UDC 575.633
https://doi.org/10.2298/GENSR1701051I
Original scientific paper

DNA AND BIOCHEMICAL ANALYSIS OF A POTENTIAL *OPAQUE2* MAIZE POPULATION

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Ignjatovic-Micic D., A. Nikolic, A. Nikolic, M. Kostadinovic (2017): *DNA and biochemical analysis of a potential opaque2 maize population*.-Genetika, Vol 49, No.1, 51-61.

Maize has low nutritional value because it is poor in essential amino acids lysine and tryptophan, but different mutations have been identified that increase their content. Two high lysine/tryptophan populations from Maize Research Institute genebank (IP1 and IP2) were identified in a previous research. In both populations, analysis with umc1066 *opaque2* specific marker detected a recessive (*o2*), a dominant (*O2*) and an unknown allele (UA). However, IP2 lacked homozygous recessive *o2o2* genotypes. The aim of the present research was to determine by DNA and biochemical analysis if UA allele was a recessive allele and/or if high tryptophan content was due to the *o2* or some other mutation. Tree more opaque accessions with different mutations - IP3*o5*, IP4*o14* and IP5*floury* (no data on type of mutation) were used in biochemical analysis for comparison with IP1 and IP2. Kernels were divided into two samples – with hard and with soft kernels. The UA allele sequencing revealed that it was a dominant allele with four GCCAGA repeats. SSR analysis showed presence of *o2* in IP1 in both hard and soft kernels. Decrease in 22 kDa, 19 kDa and 27 kDa zeins in soft kernels was observed only in IP1 and IP2. Tryptophan content was high in soft kernels of IP1 (0.081) and IP2 (0.085), and in both hard and soft kernels of IP3*o5* (0.083 and 0.085, respectively). It can be concluded that IP1 is an *o2* mutant and that IP2 carries a high tryptophan mutation other than *o2*, *o5*, *o14* or *floury*.

Key words: maize, *opaque2*, sequencing, SSR, tryptophan, zeins

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INTRODUCTION

Maize kernel has low nutritional value because it is poor in essential amino acids lysine and tryptophan. Different mutations have been identified that increase the content of these amino acids, including the best characterized *opaque2* and *floury 2* (MERTZ *et al*., 1964; NELSON *et al*., 1965). However, these mutant genotypes have soft (opaque) kernels leading to lower yields and higher susceptibility to ear rots and stored grain pests. The recessive *opaque2* mutation (*o2*) was the most suitable one for genetic manipulations and a lot of effort was put in improving kernel hardness of high lysine *o2* genotypes. The result was development of Quality Protein Maize (QPM), which is high lysine/tryptophan maize (lysine and tryptophan are highly correlated and the value of lysine is three times that of tryptophan) with hard kernels and good agronomic performances (VIVEK *et al*., 2008).

Maize kernel hardness is determined by the relative amounts of hard (vitreous) and soft (opaque) endosperm. Positive correlation between zein storage proteins and kernel virtuousness was found (MOOSE *et al*., 2004). Zeins (prolamines) are a mixture of alcohol-soluble proteins and based on their structure can be divided into α, β, γ and δ zeins (ESEN, 1987). Furtheron, α zeins encompass 19 and 22 kDa proteins, γ zeins 16, 27 and 50 kDa proteins, while β and δ zeins refer to 15 and 10 kDa proteins, respectively. Zeins are deposited in protein bodies (PB) in endosperm cells (WOO *et al*., 2001; LARKINS and HURKMAN, 1978), with α and δ zeins being stored in the center of PBs and γ and β zeins deposited in the peripheral region (LENDING and LARKINS, 1989). The kernel vitreousness is influenced by PB composition and the spatial organization of α, β, γ and δ zeins (HOLDING and LARKINS, 2006). Because zeins are essentially devoid of lysine and tryptophan, their high-level accumulation results in poor grain-protein quality.

There are a number of mutants that have soft kernels that do not transmit light and are thus termed opaque. Several of these mutants have been shown to alter some aspects of zein synthesis and PB structure. The *opaque2* protein is a basic leucine (Leu)-zipper transcriptional activator, which positively regulates the expression of 22 kDa α zein genes as well as some other genes (SCHMIDT *et al*., 1990). The abundance of other zeins is also reduced in *o2*, especially 19 kDa α zein. The reduction of zeins is accompanied by an increase of non-zein proteins rich in lysine and tryptophan. Similar changes were found in *fl2* mutants, while in some other opaque mutants such as *o1* and *fl1* no changes in lysine content were detected (MORTON *et al*., 2015).

