvaranje QPM. Rezultati ovog rada su deo projekta Instituta za kukuruz koji za cilj ima prevođenje standardnih u QPM linije adaptirane na umerene klimatske uslove pomoću molekularnih markera. Analizirana je genetička i biohemijska varijabilnost između standardne (ZPL 5) i QPM (CML 144) linije i ispitana je efikasnost tri SSR markera specifična za *opaque2* radi odabira odgovarajućeg markera za naš program. Phi057 i umc1066 mogu da razlikuju homozigotne i heterozigotne biljke i biće korišćeni kao markeri za selekciju. Genetička sličnost između linija je bila 0.05 što potvrđuje dobar izbor roditeljskih komponenti za stvaranje hibrida. Sadržaj triptofana je bio 0.093 i QI je bio 0.85 kod CML 144, što je duplo više u odnosu na 0.054 i 0.48 kod ZPL 5. I triptofan i QI su bili iznad praga za QPM, triptofan je bio viši za 24% i QI za 0.625%, čineći ga dobrim donorom *o2*. Ovi rezultati predstavljaju osnovu za program inkorporacije *o2* u lokalne genotipove kukuruza selekcijom pomoću molekularnih markera.

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