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Original scientific paper

MOLECULAR CHARACTERIZATION OF CHEMICAL MUTAGENESIS INDUCED DIVERSITY IN ELITE MAIZE GERMPLASM

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Three classical breeding Iowa Super Stiff Stalk (SSS) inbred lines B37, B73 and B84, one Lancaster inbred Oh43 and mutant lines obtained by chemical mutagenesis followed by mutation breeding as follows: two of B37 and four of Oh43 were selected for molecular characterisation. The mutant inbred lines were chosen because in addition to the improved GCA and SCA for grain yield, proven by their predominance in the Bulgarian breeding programs, they showed shifts in the flowering time as compared to the initial inbreds. Molecular markers (microsatellites and other PCR-based DNA markers) were used for characterization of maize genotypes and determination of the induced by chemical mutagenesis genetic variability in maize germplasm. The tested nine SSR markers (*umc1001, umc1014, umc1057, umc1181, umc1015, umc1029, umc1003, umc1033 and umc1035*) can discriminate between the initial classical breeding inbred lines and the originating mutant inbreds. Allelic

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diversity was also studied by PCR amplification with specifically designed primers in the coding regions and flanking sequence of two genes: *dwarf8 (d*8; chromosome 1, 198.5 cM), and *indeterminate1 (id*1; chromosome 1, 175.0 cM). These are considered candidate genes for variation in plant height and/or flowering time, based on mutant phenotypes and chromosomal locations near major QTLs. Single nucleotide polymorphisms and indels were detected in the region flanking the SH2 domain of *dwarf8* gene in some of the mutant inbreds as a result of SSCP and sequencing analyses. However, these polymorphisms could not be associated with the observed variations in flowering time. PCR analysis of the promoter region *dwarf8* showed a variant fragment of about 1kb in the inbred line Oh43 that was not present in any other initial and mutant inbred lines included in the study. PCR amplification of the 5' end of the Id1 coding sequence revealed polymorphic bands in the mutant lines XM535, XM521, XM250-1, XM98-8 and XM85-105, as well as in the classical breeding line B73. The data, presented here demonstrate the usefulness of chemical mutagenesis for generation of genetic diversity within the elite maize germplasm. Some of this variation may affect the major genes in the QTLs. Our initial data revealed mutagenesis induced polymorphisms in the coding sequences of two important for the determination of flowering time transcription factors. Further molecular analyses of the proposed model systems may complement the trait association efforts and will help to directly identify the major genes in the QTLs.

Key words: maize, quantitative traits, chemical mutagenesis, SSR, SSCP, molecular markers, diversity, flowering time

INTRODUCTION

Transition from classical to new genetic technologies including biotechnology in maize breeding programs of Maize Research Institute (MRI)-Kneja was done in the last years. The new biotechnology techniques are being developed in collaboration with the Agrobioinstitute (ABI).

Common feature of all programs for heterosis breeding of maize is the utilization of mainly recombinations derived variation (HALLAUER and MIRANDA, 1982; HRISTOV *et al*., 1991). According to SEGAL (1983)**,** there is sufficient stock of elite maize germplasm in the world at present and the sustainable success of the breeding programs depends on how effectively the elite germplasm is distributed and utilized in the breeding programs of each country.

Since 1973, a breeding program aiming at the utilization of chemical mutagenesis and mutation breeding for acceleration of breeding by induction of additional genetic variation in elite lines and hybrids, resulting from the best maize breeding achievements in the world, was initiated at the MRI-Kneja (HRISTOV, 1985; HRISTOV and IVANOVA, 1989; CHRISTOVA *et al*., 1993; CHRISTOV and CHRISTOVA, 1995; CHRISTOVA and CHRISTOV, 1995;). The first maize hybrid created by means of chemical mutagenesis and mutation breeding, resulting from the above program, was recognized in 1980s (MICKE, 1988). The results of the studies concerning the mutagen choice and the optimum doses for induction of mutants having high productivity and combining ability were reported elsewhere

