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IDENTIFICATION OF CHROMOSOME REGIONS DETERMINING KERNEL HIGH OIL CONTENT IN MAIZE (*Zea mays* L.) SYNTHETIC POPULATIONS

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Chromosome regions determining kernel high oil content were identified by RFLP analysis of individual plants from C0 and C9 selection cycles of two maize synthetic populations - DS7u and YuSSSu. Identification of chromosome regions was done with informative RFLP markers (that were identified with BSA earlier) using a single plant approach – analysis of individual plants. This analysis revealed the number of plants carrying alleles that endured frequency alterations during selection process. Statistical analysis ($\chi 2$ test) revealed chromosome regions that comprise putative QTLs affecting expression of kernel high oil content of analyzed maize populations. Four regions on chromosomes 1, 6, 7 and 8 were identified in both DS7u and YuSSSu

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populations. Additional four regions on chromosome 4, 9 and 10 were detected only in DS7u population.

Key words: maize, kernel oil content, RFLP, $\chi 2$ test, chromosome regions

INTRODUCTION

Energy value of maize depends on kernel starch and oil content, as well as on their ratio. High oil maize has higher metabolic energy value than standard types of maize, since this energy value in oil is 2.25 times higher than in carbohydrates (ATWELL et al., 1988; GROSS and KERR, 1992). High oil maize is mostly used in domestic animal nutrition (LAMBERT et al., 1998). The most important advantage of high oil maize genotypes is their kernel high energy density (energy per kilogram) necessary for nutrition of animals which need high energy diets (DALE and WHITTLE, 1991; ADAMS et al., 1994). The minimum limit of oil content for high oil genotypes was set at the level of 6%, because lower kernel oil content has no significant positive effects in domestic animal nutrition when compared to standard quality kernel maize hybrids (DUMANOVIĆ, 1995).

Breeding for high oil maize was initiated in Serbia during the 1950-ies (DUMANOVIĆ and MIŠOVIĆ, 1961). In Maize Research Institute „Zemun Polje“relevant number of high oil hybrids and inbreds was developed. Although some of these hybrids had the same yield as the best hybrids with standard kernel types there was no sufficient interest for their production (DUMANOVIĆ, 1995). Besides hybrids and inbreds several high oil synthetic populations were developed. DS7u population was obtained by recombination of Yugoslavian, Canadian and American inbreds and hybrids, while YuSSSu population derives from Iowa Stiff Stalk Synthetic – BSSS(R) C5. During nine cycles of selection oil content was increased for 8.52 or on average for 0.94 in DS7u, i.e. for 8.18 or on average for 0.91 absolute percents per cycle in YuSSSu. It was concluded that these populations detained satisfactory variability in kernel oil content after nine cycles of selection and that further increase can be expected (MIŠEVIĆ et al., 1985; DUMANOVIĆ, 1995; SARATLIĆ, 1994, 1995).

Kernel oil content is considered a quantitative trait controlled by many genes with small effects (DUDLEY, 1977). The number of genes controlling this trait estimated by quantitative genetic and biochemical methods was found to be between 20 and 69 (SPRAGHUE and BRIMHALL, 1949; DUDLEY and LAMBERT, 1992). However, development of molecular marker techniques enabled more accurate genetic dissection of kernel high oil content inheritance. RFLP (*restriction fragment length polymorphism*) markers, used in many experiments, identified potential genes on different chromosomes depending on the material and cycle of selection that were analyzed (SUGHROUE and ROCHEFORD, 1994; GOLDMAN et al., 1994; BERKE and ROCHEFORD 1995). DAMON and ROCHEFORD (2001) used SSR (*simple sequence repeats*) markers for mapping QTLs (*quantitative trait loci*) for economically important traits. They found QTLs with major effects on kernel oil

content on chromosomes one, six, seven, eight and ten. In the work of CAI et al. (2001) using AFLP (*amplified fragment length polymorphism*), SSR and RFLP markers QTLs for oil content were identified on chromosomes one and four.

The objective of this paper was to identify chromosome regions that carry potential gene(s) for determining high kernel oil content in two high oil synthetic populations created at MRI - DS7u and YuSSSu. Identification of chromosome regions was done with informative RFLP markers that were identified by bulk segregant analysis – BSA (MARKOVIĆ et al., 2007), using single plant approach – analysis of individual plants from the bulked samples. Analysis of individual plants with informative molecular markers and $\chi 2$ test of the results were used for verification of the linkage between the marker and potential gene(s), i.e. identification of the target chromosome regions.