In a pre-breeding program aimed at identification of accessions from Maize Research Genebank (MRI) with beneficial grain quality properties that could be used for improving commercial germplasm, two introduced populations from Iran (IP1 and IP2) with high tryptophan content were identified (IGNJATOVIC-MICIC *et al*., 2014). SSR analysis with specific *o2* primer pair (umc1066) revealed presence of three alleles within the populations – dominant, recessive and an allele positioned between the dominant and the recessive alleles (designated as unknown allele – UA). Also, the absence of homozygous *o2* genotypes required for high tryptophan content and a small percent of heterozygous *O2/o2 genotypes* (8%) were found in IP2. It was assumed that the unknown allele could also be a recessive one or that IP2 could carry a mutation other than *o2*. The objective of the research presented herein was to determine if the UA was a recessive *opaque2* allele and to compare zein profiles obtained for these two populations and some other opaque mutants, in order to establish the nature of IP2 high tryptophan content.

MATERIALS AND METHODS

Plant material

Five accessions from MRI gene bank collection were used in this experiment. All of them were introduced populations (IP) - IP4347 (IP1), IP4353 (IP2), IP1315*o5* (IP3*o5*), IP560*o14* (IP4*o14*) and IP6612*fl* (IP5*fl*). IP1 and IP2 were found to be high tryptophan genotypes, but without distinct confirmation of the type of mutation. IP3*o5* is an *opaque5* genotype, IP4*o14* is an *opaque14* and IP5*fl* is a floury genotype (there is no data on what floury mutation is in question).

IP1 and IP2 were subjected to both biochemical and DNA analysis, while the other three populations were subjected only to biochemical analysis. Oh43 and Oh43*o2* inbred lines were used as standards for all analyses except for sequencing. For sequencing, CML144 QPM line and a commercial ZP 5 inbred line were used as checks.

Identification of kernel types

For each genotype 200 kernels were visually scored using light table (VIVEK *et al*., 2008). The scoring scale defined kernel types, from type 1 (completely translucent, with no opaqueness) to type 5 (completely opaque). Kernels with 25% opaqueness were scored as type 2, while types 3 and 4 were 50% and 75% opaque kernels, respectively.

For zein, tryptophan and SSR analysis two groups of samples were formed within each population. The first group consisted of hard (translucent) kernel types 1 and 2. The second one consisted of soft (opaque) kernel types 3, 4 and 5.

Zein analysis

Ten soft and ten hard kernels for each population (two samples per population) were pooled and milled using a coffee grinder and stored in 2 ml tubes. 200 mg of endosperm powder were mixed with 1 ml extraction buffer (70% ethanol, 2% β - mercaptoethanol) and incubated at 65°C for 15 minutes, vortexing for 15 seconds every 3-5 minutes. After centrifuging at 13 000 rpm for 10 min, supernatant was transferred into a new tube. To resolve the proteins on the basis of their size by SDS-PAGE, samples were prepared as follows: 10 µl of extract, 50 µl of 3X Sample buffer (0.2 M Tris-HCl, pH 6.8, 4.5% SDS, 12% β - mercaptoethanol, 30% glycerol, 0.06% Bromphenol Blue) and 90 µl of water. The samples were boiled for 3 minutes, briefly cooled at room temperature and 10 µl per sample were loaded on the polyacrylamide gels (5% stacking and 15% running gel) with PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermo Fisher Scientific). The gels were run at 200 V in Mini Protean Tetra-cell (BioRad) until the dye reached the bottom (about 1 hour), stained in staining solution (2 g Coomassie Brilliant Blue R 250, 500 ml ethanol, 70 ml glacial acetic acid, 430 ml water) over-night, followed by five washes of at least half an hour each in destaining solution (400 ml ethanol, 70 ml glacial acetic acid, 530 ml water) until the bands were clearly visible and no background was present. Gels were photographed with a digital camera (D-60, Nikon).