(CHRISTOV and CHRISTOVA, 1995). A principal scheme of Recurrent Reciprocal Mutation Breeding (RRMB) was developed on the base of these studies (HRISTOVA and HRISTOV, 1984a; 1984b; HRISTOVA and HRISTOV, 1987a; 1987b; HRISTOV and HRISTOVA, 1987; CHRISTOVA *et al.*, 1993; CHRISTOVA and CHRISTOV, 1995)**.** The RRMB scheme was specially designed breeding technology for fixing chemically induced mutant genes controlling the quantitative traits and combining ability in mutant lines and selecting the highly competitive mutant maize hybrid combinations.

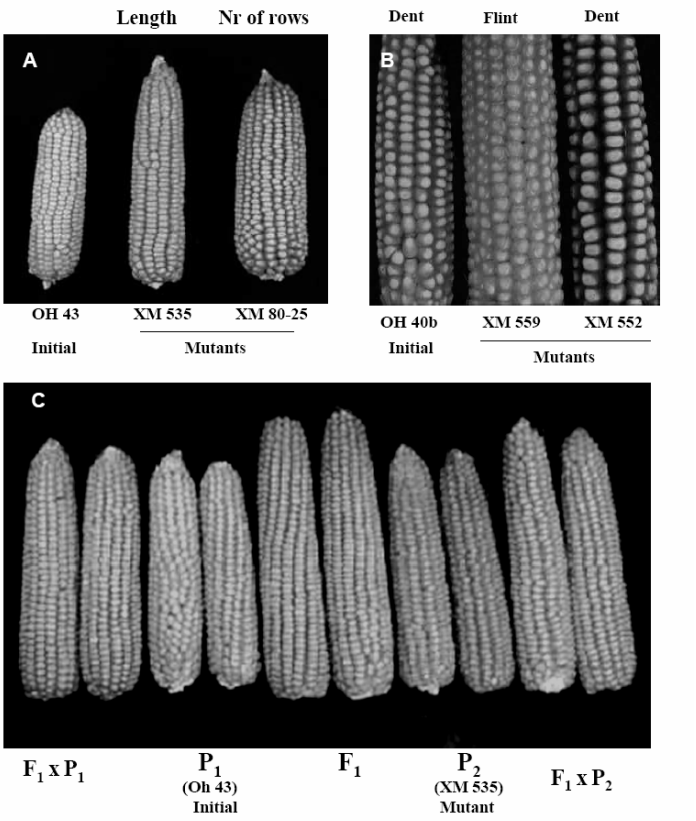

Fig. 1. Genetic variation generated by the application of chemical mutagenesis and mutation breeding. a) Variation in quantitative characters "ear length" and "Nr. of rows"; b) Variation in the kernel type; c) Expression of heterosis in crosses between the mutant and initial line

The induced by the chemical mutagenesis phenotype variations included important quantitative traits such as increased grain yield, high protein content, reduced flowering time, changes in the grain type, improved general and specific combining ability and other traits of agronomic importance in comparison to the initial inbred lines (Fig. 1 a,b). It is important to note that in crosses of mutant by initial inbred lines a heterosis effect is observed (Fig. 1c).

In this paper we report on molecular characterisation (SSR markers and SNP polymorphisms in the major candidate genes involved in the flowering time) of the diversity generated in the elite maize germplasm by the application of chemical mutagenesis and RRMB.

MATERIALS AND METHODS

Plant material. - All mutant inbreds included in the present study were obtained by chemical mutagen treatment of elite inbred lines, followed by RRMB breeding and stabilized by at least 5 generations of selfing. A scheme, depicting the initial inbred lines and the mutant inbred lines including the pedigree information is shown on Fig. 2. The sample included three classical breeding Iowa Super Stiff Stalk (SSS) inbred lines B37, B73 and B84, and mutant lines XM85-105 and XM87-136 originating from B37, one Lancaster inbred Oh43, and the mutant inbred lines XM-521, XM-535, XM-250-1, and XM-98-8, originating from Oh43 respectively (Fig. 2).

