

VARIABILITY ANALYSIS OF NORMAL AND *opaque2* MAIZE INBRED LINES

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Nutritional value of maize is poor due to deficiency of two essential amino acids – tryptophan and lysine. It was shown that *opaque2* (*o2*) mutations can nearly double the lysine and tryptophan content of the endosperm compared with the normal type. Maize Research Institute Gene bank maintains a collection of *opaque2* inbred lines developed in the seventies, primarily based on kernel hardness and analytical methods. In order to describe these lines in more detail they were analyzed for tryptophan content and subjected to SSR analysis with *opaque2* markers, a marker for endosperm hardness modifier gene and the most significant amino acid modifier markers. Also, a pathogenicity test for inbred lines tolerance to *Fusarium* spp., which is one of the most important maize

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pathogens in our region and a causer of maize stalk, root and ear rot, was performed. Differences in tryptophan content between normal and *opaque2* lines were significant. All primers used for distinguishing alleles between normal and *opaque2* inbred lines gave positive results. Each genotype gave a specific allelic pattern with amino acid modifier gene primers, without any obvious correspondence to the tryptophan content. Phytopathogenicity test showed on average higher susceptibility to *Fusarium graminearum* of *opaque2* genotypes. These results gave an insight into the applicability of the methods in describing *opaque2* lines to be converted into quality protein maize QPM - genotype in which *opaque2* has been incorporated along with associated modifiers.

Key word: maize, *opaque2*, phytopathogenicity test, SSR, tryptophan

INTRODUCTION

Maize is one of the most important crops in human and animal nutrition. However, like most other cereals, its nutritional value is poor due to deficiency of two essential amino acids – tryptophan and lysine. It was shown that *opaque2* (*o2*) mutations can nearly double the lysine content of the endosperm compared with the wild type (MERTZ *et al.*, 1964). Isolation and characterization of *opaque-2* gene showed that this gene is responsible for a transcriptional factor which regulates expression of zein genes and a gene encoding a ribosomal inactivating protein (SCHMIDT *et al.*, 1990; LOHMER *et al.* 1991; BASS *et al.*, 1992). The *o2* mutation reduces α -zein content by one-half and enhances the synthesis of a number of non-zein proteins (DAMERVAL and DEVIENNE, 1993; HABBEN *et al.* 1993).

Incorporation of *opaque2* into high yielding commercial cultivars failed, because of its numerous agronomic and processing problems – reduced grain yield, soft endosperm, chalky and dull kernel appearance and susceptibility to ear rots and stored grain pests. These drawbacks have been corrected in genetically improved, hard endosperm quality protein maize (QPM). QPM is a genotype in which *opaque2* has been incorporated along with associated modifiers. The modifiers comprise two distinctive genetic systems. The first one consists of endosperm hardness modifier genes and the second of amino acid modifier genes.

The endosperm hardness modifier genes convert the soft/opaque mutant endosperm to a hard/vitreous endosperm with little loss of protein quality. It has been shown that the increased levels of gamma zein probably contribute to the recovery of a hard endosperm phenotype, as the QPM grains have approximately double amount of gamma zein in the endosperm relative to the *o2* mutants (WALLACE *et al.*, 1990). The endosperm hardness modifier genes, as well as the *opaque2* mutation, can be selected for by projecting light through the grains using a light box (VIVEK *et al.*, 2008).

The amino acid modifier genes affect the relative levels of lysine and tryptophan content in the grain endosperm. The lysine levels in normal and QPM maize average 2% and 4% of total protein in whole grain flour respectively, but range across genetic background from 1.6 – 2.6% in normal maize and 2.7 – 4.5% in their converted *o2* counterparts (MORO *et al.*, 1996). Multiple genes have been identified in controlling amino acid content – at least three in controlling the levels of protein synthesis factor correlated with lysine levels and additional nine implicated in free amino acid content were identified (WANG *et al.*, 2001; WU *et al.*, 2002).