MATERIAL AND METHODS

Plant material

Two hundred plants from each cycle of selection of DS7u and YuSSSu populations were grown in field during two years. Each plant was selfed and the kernels were used for oil content determination. Leaves of 50 plants per cycle were used for marker analysis.

Oil content determination

From each of the 50 plants chosen for marker analysis 30 kernels from the middle part of the ear were dried at 40-45^{0}C until the moisture content decreased to 5%. Nuclear Magnet Resonance (NMR) method according to ALEXANDER et al. (1974) was used for measuring kernel oil content. Statistical analysis of the data was done using Microsoft Excel computer program. Student's t-test was performed in order to ascertain the significance of oil content difference between C0 and C9 cycles of selection of DS7u and YuSSSu populations.

Marker analysis

Marker analysis was performed with RFLPs probes. DNA isolation from the plant samples was done by the modified CTAB method according to SAGHAI-MAROOF et al. (1984). DNA was digested with three restriction enzymes - EcoRI, BamHI and HindIII. Restricted fragments were separated on 1% agarose gels and Southern Blotted onto positively charged nylon membranes. Membranes with individual plants' DNA were hybridized with probes proven to be informative after BSA. All the probes were labeled with dig-11-DTP by PCR (*polymerase chain reaction*) reaction. Hybridized probes were detected by chemiluminiscent reaction between *Anti-digoxygenin Fab* fragments conjugated with alkaline phosphatase and CSPD substrate (*The Dig System User's Guide for Filter Hybridization, Boehringer Mannheim*, 1995).

RFLP films were scanned and band (allele) profiles were defined for each sample. Individual plant RFLP analysis revealed the number of plants which carry

alleles that went through frequency change during selection process. In order to determine the significance of allele frequency increase or decrease during selection for high oil content (i.e. if the marker signifies the chromosome region involved in determination of kernel high oil content) $\chi 2$ test was performed on the individual plant analysis results.

RESULTS

Statistical analysis of NMR results revealed highly significant differences in kernel oil content between C0 and C9 cycles of selection in both populations. The average oil content per cycle of selection was 3.94 (C0) and 9.63 (C9) in YuSSSu, while in DS7u these values were 4.46 (C0) and 11.5 (C9). Student t-test was used to verify the significance of detected differences in oil content. The results of NMR analysis are presented in Picture 1 and the results of statistical analysis in Table 1.

Table 1 Statistical analysis of NMR results for kernel oil content

		CV	σ^2	Average oil %	t - test
DS7u	C0	19.84	0.875	3.94	
	C9	6.15	0.503	9.63	**
YuSSSu	C0	7.22	0.081	4.46	
	C9	9.92	0.912	11.5	**

CV – variation coefficient
σ^2 - standard deviation
** - $P< 0.01$

The bulk segregant analysis revealed eight informative probes, i.e. probes that detected changes over 10% in allele frequencies during selection for high kernel oil content. Bnl6.32 (bin1.12), umc21 (bin6.05), umc254 (bin7.04) and npi268 (bin8.07) were informative in both DS7u and YuSSSu, while umc156 (bin4.06), csu147 (bin9.04), php20075 (bin10.01) and umc44 (bin10.06) were informative only in DS7u (MARKOVIĆ et al., 2007).

Analysis of individual plants with informative RFLP probes identified the number of plants carrying alleles determined by BSA. $\chi 2$ test revealed the significance of the change in the number of plants with the BSA identified alleles, i.e. if accumulation of these alleles during nine cycles of selections was high enough to be correlated with kernel high oil content. Results of the $\chi 2$ test are presented in Tables 2 and 3.