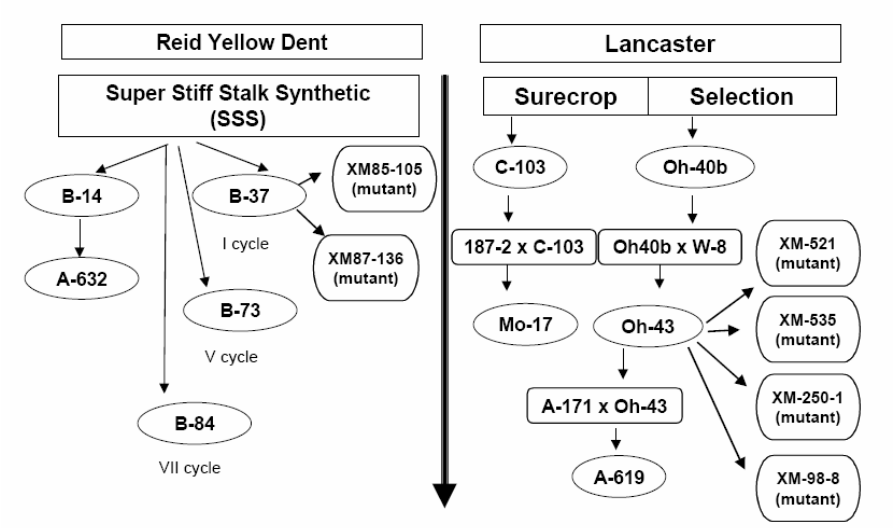

Fig. 2. Evolutionary links of classical and mutant maize inbreds included in the study

Field tests were conducted at the ABI experimental fields in Kostinbrod (near Sofia), Bulgaria. The flowering time was measured in terms of the number of days to pollen shed. The parameters were recorded for 10 individual plants from each inbred line in two consecutive years 2001 and 2002.

Microsatellite markers and PCR amplification. - Microsatellite analysis was performed using a total of 9 SSR markers: *umc1001*, *umc1003*, *umc1014*, *umc1015*, *umc1029*, *umc1033*, *umc1035*, *umc1057*, and *umc1181*. The primer sequence information and the detailed protocol for maize SSR amplification are available at http://www.agron.missouri.edu.

PCR reactions were performed in a volume of 23µl each, containing1x PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100), 6 pMol of each forward and reverse primers, 400µmol dNTPs, 1 unit Taq polymerase, 2.5 mM $MgCl_2$ and 50 ng DNA template. Thermocycling conditions were the following: 95° C 1 min., 65° C 1 min. and 72° C 1.5 min. for one cycle and then a one-degree decrement for the annealing temperature, each repeated once, until the annealing temperature is 55° C followed by 30 cycles at 95° C 1 min., 55° C 1 min. and 72° C 1.5 min. In the last cycle, extension time at 72°C was increased to 10 min.

Microsatellite fragments were resolved in ethidium bromide stained 4% Wide Range agarose gels using 50bp ladder as molecular weight marker.

The primers for PCR amplification of fragments from maize *dwarf 8 (d8)* and *indeterminate 1 (id1)* genes were designed from the published sequences, Genbank accessions AF413120 and AF058757 respectively. The fragments from the coding sequences were amplified with the primer pairs 5'-CGCTTCAC-CGAGTCGCT-3' and 5'-TCCGACATGACCTGGTCCGT-3' for *d8*, and 5'-AACAGAGAGAGACAAGCGCA-3' and 5'-AGCTTCCAGGGGAGGTTGT-3' for *id1*. The PCR reactions were performed in a volume of 23 µl each, containing 6 pmol of each forward and reverse primer, 200 µmol dNTPs, 1 unit Taq polymerase, 1.5 mM $MgCl_2$ and 100 ng DNA template. Thermocycling was done for 30 cycles at 95° C 1 min., 60° C 1 min. and 72° C 30 sec. (1 min for *id1*) followed by a final extension at 72° C for 7 minutes. Fragment of the *d8* promoter region was amplified with the primers 5'- GGCCTTAGTTGCATTGATCT-3' and 5'-CCGCCATCATCTTGTC-3' for 30 cycles with the following cycling conditions: 95° C 1 min., 58° C 1 min. and 72° C 1 min followed by a final extension at 72° C for 7 minutes.