Tryptophan content analysis

Thirty soft and thirty hard kernels per population were used for tryptophan content determination. Kernels were dried in a thermostat at 65°C overnight (16-18 hours) and milled in a Cyclone sample mill - Simmons Fastener, USA. The flour was defatted by hexane treatment during four hours in Soxhlet extractor.

Tryptophan content was determined using the colorimetric method (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 glyoxylic acid and ferric chloride dissolved in sulfuric acid. After incubation at 65°C for 30 min, absorbance was read at 560 nm. Tryptophan content was calculated using a standard calibration curve, developed with the known amounts of tryptophan, ranging from 0 to 30 μg ml^{-1}.

DNA sequencing

Genomic DNA was extracted from maize leaf samples using CTAB method according to MURRAY and TOMPSON (1980) with few modifications. The sequences of dominant, recessive and the UA alleles were analysed by PCR followed by DNA sequencing. The PCR amplifications were carried out using umc1066 primer in the reaction mixtures containing (in total volume of 20μL): approximately 100ng of DNA, 1.25U of Taq DNA Polymerase (Thermo Scientific), 1xPCR buffer (Thermo Scientific), 3mM $MgCl_2$, 200 μM each dNTP and 0.25μM of each primer. The amplification was performed as follows: initial denaturation at 94°C for 2min, 35 cycles of 94°C for 1min, 62°C for 1min and 72°C for 2min, and final elongation at 72°C for 10min. The obtained products were purified using Thermo Scientific, GeneJET Gel Extraction Kit. Purified PCR products were sequenced using the ABI Prism BigDye Terminator Kit (Applied Biosystems) and primers used for PCR amplification on 3130 Genetic Analyzer (Applied Biosystems). Sequences were analyzed using the Sequencing Analysis software v5.2 (Applied Biosystems).

SSR analysis

PCR amplification was performed on the DNA isolated from bulked hard and bulked soft kernels per population, with primer specific for *opaque2* gene - umc1066. The amplification reaction was carried out in 20 μl reaction volumes containing 1x enzyme buffer, 3 mM $MgCl_2$, 200 μM dNTPs, 0.25 μM primers, 1.25 U *Taq* polymerase and 50 ng of DNA. The amplification profiles were: an initial denaturation at 94°C/2min, followed by 40 cycles each of denaturation at 94°C/1min, annealing at 60°C/2min and extension at 72°C/2min, with final extension at 72°C/10min. Amplified fragments were separated on 8% polyacrylamide gels. After electrophoresis gels were stained with ethidium bromide and photographed using BioDocAnalyze (Biometra) gel documentation system. The amplification products were determined based on the positions of the bands relative to the normal (dominant allele) and QPM line (recessive allele).

RESULTS AND DISCUSSION

Kernel opaqueness (softness) can indicate presence of increased lysine/tryptophan content, although there are some opaque mutants that show little quantitative or qualitative differences in zein accumulation and lysine/tryptophan content (MORTON *et al*., 2015). The analysed opaque genotypes had different percentage of vitreous (hard) and soft kernels. The percentage of kernel types within each population is presented in Fig 1. Vitreous kernels (type 1 and 2) were predominant in all populations (from 80 to 94%), except in IP5*fl* in which only completely opaque kernels (type 5) were found. While in IP1, IP3*o5* and IP4*o14* types 3, 4 and 5 were present in a small percentage (20%, 7% and 12%, respectively), IP2 was devoid of types 4 and 5. In this population only types 1, 2 and 3 were found (53%, 41% and 6%, respectively), what is in accordance with results from previous research (IGNJATOVIC-MICIC *et al*., 2014). Based on

these results, two samples (from translucent and opaque kernels) were formed for all populations except for IP5*fl*.