Within the *opaque2* gene sequence three different internal repetitive elements (SSR markers) have been identified – phi057, phi112 and umc1066. The internal position of these markers enables direct selection for the *opaque2* gene without false positive and false negative results in breeding programs, i.e. enables effective marker assisted selection – MAS (BABU *et al.*, 2005; DANSON *et al.*, 2006). However, to bring to bear the full effectiveness of QPM genotype MAS, a suite of effective markers linked to modifying loci needs to be identified.

Maize Research Institute Genebank maintains a collection of *opaque2* inbred lines developed in the seventies on the basis of kernel hardness and analytical method (DENIĆ *et al.*, 1979). This material is a valuable breeding source for development of QPM. As natural variability is a fundamental condition for the genetic gain in improvement breeding programs it is important to describe available lines in more details. Lines were analyzed for tryptophan content and subjected to SSR analysis with *opaque2* markers, a marker for endosperm hardness modifier gene and the most significant amino acid modifier markers. Also, a pathogenicity test for tolerance to *Fusarium* spp., which is one of the most important pathogens on maize in our region and a causer of maize stalk, root and ear rot, was performed. It was shown that susceptibility to ear and kernel rots is one of the most important drawbacks of *opaque2* maize (NASS and CRANE, 1970; LOESCH *et al.*, 1976). Collected parameters will assist to assort *opaque2* lines to be converted into quality protein maize (QPM).

MATERIAL AND METHODS

Material Five normal and nineteen *opaque2* inbred lines were analyzed for tryptophan content. Based on these results two normal (B73 and Oh43) and eight *opaque2* lines (B73o2, Oh43o2, 802, 803, 817, 804, 815 and 822) were chosen for SSR analysis, as a subgroup for developing the protocols. One QPM line from CYMMIT (CML144) was used as a positive control. Three *opaque2* inbred lines were chosen for SSR analysis based on tryptophan content and quality index (QI) similar to the threshold values. The choice of another five lines was based on values higher than threshold levels. The threshold values for tryptophan content are 0.07 (endosperm) and 0.075 (whole grain), while for QI they are 0.7

and 0.8, respectively. The same inbred lines used for SSR analysis, except CML144, were also used for phytopathogenicity test.

Tryptophan content determination Tryptophan content was determined using a modified colorimetric method by HERNANDEZ and BATES (1969). The protocol is based on the Hopkins-Cole reaction, in which one molecule glyoxylic acid and two molecules tryptophan form a colored compound with a maximum absorption at 560nm. Briefly, a random sample of 30 seeds (endosperm and whole grain) was grounded to a fine powder. The grounded samples were defatted and digested with papain. Colorimetric reaction was developed using $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ dissolved in glacial acetic acid and 30N H_2SO_4 . Absorption was read at 560nm in a spectrophotometer. Tryptophan % was calculated using a standard (calibration) curve, developed with known amounts of tryptophan, ranging from 0 to 30 $\mu\text{g/ml}$. Besides tryptophan content quality index (QI), defined as tryptophan to protein ratio in the sample, was also calculated.

SSR analysis SSR analysis was done with three primers specific for *opaque2* gene (phi057, umc1066 and phi112), six primers for amino acid modifiers (bnlg2248, phi072, bnlg1633, bnlg1382, phi075 and mmc0241) and one primer for endosperm hardness modifier gene (umc1216). The amplification reaction was carried out in 20 μl reaction volume containing 1x enzyme buffer, 3mM MgCl_2 , 200 μM dNTPs, 0.25 μM primers, 1.25U *Taq* polymerase and 50ng of DNA. The amplification profiles followed were: an initial denaturation at 94 $^\circ\text{C}/2\text{min}$, followed by 40 cycles each of denaturation at 94 $^\circ\text{C}/1\text{min}$, annealing at 60 $^\circ\text{C}/2\text{min}$ and extension at 72 $^\circ\text{C}/2\text{min}$, with final extension at 72 $^\circ\text{C}/10\text{min}$. Amplified fragments were first separated on 4% agarose gels in TBE buffer. Fragments that could not be read on agarose gels were also separated at 12% polyacrylamide gels. After electrophoresis gels were stained with ethidium bromide and photographed.