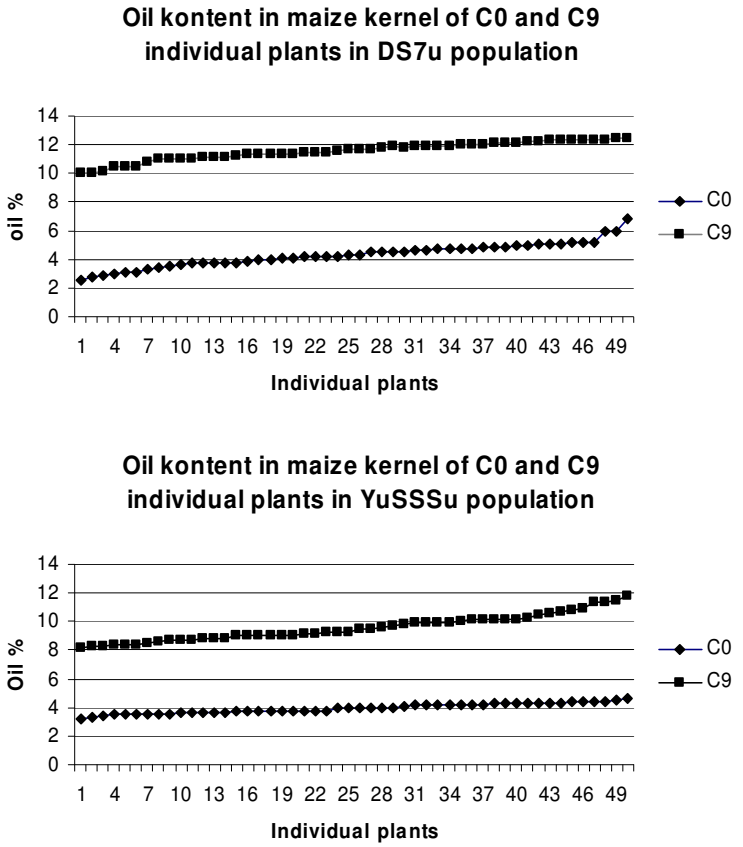

Figure 1.Results of NMR analysis for kernel oil content in a) DS7u and b) YuSSSu populations

All the chromosome regions identified with BSA were confirmed with RFLP analysis of individual plants, but differences in the target alleles were detected. Only 33, i.e. 57.9% out of 57 RFLP alleles identified as informative (frequency changes over 10%) by BSA, were confirmed by individual plant analysis. The biggest discrepancies were found for bnl6.32/EcoRI, npi268/EcoRI and csu147/EcoRI in DS7u population, where none out of three, none out of two and one out of four informative alleles, respectively, were confirmed by $\chi 2$ test. The highest coincidence was found for bnl6.32/HindIII, umc254/HindIII,

npi268/BamHI and umc156/HindIII combinations in DS7u, i.e. umc254/BamHI and npi268/HindIII combinations in YuSSSu, where all informative alleles were vouched by individual plant analysis and $\chi 2$ test. All the other probe/enzyme combinations had one to two alleles aborted as informative after individual plant analysis. Combination umc254/EcoRI in YuSSSu revealed only one informative allele by BSA (out of 6 alleles), but after individual plant analysis two significant alleles were found.

Table 2 Comparison of BSA and individual plant RFLP analysis for identification of molecular markers indicating chromosome regions involved in kernel high oil content expression in DS7u maize population

Probe	Enzyme	Total # of alleles	BSA		Individual plants	
			# of informative alleles	Frequency changes	# of identified alleles	$\chi 2$
Bnl6.32	EcoRI	3	3	18.8 – 21.2	0	-
	BamHI	3	3	16.1 – 17.9	1	5.0*
	HindIII	4	4	7.6 – 49.2	4	4.0* - 9.31**
Umc21	EcoRI	4	4	10.5 - 35	3	5* - 10.8**
	BamHI	3	2	55.9 – 59.5	1	25**
Umc254	HindIII	5	1	12.8	1	8.0**
Npi268	EcoRI	3	2	15.9 - 19	0	-
	BamHI	4	2	47.5 – 48.9	2	6.67* - 19**
	HindIII	5	3	10.8 – 17.7	2	3.85* - 9.0**
Umc156	HindIII	4	1	5.2	1	5.33*
Csu147	EcoRI	4	4	10.1 – 39.5	1	14.73**
	BamHI	4	2	13.7 – 16.6	1	8.0**
Php2007	HindIII	2	2	31.8 – 31.8	1	11.84**
Umc44	HindIII	4	3	13.7 – 28.9	1	14**

Significance $\chi 2$: * ($P<0.05$), ** ($P<0.01$)

Table 3 Comparison of BSA and individual plant RFLP analysis for identificatiom of molecular markers indicating chromosome regions involved in kernel high oil content expression in YuSSSu maize population

Probe	Enzyme	Total # of alleles	BSA		Individual plants	
			# of informative alleles	Frequency changes	# of identified Alleles	$\chi 2$
Bnl6.32	BamHI	3	3	7.7-19.6	1	5.33**
	HindIII	3	3	12.6 – 28.8	1	14**
Umc21	EcoRI	3	3	25.6 – 52.8	1	16.13**
	BamHI	3	2	40.2 – 48.4	1	18.61**
Umc254	EcoRI	6	1	21.1	2	4.0* - 5.3**
	BamHI	5	2	15.9 - 19	2	4.0* - 5.4**
Npi268	EcoRI	3	3	14.3 – 47.4	2	8.7* - 17.0**
	BamHI	3	3	11.6 - 41	2	7.0* - 8.3**
	HindIII	4	2	30.6 – 32.6	2	9.1* - 16.0**

Significance $\chi 2$: * ($P<0.05$), ** ($P<0.01$)

DISCUSSION

BSA is a method that enables effective analysis of a large number of loci and identification of informative markers for individual plant analysis. Using 57 RFLP probes eight chromosome regions were identified that comprise putative QTLs affecting expression of kernel high oil content in the analyzed maize populations, i.e. four in common for both DS7u and YuSSSu and additional four in DS7u. This number would probably be higher if more RFLP probes, evenly covering the whole genome, were used (MARKOVIĆ et al., 2007).