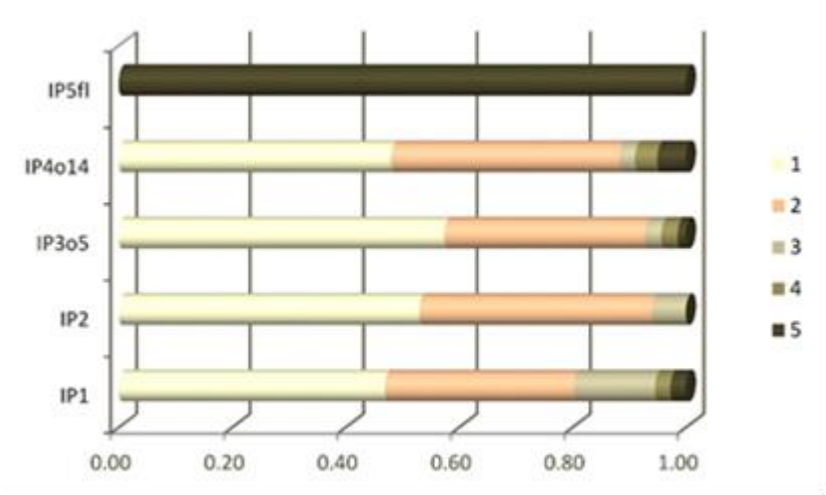

Fig. 1. Kernel type distribution within the analysed introduced populations. Legend: 1 – 100% translucent; 2 – 25% opaque; 3 – 50% opaque; 4 – 75% opaque; 5 – 100% opaque.

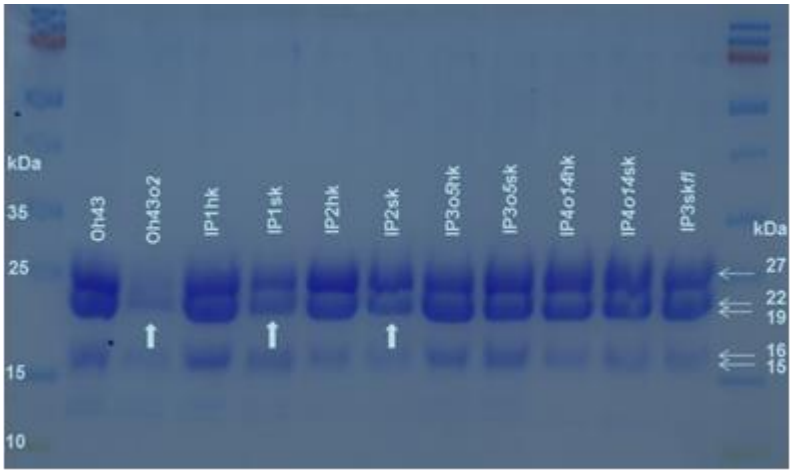

Fig 2. Zein profiles of the analysed hard (types 1 and 2) and soft (types 3, 4 and 5) kernels of the introduced populations (IP). First and last lanes: PageRuler™ Prestained Protein Ladder, 10 to 180 kDa. Abbreviations: hk – hard kernels, sk – soft kernels. Arrows under Oh43*o2*, IP1sk and IP2sk point to the differences in zein proteins of soft kernels in comparison with hard kernels. Arrows on the right identify positions of specific zein proteins.

Zein profiles for the analysed opaque genotypes are presented in Fig 2. *O2* gene encodes a bZIP transcription factor that regulates α zein genes and its recessive *o2* mutation has almost Zein profiles for the analysed opaque genotypes are presented in Fig 2. *O2* gene encodes a bZIP transcription factor that regulates α zein genes and its recessive *o2* mutation has almost two-fold increase in lysine and tryptophan content due to a decrease in zein synthesis and an increase in accumulation of non-zein proteins (COLEMAN and LARKINS, 1999). Significant reduction in all zein proteins can be noticed in *opaque2* mutant control inbred line Oh43*o2*. Similar changes are present in IP1sk and IP2sk compared to their hard kernel counterparts**,** but with more abundant presence of 22 kDa α zein and 27 kDa γ zeins. Tryptophan content (Fig 3) in IP1hk and IP1sk kernels was 0.068 and 0.081, respectively. IP2 showed somewhat higher tryptophan contents in both types of kernels. In hard kernel sample it was 0.072 and in opaque kernel type 0.085, although in this population only type 3 kernels were present.