Fusarium resistance *Fusarium* resistance was estimated according to pathogenicity test described by A. Mesterhazy (personal communication). A random sample of 30 sterilized seeds per each genotype was placed in Petri dishes on filter paper and inoculated with 30ml of *Fusarium graminearum* (isolate MRIZP- 799) suspension, previously prepared on Chapek liquid medium. Seed susceptibility was scored after seven days of incubation on 25 $^\circ\text{C}$. The number of germinated seeds (%) and disease severity, i.e. severity of seedlings rot, was estimated. For disease severity estimation rating scale 1 – 5 was used (1- no symptoms, 5 – mycelium covered seed, seedling growth stopped).

RESULTS AND DISCUSSION

Tryptophan content In five normal maize inbred lines tryptophan percentage in the endosperm ranged from 0.034 to 0.059 and QI ranged from 0.268 to 0.606. In the whole grain these values were 0.053 to 0.076 and 0.382 to 0.647, respectively.

Ten out of nineteen *opaque2* inbred lines had both tryptophan and QI over the threshold limits. Remaining nine *opaque2* inbred lines had one or two

measured values below the threshold levels. This refers mostly to QI. However, three *opaque2* inbred lines had tryptophan percent in the endosperm somewhat below the threshold levels (0.060, 0.063 and 0.067).

Results of tryptophan content analysis for inbred lines chosen for SSR analysis and pathogenicity test are given in Table 1.

Table 1 Tryptophan content (Try %) an quality index (QI) in the endosperm and whole grain of the analyzed normal and *opaque2* maize inbred lines

Sample	Endosperm		Whole grain	
	Try %	QI	Try %	QI
B73	0.059	0.606	0.076	0.647
Oh43	0.054	0.429	0.071	0.529
B73o2	0.094	1.027	0.128	1.072
Oh43o2	0.093	1.044	0.103	0.857
802	0.096	0.979	0.104	0.793*
803	0.096	1.116	0.103	0.829
817	0.104	1.055	0.136	0.985
815	0.074	0.834	0.089	0.802
822	0.072	0.859	0.109	0.861
804	0.070	0.698*	0.101	0.833

* below threshold levels

SSR analysis All primers used for distinguishing alleles between normal and *opaque2* inbred lines gave positive results. Primer phi112 revealed amplification products in two normal maize lines and a null allele in seven *opaque2* lines (Figure 1). Primer umc1066 showed a common allele for all the *opaque2* lines. With primer phi057 normal lines, *opaque2* lines with threshold levels of tryptophan and *opaque2* lines with high levels of tryptophan could be distinguished. With all three primers seven *opaque2* inbred lines (and the QPM line) showed different allelic profiles compared to two normal maize inbred lines, while B73o2 revealed the same allele as normal maize with phi112 and a specific allelic pattern with phi057.

Primers used for identifying modifier alleles did not distinguish between normal and *opaque2* lines, as well as between lines with threshold and high levels of tryptophan content in the endosperm. Primer bnlg1382 did not give any bands on the gel, probably due to poor amplification. Primer umc1216 was monomorphic and all the other primers revealed two to seven alleles (Table 2). The results were the same on both agarose and polyacrilamide gels. An illustration of a modifier gene amplification (bnlg2248) is given in Figure 2.

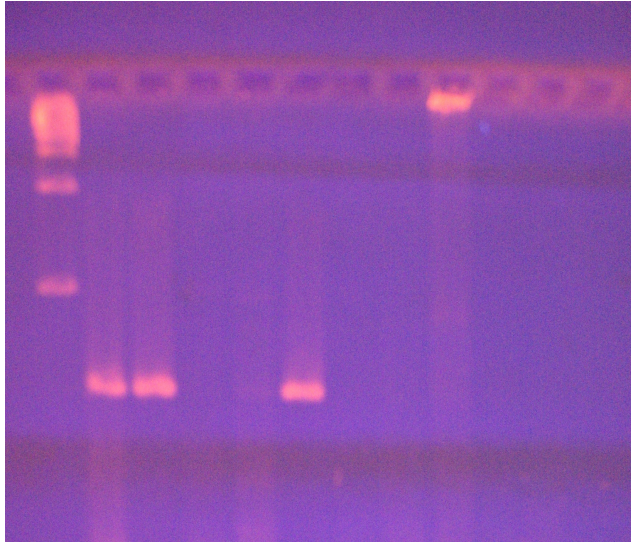


Figure 1 SSR profile of *opaque2* marker phi112. Lanes: 1 – 100kb DNA ladder; 2 – B73, 3 – Oh43 (normal maize); 4 – 802, 5 – 803, 6 – 804, 7 – 817, 8 – 815, 9 – 822, 10 – B73o2, 11 – Oh43o2 (*opaque2* maize); 12 – CML144 (QPM).