The PCR fragments were resolved in ethidium bromide stained 1% TAE agarose gels using 50bp ladder as molecular weight marker.

Phylogenetic Analysis. - The diversity indices were calculated from the SSR data by the computer program MICROSAT (MINCH *et al.*, 1997). To construct phylogenetic tree the FITCH program in the PHYLIP package (FELSENSTEIN, 1993) was used with the log-transformed proportion of shared alleles distance as implemented in the computer program MICROSAT. In FITCH, the J option was used to randomize the input order of samples. The tree was visualized by the Rod Page's TreeView program.

SSCP Analysis of PCR products. - The SSCP analysis of the PCR products was done according to the published protocol (ORITA *et al.*, 1989) with minor modifications. In brief, 1 µl of the PCR product was denatured by adding 20 µl of loading dye (95 % formamide; 10 mM NaOH; 0.25 % bromophenol blue and 0.25 % xylenecyanol), followed by heating at 95°C for 5 min and immediate cooling on ice. The gel electrophoresis was performed in nondenaturing 15 % polyacrylamide gel containing 1 x TBE buffer and 5 % glycerol overnight at 4º C, with 200 V constant voltage. After the electrophoresis the bands were visualized using silver staining kit – Amercham-Pharmacia according to the provided manual.

RESULTS AND DISCUSSION

Microsatellite and phylogenetic analyses. - Microsatellites markers were applied for characterization of maize genotypes and determination of the induced by chemical mutagenesis genetic variability in maize germplasm.

Ten inbred lines (four classical breeding lines and six mutant inbreds, Fig. 2) were analysed using 9 maize microsatellites (*umc1001, umc1014, umc1057, umc1181, umc1015, umc1029, umc1003, umc1033 and umc1035*). The gene diversity, number of alleles and allele length of the 9 microsatellite markers assayed in the 10 maize classical breeding and mutant inbred lines are shown on Table 1.

Table 1. Gene diversity, number of alleles and allele length of 9 microsatellite markers assayed in 10 maize classical breeding and mutant inbred lines

SSR locus	Chromosome location (bin)	Gene diversity	Nr of alleles	Allele size range (bp)	Major allele size (bp)
umc1001	7.04	0.70	4	120 - 172	142
umc1003 (zpu1)	2.04- 2.05	0.32	2	114 - 118	114
umc1014 (pl1)	6.04	0.74	4	90 - 158	90, 158
umc1015	7.03	0.66	3	108 - 150	120
umc1029	7.04	0.61	3	122 - 146	122, 134
umc1033	9.02	0.64	4	90 - 158	90
umc1035	1.06	0.69	4	124 - 220	180
umc1057 (cko1)	3.02	0.50	2	88 - 94	88, 94
umc1181 (tub3)	unknown	0.42	2	92, 96	92
Total - 9			28		
Mean		0.59	3.11		

Most of the tested SSRs were highly polymorphic among the investigated inbred lines. The number of the observed SSR alleles ranged from 2 to 4. The SSR locus *umc1014* showed the highest gene diversity (0.74) and the locus *umc1003* showed the lowest diversity (0.32) among the tested inbreds. To compare the molecular diversity generated by the application of mutation breeding to that obtained by means of classical breeding, we sampled two populations of inbreds. One of the populations included the 4 classical breeding lines (one Lancaster and 3 Stiff Stalk lines). The second population included the four mutant lines originating from Oh43 and 2 mutant lines originating B37. The mean gene diversity for all applied SSR markers was separately calculated for the classical and mutant line populations. The diversity in the population of mutant inbreds (0.60 ±0.04) was slightly higher than that of the classical inbreds (0.55 ±0.08) but the difference was not statisti-

cally significant according to the Student's t-test. The latest allowed us to speculate that the molecular diversity, generated by the application of mutation breeding is comparable with that generated by the extensive recombinations in the classical breeding schemes over much longer periods of time. However, a more detailed investigation including greater number of classical and mutant inbred lines and more SSR markers is needed to empirically prove this initial hypothesis.