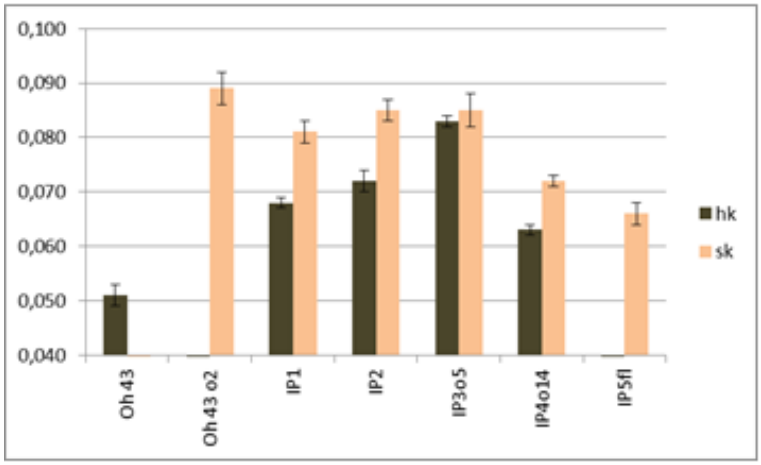

Fig. 3. Tryptophan content (%) of the analysed hard (types 1 and 2) and soft (types 3, 4 and 5) kernels of the introduced populations. Legend: hk - kernel types 1 and 2, sk – kernel types 3, 4 and 5.

No differences in zein profiles were detected between hard and soft kernel types within IP3*o5* and IP4*o14* populations. However, tryptophan content in IP3*o5* was high (0.083 and 0.085 in hard and soft kernels, respectively). It was shown that mutations of the *opaque5* locus condition opaque kernels without notable changes in protein content or amino acid composition (HUNTER *et al*., 2002). This locus encodes monogalactosyldiacylglycerol synthase and specifically affects galactolipids necessary for amyloplast and chlorophyll function (MYERS *et al*., 2011). The zein profile of IP3*o5*hk, similar to that of the hard kernel control inbred, is thus in accordance with results from previous researches, but its high tryptophan content in both types of kernels might indicate its effect on the content of this amino acid. In HUNTER *et al*. (2002), lysine content of the *o5* mutant was increased for approximately 30% compared to the wild type.

Tryptophan content in IP4*o14* was low in both hard and soft kernels (0.063 and 0.072, respectively). We could not find any data on this mutation, but according to its zein profile and

tryptophan content its soft, opaque kernels cannot be explained by changes in zein accumulation. Finally, the zein profile of IP5*fl*, which had only type 5 opaque kernels, was similar to that of the normal kernel control inbred line and its tryptophan content was low (0.066). According to these results, this floury mutation cannot be *floury2*, *floury3* or *floury4*, which cause reduction in zein accumulation and increase in lysine/tryptophan content (HOLDING *et al*., 2007; WANG *et al*., 2014). It could be *floury1*, an opaque endosperm mutant that shows little quantitative or qualitative differences in zein protein accumulation and which encodes an ER membrane protein necessary for correct α zein placement with the protein body core (HOLDING *et al*., 2007).

Referent *O2* allele sequence (a dominant allele)