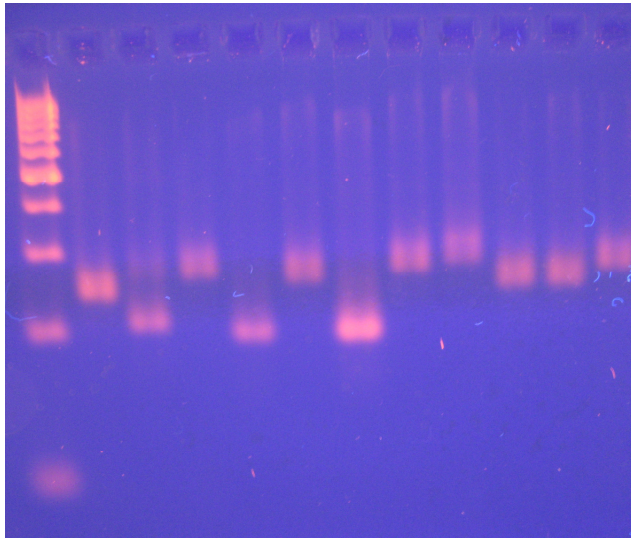


Figure 2 SSR profile of modifier gene marker bnlg2248. Lanes: 1 – 100kb DNA ladder; 2 – B73, 3 – Oh43 (normal maize); 4 – 802, 5 – 803, 6 – 804, 7 – 817, 8 – 815, 9 – 822, 10 – B73o2, 11 – Oh43o2 (*opaque2* maize); 12 – CML144 (QPM).

Fusarium resistance Two *opaque2* inbred lines – 802 and 804 were the most susceptible lines to *Fusarium graminearum*, with 10 and 6.7 percents of germinated seeds, respectively and score 5 for pathogenicity intensity. The germination percent was between 23.3 and 46.6 for all the other *opaque2* lines. Six *opaque2* lines had pathogenicity intensity score 5, except Oh43o2 (score 3) and 822, which had the lowest score (2).

Normal maize inbred line B73 had the highest germination percent of all the analyzed lines (60%). Germination percent in its *opaque2* version B73o2 decreased for 33.4% and the pathogenicity intensity score increased from 3 to 5. Another normal maize inbred line Oh43 gave exactly the same results as its *opaque2* version Oh43o2, i.e. germination percent of 46.6% and pathogenicity intensity score 3.

Results of pathogenicity test are given in Table 3.

Table 3 Germination percent (%) and pathogenicity intensity scores of normal and *opaque2* maize inbred lines inoculated with *Fusarium graminearum*

Genotype	Germinated seed (%)	Non-germinated seed (%)	Pathogenicity intensity (scale 1-5)
B73	60	40	3
Oh43	46.6	53.4	3
Average	53.3	46.7	3
B73o2	26.6	73.4	5
Oh43o2	46.6	53.4	3
802	10	90	5
803	33.4	66.6	5
817	46.6	53.4	5
804	6.7	93.3	5
815	23.3	76.7	5
822	30	70	2
Average	27.9	72.1	3.57

DISCUSSION

Both essential amino acids lysine and tryptophan are increased in *opaque2* and QPM maize genotypes (VILLEGAS et al., 1992). Since these two amino acids are highly correlated, tryptophan is usually used as a single parameter for evaluating the nutritional quality of the protein. Normally, the value of lysine is four times that of tryptophan, hence it can be easily estimated.