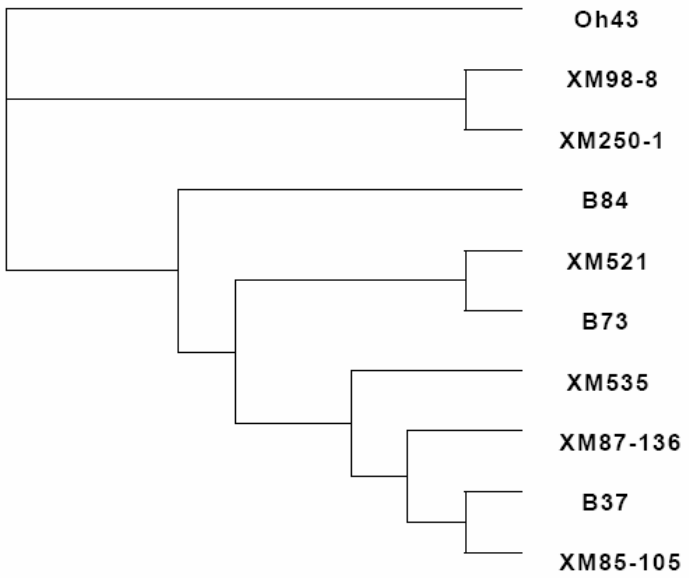

Fig. 3. Phylogenetic tree of classical and mutant maize inbred lines based on allelic profiles of 9 microsatellite markers

A phylogenetic tree generated from the SSR data is shown on Fig. 3. As expected, the SSS lines formed a clade and the two mutant inbreds, originating from B37 clustered close to the initial line. However, different situation was observed with the mutant lines originating from Oh43. Two of them, XM 521 and XM 535 clustered in the clade of the SSS lines, and the other two, XM 250-1 and XM 98-8 formed a separate clade between Oh43 and the SSS cluster. A number of studies have correlated the genetic distance calculated from the phylogenetic analyses of molecular markers, with the expression of heterosis effects (MELCHINGER *et al.*, 1990; DHILLON *et al.*, 1993; BURSTIN *et al.*, 1995; TSAFTARIS, 1995; LANZA *et al.*, 1997; MARSAN *et al.*, 1998; MELCHINGER *et al.*, 1998). The heterosis effect that was observed in the crosses of XM535 with the initial line Oh43 (Fig. 1c) is compatible with these findings. A more detailed study using more microsatellites and larger number of elite inbreds from both SSS and Lancaster groups is undergoing to estimate more precisely the genetic distances and correlate them with so called "mutant heterosis" observed in the crosses of mutant by initial inbreds.

Some of the mutant inbreds included in the present study showed differences in the flowering time as compared to the initial lines (Table 2). Therefore we analysed the genes *dwarf8 (d*8; chromosome 1, 198.5 cM), and *indeterminate1 (id*1; chromosome 1, 175.0 cM) by PCR with specifically designed primers. The latest are considered to be candidate genes for variation in plant height and/or flowering time, based on mutant phenotypes and chromosomal locations near major QTLs (REMINGTON *et al*., 2001).