AGGAGATCCCGGGCCCTTCTGGGAGCTGCTACCACCGCCAGC5(GCCAGA)GCGAGAGCAGCCTCCGTAACCG
GCATCGTCGTCGGC

Dominant *o2* allele sequence

Zp5

AGGAGATCC**T**CGGGCCCTTCTGGGAGCTGCTACCACCGCCAGC**5(GCCAGA)**GCGAGAGCAGCCTCCG**G**TAA
CCGGCATCGTCGTCGGC

IP1

AGGAGATCCTCGGGCCCTTCTGGGAGCTGCTACCACCGCCAGC**5(GCCAGA)**GCGAGAGCAGCCTCCGGTAAC
CGGCATCGTCGTCGGC

IP2

AGGAGATCCTCGGGCCCTTCTGGGAGCTGCTACCACCGCCAGC**5(GCCAGA)**GCGAGAGCAGCCTCCGGTAAC
CGGCATCGTCGTCGGC

Recessive *o2* allele sequence

CML144

AGGAGATCC**T**CGGGCCCTTCTGGGAGCTGCTACCACCGCCAGC**2(GCCAGA)**GCGAGAGCAGCCTCCG**G**TAA
CCGGCATCGTCGTCGGC

IP1

AGGAGATCC**T**CGGGCCCTTCTGGGAGCTGCTACCACCGCCAGC**2(GCCAGA)**GCGAGAGCAGCCTCCGGTAAC
CGGCATCGTCGTCGGC

IP2

AGGAGATCC**T**CGGGCCCTTCTGGGAGCTGCTACCACCGCCAGC**2(GCCAGA)**GCGAGAGCAGCCTCCG**G**TAA
CCGGCATCGTCGTCGGC

Unknown (UA) *o2* allele sequence

IP1

AGGAGATCCTCGGGCCCTTCTGGGAGCTGCTACCACCGCCAGC**4(GCCAGA)**GCGAGAGCAGCCTCCGGTAAC
CGGCATCGTCGTCGGC

IP2

AGGAGATCCTCGGGCCCTTCTGGGAGCTGCTACCACCGCCAGC**4(GCCAGA)**GCGAGAGCAGCCTCCGGTAAC
CGGCATCGTCGTCGGC

Fig. 4 Umc1066 SSR allele sequences within the *opaque*2 gene of IP1 and IP2

Umc1066 SSR allele sequences within the *opaque*2 gene of the analysed populations are given in Fig 4. The referent sequence was taken from the Maize Genetics and Genomics Database – MGDB (http://www.maizegdb.org/data_center/ssr?id=193779). The sequencing revealed that the

referent sequence (a dominant allele) as well as dominant alleles from the *O2* control inbred line ZP 5, IP1 and IP2 had five perfect GCCAGA repeats. All recessive alleles (CML144 control inbred line, IP1 and IP2) had two perfect GCCAGA repeats. The unknown allele in IP1 and IP2 previously found by PCR and gel electrophoresis (IGNJATOVIC-MICIC *et al*., 2014) had four perfect GCCAGA repeats. In YANG *et al*., (2004) two alleles were found at the umc1066 site among one *O2/O2* and 14 *o2/o2* inbred lines with different endosperm phenotypes, divergent ecotypes and genotype with or without modifier genes – a recessive allele with two perfect GCCAGA repeats and a dominant allele with three perfect repeats. It can be concluded two GCCAGA repeats are characteristic for the recessive allele and that dominant alleles can have three or more GCCAGA repeats. Thus, the unknown allele in IP1 and IP2 is a dominant allele.

SSR analysis of IP1 and IP2 hard and soft kernels with specific umc1066 marker is presented in Fig. 5. Recessive *opaque2* allele was detected in both types of kernels in IP1, while this allele was completely absent in IP2. In the previous analysis of the individual kernels from these populations (IGNJATOVIC-MICIC *et al*., 2014), 37% and 0% of recessive homozygous *o2o2* kernels were found in IP1 and IP2, respectively. The unknown allele was present in the heterozygote *O2UA* form in both populations (10.5% in IP1 and 40% in IP2), while homozygous *UAUA* was found only in IP2 (3.5%). Dominant alleles in IP1 can be seen on the electrophoregram given in Fig. 5, although they are present in much lower frequency, what might be due to the heterozygous *O2o2* normal kernels, as well as to the use of bulk analysis. On the other hand, absence of the recessive *o2* allele in IP2 implies presence of some other high lysine/tryptophan mutation.

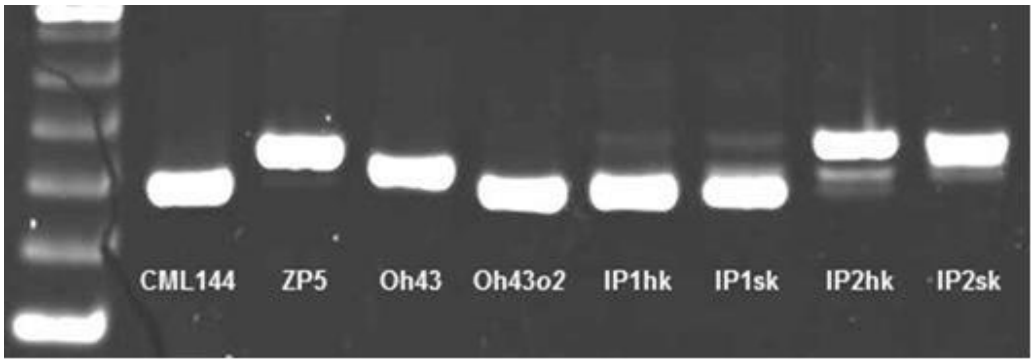

Fig. 5. SSR analysis of IP1 and IP2 with *opaque2* specific umc1066 primer pair. Lane 1 - 20 bp molecular weight ladder (lowest band 100bp, highest band 200bp); hk – hard kernels; sk – soft kernels.