Protein, tryptophan and QI have to be above the acceptable limits when interpreting the results for making selection for QPM (VIVEK et al., 2008). These limits were respected in the analysis of the *opaque2* lines from MRI gene bank, as QPM is the agronomically improved version of *opaque2* genotypes. In all the

analyzed samples protein content was over 0.8%, which is the limit for all QPM and non-QPM genotypes. Differences in tryptophan content between normal and *opaque2* lines were as expected. Ten out of 19 *opaque2* lines had all four values over the threshold limits, while in nine lines at least one of the values was under the limits – mostly QI. However, genotypes with lower values should not be excluded right away from assorting *opaque2* lines to be converted into quality protein maize, as agronomical characteristics of the lines play as much important role.

Three SSR markers – phi057, phi112 and umc1066, located as internal repetitive elements of *opaque2* gene, have been identified (LOPEZ *et al.*, 2004; BANTTE and PRASANNA, 2003). These primers, also known as opaque endosperm 2, could be used for foreground marker assisted selection (MAS) to identify the gene of interest without extensive phenotypic assays. Phi112 primer showed a null fragment with seven *opaque2* lines and a band size between 150bp and 200bp with normal maize lines. 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, i.e 150bp respectfully, for non-QPM lines analyzed. The presence of the band in B73o2 indicates possible pollen contamination from the normal maize during seed maintenance. Phi112, which exhibits dominant polymorphism, cannot be used for discriminating homozygous and heterozygous backcross progeny. Nevertheless, this marker could be of use in checking the seed purity during routine field maintenance of *opaque2* or QPM inbred lines. Phi112 electrophoregram is given in Fig 1.

Phi057 revealed differences between normal maize and *opaque2* lines. B73 and Oh43 had a somewhat lower band compared to the *opaque2* band. The bands were around 170bp size. This primer distinguished also between *opaque2* lines with high level and threshold levels of tryptophan. In work of DANSON *et al.* (2006) phi057 showed a band of 165bp for QPM and of 159bp for non-QPM lines. In BABU *et al.* (2005) QPM lines gave a band of about 170bp and normal lines a band of about 160bp. This marker can be recommended for discriminating between homozygous and heterozygous backcross progeny.

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. In our work umc1066 could distinguish between normal and *opaque2* lines, giving a slightly higher band in normal maize. The bands were around 150bp size. According to BABU *et al.* (2005), as well as to our results, this primer could also be used for distinguishing between homozygous and heterozygous progeny during MAS process.

It is well established that the concentration of protein synthesis factor elongation factor 1 α (eEF1A) is consistently highly correlated with lysine content ($r=0.9$) of maize endosperm flour (MORO *et al.*, 1996). In higher eukaryotes eEF1A is typically encoded by a multigene family. In maize there are 10 to 15 genes, five of which are expressed in endosperm (CARNEIRO *et al.*, 1999). WU *et al.* (2002)

identified two significant QTLs (on chromosomes 2S and 4S) and two suggestive QTLs (on chromosomes 5S and 6S) for eEF1A. In our work normal inbred lines and *opaque2* lines (with threshold level and high level of tryptophan content) were screened with primers which were used for identification of eEF1A QTLs. The aim of the screening was to determine whether these primers have the potentiality to be used in marker assisted selection. The results were negative. Each genotype had its specific allelic pattern without any obvious correspondence to the tryptophan content. An example is given in Fig2.

Another measure of grain protein quality in maize, besides eEF1A, is free amino acid (FAA) content. Increased levels of FAA that nearly double the lysine content of maize endosperm were detected in *opaque2* mutants (MAURI *et al.*, 1993). Ten significant and one suggestive QTL for FAA were identified on all ten chromosomes (WU *et al.* 2002). The most pronounced example of loci affecting FAA content is QTL on chromosome 2L (identified with primer bnlg1633). This QTL is coincident with genes encoding two Asp kinase enzymes, which control important steps in metabolic pathways for amino acid biosynthesis and lys degradation. We used primer bnlg1633 for screening the maize inbred lines, with the same aim as for screening with eEF1A primers. The results were also negative - each genotype had its specific allelic pattern.