Table 2. Time to pollen shed in initial and mutant maize inbreds (mean for 2001 and 2002)

Inbred Line	Time to flower (days)	Phenotype relative to initial line	6 bp Deletion flanking SH2
Oh43	87 ± 2 d.	NA*	-
XM521	89 ± 3 d.	-	+
XM535	87 ± 3 d.	-	+
XM250-1	76 ± 1 d.	-11 ± 3 d.	-
XM98-8	77 ± 2 d.	-10 ± 4 d.	-
B37	94 ± 3 d.	NA	-
B73	88 ± 1 d.	-6 ± 4 d.	-
B84	91 ± 2 d.	-	-
XM85-105	82 ± 1 d.	-10 ± 4 d.	-
XM87-136	83 ± 2 d.	-9 ± 5 d.	-

*NA-not applicable

PCR analyses of dwarf 8 and indeterminate1 genes. - PCR analysis of *dwarf 8* promoter region showed a fragment of about 1kb in addition to the expected fragment of 661 bp in the inbred line Oh43 (Fig. 4a). No variation in the length of the amplified PCR fragment was observed in the rest of the initial and mutant inbred lines included in the study (data not shown). This result is compatible with a heterozygous state of the previously reported 485 bp MITE insertion (THORNSBERRY *et al*., 2001) in the Oh43 stock used in the present study.

A 122 bp PCR fragment flanking the SH2 domain of *d8* gene (Fig. 4a) was amplified by PCR and subjected to SSCP analysis for detection of SNP polymorphisms in the amplified region. The SSCP analysis showed different patterns in the mutant lines XM535 and XM521 in comparison to the initial line Oh43. On the other hand, the mutant inbreds XM250-1 and XM98-8, showed SSCP pattern that was different from those of Oh43, XM535 and XM521 and the same as the pattern shown by all other analysed lines (Fig. 4b). The PCR fragments were then cloned and sequenced and the sequence alignment (Fig. 4c) showed that the strain of Oh43 included in the present study had a single nonsynonimous nucleotide substitution and was missing the 6 bp deletion found in the published Oh43 sequence (AF413120). As the stock of Oh43 included in the present study have been maintained in Bulgaria for more than 20 years, one explanation for the result might be that the 6 bp deletion have been lost by recombination due to selective pressure during the period of adaptation to the Bulgarian climate. However further analyses

are needed to prove or disprove this possibility. The mutant inbreds XM535 and XM521 showed the same sequence pattern as the published Oh43 sequence and no SNPs were found in the mutant inbreds XM250-1, XM98-8 and the rest of the inbred lines included in the study (Fig. 3c, Table 2). The 6 bp deletion in the region, flanking the SH2 domain of maize *d8* gene was previously reported to be associated with 10 days reduction in the flowering time (THORNSBERRY *et al.*, 2001). Nevertheless, summarizing the data from the *d8* gene analyses and the flowering time obtained in the present study (Table 2), we can conclude that the variation in the *d8* sequence could not be associated with the variation of flowering time observed in the mutant inbreds analysed in the present study.