The results of SSR, tryptophan and zein analyses are summarised in Table 1. Considering these results, as well as the fact that UA is a dominant allele, it can be concluded that IP1 is and IP2 is not an *opaque2* mutant. However, IP1sk showed lower decrease in zeins compared to Oh43*o2* control inbred line, primarily in 27 kDa γ zeins. This could probably be explained by the presence of *opaque2* modifier genes, which increase gamma zein proteins resulting in hard kernels (Lopes and Larkins 1995). The action of modifier genes can be surmised from the distribution of kernel types – 80% types 1 and 2, 14% type 3and 6% types 4 and 5 (Fig 1).

Table 1. Summary of the biochemical and SSR analysis of the introduced opaque populations

Genotype	Trp[a] content		Zein profile changes in soft kernels			Presence of *o2*	
	hk[b]	sk[c]	19 kDa	22 kDa	27 kDa	hk	sk
IP1	low	high	absent	decreased	decreased	yes	yes
IP2	low	high	absent	decreased	decreased	no	no
IP3o5	high	high	no change	no change	no change	-	-
IP4o14	low	low	no change	no change	no change	-	-
IP5fl[d]	-	low	no change	no change	no change	-	-

[a]Trp – tryptophan; [b]hk – hard kernels; [c]sk – soft kernels; [d]data for IP5 are given in comparison with normal kernel inbred line Oh43

Although not carrying the recessive *o2* mutation, IP2 had similar tryptophan content and zein profile as IP1. Compared to the other analysed mutants, IP2 had different biochemical characteristics and thus it cannot be *o5*, *o14* or the analysed floury mutation (which could be *fl1*, as described before). More in-depth analyses, such as immunoblotting and electron microscopy of protein bodies, should be performed in order to identify this high tryptophan mutation and further define its potential for use in breeding programs. However, IP1 could be recommended as a source of high lysine/tryptophan for breeding QPM, due to the presence of *o2o2* and desirable endosperm modifier genes.

ACKNOWLEDGEMENT

This research was supported by the Ministry of Education, Science and Technological Development of Republic of Serbia through the project TR31028 Exploitation of maize diversity to improve grain quality and drought tolerance.

Received April 14th, 2016
Accepted December 15th, 2016

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DNK I BIOHEMIJSKA ANALIZA MOGUĆE *OPAQUE2* POPULACIJE KUKURUZA

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Izvod

Hranljiva vrednost kukuruza je niska zbog niskog sadržaja esencijalnih aminokiselina lizina i triptofana, ali identifikovane su različite mutacije koje doprinose povećavanju njihovog sadržaja. U ranijim istraživanjima, kod dve populacije iz Banke gena Instituta za kukuruz (IP1 i IP2) utvrđen je visoki sadržaj ovih aminokiselina. Kod obe populacije, *opaque2* specifičnim umc1066 markerom nađen je recesivan (*o2*), dominantan (*O2*) i nepoznati alel (UA). Međutim, kod IP2 nisu identifikovani recesivno homozigotni genotipovi (*o2o2*). Cilj ovog rada je bio da se DNK i biohemijskom analizom utvrdi da li je UA recesivan alel i/ili je visok sadržaj triptofana posledica *o2* ili neke druge mutacije. Tri dodatne populacije sa drugim mutacijama - IP3*o5*, IP4*o14* and IP5*floury* su korišćene za biohemijsku analizu radi poređenja sa IP1 i IP2. Za analizu su odabrana po dva uzorka - sa tvrdim i mekim zrnom. Sekvenciranjem je pokazano da je UA dominantni alel sa četiri GCCAGA ponovka. SSR analizom je utvrđeno prisustvo *o2* kod IP1 u oba uzorka. Smanjenje 22 kDa, 19 kDa and 27 kDa zeina kod uzorka mekog zrna je nađeno samo kod IP1 i IP2. Sadržaj triptofana je bio visok u uzorcima mekog zrna kod IP1 (0,081) i IP2 (0,085), kao i kod uzoraka mekog i tvrdog zrna kod IP3*o5* (0,083 i 0,085, redom). Može se zaključiti da je IP1 nosilac *o2* mutacije, a IP2 neke druge mutacije osim *o2*, *o5*, *o14* i *floury*.

Primljeno 14. IV 2016.
Odobreno 15. XII. 2016.