One of possible explanations for not establishing any obvious correlation between modifier alleles and tryptophan content in this experiment could be the mechanism of gene regulation affected by *o2* gene. It is presumed that the set of genes directly affected by *o2* are predominantly downregulated and consistent across genetic backgrounds. The downregulation of these transcripts causes a compensatory response such that genes indirectly affected by *o2* are predominantly upregulated and are specific to genetic background (JIA *et al.*, 2007). It is known that elongation factor 1 α is upregulated in *o2* mutants (CARNEIRO *et al.*, 1999). We could presume the same regulation pattern for FAA. Thus, genetic background would have significant impact on these genes. Different allelic patterns of analyzed genotypes in our experiment were consistent with background influence. Another explanation could be that the distance between SSR markers and QTLs does not enable routine screening between genotypes with different levels of tryptophan and lysine.

The umc1216 marker, known as 27-kD gamma zein, is an endosperm hardness modifier gene. In DANSON *et al.* (2006) this primer could distinguish between QPM and non-QPM lines, but only when SSR analysis was done using ABI3730 sequencer and genemaper3.7 software. Two peaks at 112bp and 115bp were detected for non-QPM lines and only one peak at 115bp for QPM lines. In our analysis this primer was monomorphic on both agarose and polyacrilamide gels. It is obvious that more sophisticated technology, which can detect exact fragment size, needs to be employed when allele differences are of a few base pairs.

High susceptibility of *opaque2* genotypes to *Fusarium spp.* has been determined (NASS and CRANE, 1970; LOESCH *et al.*, 1978) and is considered as

significant drawback in production of *opaque2* hybrids. In this work we tried to assess potential usability of a laboratory method, i.e. pythopathogenity test. The results were indicative, showing on average higher susceptibility of *opaque2* genotypes. Oh43 and its opaque counterpart Oh43o2 were an exception as the susceptibility was scored the same in both genotypes. Also, *opaque2* line 822 had the lowest pathogenity intensity score. In order to get more in depth results these genotypes will be scored in field plots under artificial inoculation and the relevance of pathogenity intensity test will be concluded after comparison with field results.

CONCLUSION

It could be concluded that even though some of the analyzed parameters can help in assorting *opaque2* lines for further breeding process it is still necessary to complement them with agronomical characteristics that play an important role in developing desirable genotypes. As an example, lines with lower tryptophan content or smaller QI can be a good choice only if their agronomical characteristics are good, while lines with higher tryptophan content and QI can be a poor choice if having unsatisfactory agronomic characteristics.

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I z v o d

Kukuruz ima nisku hranjivu vrednost zbog nedostatka dve esencijalne amino kiseline – triptofana i lizina. Utvrđeno je da *opaque2* (*o2*) mutacija može skoro udvostručiti sadržaj lizina i triptofana u endospermu kukuruza. Banka gena instituta za kukuruz „Zemun Polje“ poseduje kolekciju *opaque2* inbred linija razvijenih tokom sedamdesetih godina, prvenstveno na osnovu tvrdoće zrna. Sa ciljem detaljnijeg opisa ovih linija urađena je analiza sadržaja triptofana i SSR analiza sa *opaque2* markerima, markerom za gen modifikator tvrdoće endosperma i najsignifikantnijim markerima za gene modifikatore amino kiseline. Takođe je urađen test fitopatogenosti radi utvrđivanja osetljivosti *opaque2* genotipova na *Fusarium gramaenarum*, jednog od najvažnijih prouzrokovaca truleži stabla, korena i klipa kukuruza u našem regionu. Utvrđene su značajne razlike u sadržaju triptofana između normalnih i *opaque2* linija kukuruza. Svi SSR prajmeri korišćeni za razlikovanje normalnih i *opaque2* linija su dali pozitivne rezultate. Svaki od analiziranih genotipova je dao specifičnu alelnu sliku sa markerima za gene modifikatore amino kiseline, bez očigledne saglasnosti sa sadržajem triptofana. Test fitopatogenosti je, u proseku, pokazao veću osetljivost *opaque2* linija na *Fusarium graminearum*. Ovi rezultati su dali uvid u primenjivost navedenih metoda za opisivanje *opaque2* linija radi njihovog konvertovanja u kukuruz visokog kvaliteta proteina (QPM) – genotipa u koji se *opaque2* inkorporira zajedno sa odgovarajućim genima modifikatorima.

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