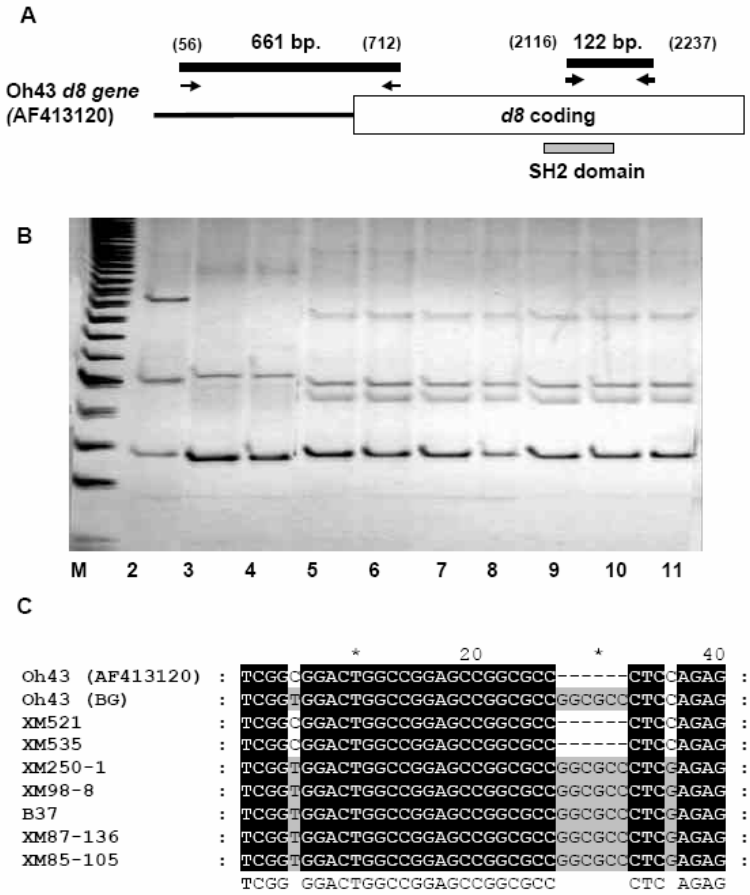

Fig. 4. PCR analyses of the *dwarf8* gene. a) Schematic representation of the primer positions; b) SSCP analysis of the region flanking the SH2 domain, Lane 1, 50 bp ladder marker, Lanes 2-11: Oh43, XM521, XM535, XM250-1, XM98-8, B37, B73, B84, XM85-105 and XM87-136 respectively; c) Sequence alignment of the region flanking the SH2 domain of *dwarf8* gene

Initial PCR analysis of a fragment including the 5' end of the *id1* coding sequence and part of the untranslated region revealed variation in the size of the amplified fragments for in the mutant lines XM535, XM521, XM250-1, XM98-8 and XM85-105 in comparison to the initial inbreds, as well as in the classical breeding line B73 (Fig. 5). A more detailed study is undergoing to search for polymorphisms in the coding and promoter sequences of the *id1* gene and other candidate genes for the determination of flowering time that could be associated with the variation observed in the mutant inbreds included in the present study.

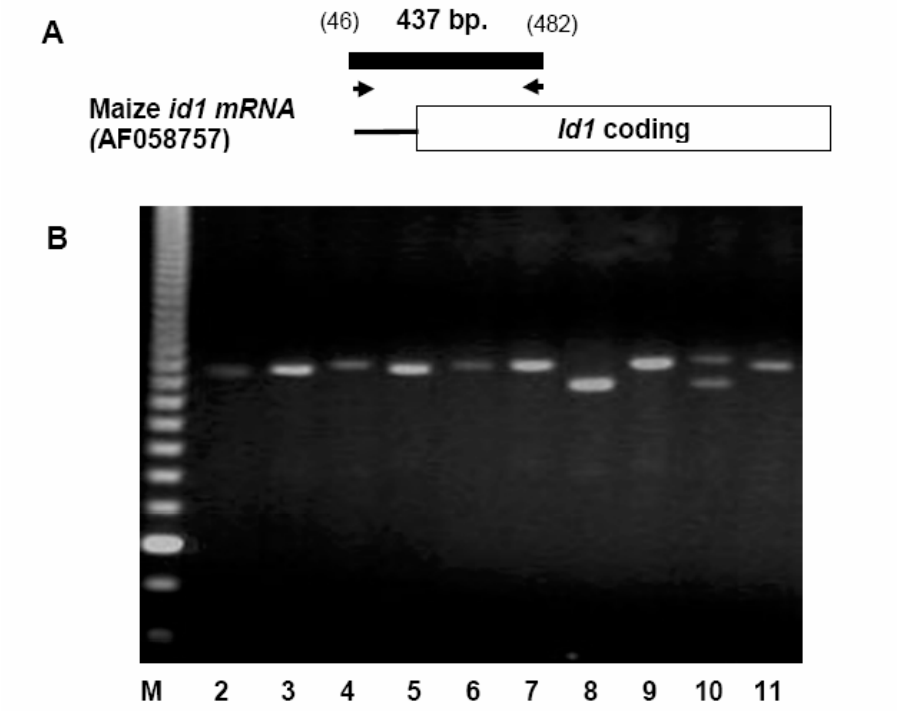

Fig. 5. PCR analyses of the *indeterminate1* gene. a) Schematic representation of the primer positions; b) 1% TAE agarose electrophoresis of the PCR fragments, Lane 1, 50 bp ladder marker, Lanes 2-11: Oh43, XM521, XM535, XM250-1, XM98-8, B37, B73, B84, XM85-105 and XM87-136 respectively