The results of BSA have to be verified by individual plant analysis, because the allele frequency changes identified in bulked samples are not always the result of increase of the number of plants carrying the target alleles. In this work all potential chromosome regions encompassing putative QTLs for high kernel oil content were verified by individual plant analysis, although the number of RFLP alleles potentially linked to high oil content was lower compared to BSA. Only in one probe/enzyme combination (umc254/EcoRI in YuSSSu) an additional allele was identified. These results indicate the limitations of BSA and the necessity of individual plant analysis for identification of alleles that could be used for QTL mapping and/or marker assisted selection.

According to published literature data high kernel oil content is controlled by more than 12 genes and this number aggravates selection for high oil and at the same time for high yielding maize hybrids (MAZUR et al., 1999). Also, the significance of chromosome 6 in high oil content inheritance was found in different experiments. Existence of regions on this chromosome involved in control of the trait, without precise identification of the target genes, was reported in SUGHROUE and ROCHEFORD (1994) and GOLDMAN et al. (1994). BERKE and ROCHEFORD (1995) identified bin6.04 as a region with significant impact on high oil content, while ALREFAI et al. (1995) identified a QTL for fatty acid inheritance at the same region. QTL for oleic and linoleic acids were found at bin6.04 and a QTL for high oil content with a major effect at bin6.05 (MIKKILINENI and ROCHEFORD, 2003). These results are congruent with results in our work.

Chromosome region bin7.04 identified in our work was also identified in GOLDMAN et al. (1994) and DAMON and ROCHEFORD (2001), while bin8.07 was identified in ALREFAI et al. (1995), BERKE and ROCHEFORD (1995) and DAMON and ROCHEFORD (2001). Also, RFLP probe bnl6.32 (bin1.12) was found to be linked to a gene with overdominant effect on high level of palmitic acid in kernel oil content of Illinois populations (ALREFAI et al., 1995). The same authors detected QTLs for high oil content using umc156 and umc44 RFLP probes, the same probes that identified chromosome regions in DS7u population.

Coincidence between the results from our work and the results presented in literature indicates that the identified chromosome regions could carry putative genes with major effects on high kernel oil content control. It also indicates the source and number of genotypes used for creation of DS7u and YuSSSu population. They were obtained by recombination of Yugoslavian, Canadian and American inbreds and hybrids (DS7u), i.e. from Iowa Stiff Stalk Synthetic – BSSS(R)C5 (YuSSSu), many of which have the same origin with populations used by the other authors.

It could be concluded, based on the results presented in this paper, that BSA and individual plant analysis with RFLP markers can be successfully used in target chromosome region identification, i.e. identification of molecular markers closely linked to genes of interest. The identified markers could be further used in marker assisted selection, an approach that should surpass difficulties of phenotypic selection, such as low heritability.

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IDENTIFIKACIJA HROMOZOMSKIH REGIONA UKLJUČENIH U KONTROLU VISOKOG SADRŽAJA ULJA U ZRNU SINTETIČKIH POPULACIJA KUKURUZA (*Zea mays* L.)

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I z v o d

Hromozomski regioni uključeni u ekspresiju visokog sadržaja ulja u zrnu kukuruza su identifikovani RFLP analizom individualnih biljaka C0 and C9 ciklusa selekcije dve sinteičke populacije kukuruza - DS7u i YuSSSu. Identifikacija hromozomskih regiona je rađena analizom pojedinačnih biljaka iz grupnih uzoraka sa informativnim RFLP markerima (koji su otkriveni analizom grupnih uzoraka u prvom delu eksperimenta). Rezultati ove analize su ukazali na broj biljaka nosioca alela kod kojih je došlo do promena u frekvenciji tokom selekcije. Statističkom analizom ($\chi 2$ test) rezultata identifikovani su hromozomski regioni nosioci potencijalnih QTL koji utiču na ekspresiju visokog sadržaja ulja u zrnu analiziranih populacija. Četiri zajednička regiona je identifikovano u obe analizirane populacije, na hromozomima 1, 6, 7 i 8. U populaciji DS7u je identifikovano još četiri regiona, na hromozomima 4, 9 i 10.

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