To our knowledge, the present study is the first attempt for molecular analyses of mutant inbreds with improved quantitative characters resulting from application of chemical mutagenesis of elite maize inbred lines followed by RRMB breeding. The results, presented here clearly demonstrate the usefulness of chemical mutagenesis for generation of genetic diversity within the elite maize germplasm. Some of this variation may affect the major genes in the QTLs. Our initial data revealed mutagenesis induced polymorphisms in the coding sequences of two important for the determination of flowering time transcription factors. Further

molecular analyses of the proposed model systems may complement the trait association efforts and will help to directly identify the major genes in the QTLs.

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MOLEKULARNA KARAKTERIZACIJA HEMIJSKIM MUTAGENIMA INDUKOVANE VARIJABILNOSTI ELITNE GERMPLAZME KUKURUZA

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U idukovanju mutacija kod kukuruza hemijskim mutagenima korišćene su javne samooplodne linije (poreklo ISSS – Iowa Super Stiff Stalk) B37, B73 i B84 i jedna Lankaster linija, Oh43. Za molekularnu karakterizaciju indukovanih mutacija odabrane su dve linije – potomstvo B37 i četiri linije, potomstvo linije Oh43. Linije su odabrane za molekularnu karakterizaciju jer , pored poboljšane GCA i SCA za prinos zrna u poređenju sa Bugarskim linijama u programu oplemenjivanja, imaju izmenjeno vreme cvetanja u odnosu na originalne linije. Molekularni markeri (SSR i drugi PCR- zasnovani markeri) su korišćeni za karakterizaciju genotipova i determinaciju genetičke varijabilnosti indukovane hemijskim mutagenima. Devet korišćenih SSR markera (*umc1001*, *umc1014*, *umc1050*, *umc1181*, *umc1029*, *umc1003*, *umc1033* i *umc1035*) su pokazali osobinu diskriminacije originalnih samooplodnih linija i dobijenih mutanata. Alelna divergentnost je ispitivana PCR amplifikacijom specifićnih sekvenci u kodirajućim i graničnim sekvencama dva gena: *dwarf8* (d8: hromozom 1, 198.5cM9, i *indeterminate1* (*id1*: hromozom 1, 175.0cM). Ovi geni su na osnovuizmenjenog fenotipa i hromozomalne lokacije u blizini glavnih QTLs, razmatrani kao geni – kandidati, koji kontrolišu visinu biljke/ili vreme cvetanja. Polimorfizam pojedinačnih nukleotida iindex su određivani u regionu koji graniči SH2 domein dwarf8 gena kod nekih linija – mutanata, koristeći rezultate SSCP i sekvenciranja. Međutim ovaj polimorfizam ne može da se veže za utvrđenom varijabilnošću u vremenu cvetanja. PCR analizom regiona promotera *dwarf8* gena utvrđeno je prisustvo 1kb DNK fragmenta samo u samooplodnoj Oh43 liniji. PCR amplifikacija 5' kraja *Id1* kodirajuće ekvence je dala polimorfnr fragmente u mutantima XM535, XM521, XM250-1, XM98-8 i XM85-105 kao i u originalnoj B73 samooplodoj liniji. Rezultati prikazani u ovom radu pokazuju korisnost korišćenja hemijskih utagena u indukovanju genetičke divergentnosti unutar elitne germplazme kukuruza. Neke od tih promena mogu da utiču na ekspresiju glavnih gena u lokusima koji kontrolišu kvantativne osobine 8QTLS). Inicijalni podaci su potvrdiliindukovani polimorfiza u kodirajućim sekvencama dva transkripciona

faktora uključena u kontrolu vremena cvetanja kukuruza. Dalje molekularne analize će pomoći u direktnoj identifikaciji glavnih gena u lokusima koji kontrolišu kvantativne osobine (QTLs).

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