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MOLECULAR GENETICS - STEP BY STEP IMPLEMENTATION IN MAIZE BREEDING

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Efficiency in plant breeding is determined primarily by the ability to screen for genetic polymorphism, productivity and yield stability early in program. Dependent on the knowledge about the biochemical bases of the trait and nature of its genetic control, trait could be modified either through mutagenesis of genes controlling it or through the transfer of already existing mutant genes, controlling desired trait to different plant genotypes by classic crossing. Objective of this report is to present partly results on the investigation of the possibilities to apply ionizing radiations (fast neutrons, γ - rays) and chemical mutagens (EI, iPMS, EMS, ENU) to get maize and wheat mutants with increased amount and improved protein quality. Besides this approach in mutation breeding, results on the very early investigation of biochemical background of *opaque* -2 mutation including use of coupled cell – free RNA and protein synthesis containing components from both wild and *opaque* – 2 maize genotypes (chromatin, RNA polymerase, microsomall fraction, protein bodies) will be presented. Partial results on *opaque* – 2 gene incorporation in different genetic background are reviewed.

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Part of report is dealing with different classes of molecular markers (proteins, RFLP, AFLP, RAPD, and SSR) application in maize genome polymorphism investigation. Besides application of different molecular markers classes in the investigation of heterosis phenomena they are useful in biochemical pathway of important traits control determination as well.

Key words: maize, mutagens, chromatin, proteins, molecular markers, heterosis

INTRODUCTION

After biological sciences development, particularly molecular biology, during last decades molecular biotechnology, based on genetic engineering are widelly used in genome structure and expression investigation. As consequence of the some results application**,** new varieties of commercially grown crops with higher yield are developed, as well as decreassing time to breed new genotypes having satisfactory resistance to different pathogens, tolerance to biotic and abiotic stress conditions etc. Inspite of the fact that both information technology and molecular biotechnology are „strategic" technologies, it is out of the question that remarkable progress in maize genetics and breeding was gained during last few decades by the use of conventional genetics and breeding methods. Although modern maize breeding is based on the concepts of SCHULL (1910) methods of breeding and evaluation have changed due to utilisation of new or improved germplasm sources, more knowledge and information on the inheritance pathway of complex traits as well as information from theoretical and computer simulation studies.

Existing or induced variation that will enable the selection of superior genotypes is at the core of a successful breeding program. KUCKUK et al. (1991) classified breeding methods into three groups: (i) selection breeding, where breeder relies on existing variation in natural populations and genotype mixtures; (ii) combination breeding where combinatorial crosses are made and F_1 is not used directly, but only to generate subsequent segregation material that will form the basis for selection and finally (iii) hybrid breeding where combinatorial crosses are made to create new genotypes that will be used as F_1 seed. Crucial items to have effective breeding is the prediction of the best hybrid within a large set of possible genotype combinations based on genetic distance of the parental lines. According to BOPPENMAIER et al. (1992) this was not possible for line combinations only between heterotic pools of European material. It is important to survey and choose germplasm as pure lines, cultivars, populations, clones, genes, DNA sequences etc (LEE,1995).

Development of a large number of molecular markers raises question whether only their application can further enhance the efficiency of maize breeding. It is important to collect more information on the reliable level of polymorphism in order to understand biological processes, including both genetic

control of storage protein and oil biosynthesis as well as other important traits in different genome combinations.

This review is covering partly our many years period results on the biochemical background investigation of genetic control of storage protein biosynthesis in maize endosperm, induced and existing genetic polymorphism determination by biochemical and other molecular markers application with general aim to find out what kind of genome polymorphism - at the level of genome structure or expression is reliable criteria for succesful breeding program creation.

1. MUTATION BREEDING

1.1 Important advantage of mutations is that it may be introduced into the best commercial varieties with acceptable fulfil the demands on yield, straw-stiffness, desease resistance, winter hardness or other critical properties, to create a new variation from which selection can take place in any property whose improvement is desired. In principle, mutation breeding with these purposes may go one of two possible ways: 1- *induced mutation* and 2 – *biochemical plant breeding with special reference to existing mutants*

1.2 Induced mutations

It is known that different genes may vary strongly with respect to their relative sensitivity to different mutagenic agents (HAGBERG et al, 1958) and theoretically it is possible to develop more or less specific mutagenic agents, that preferably provoke a change or variation in the gene or genes controlling the property to be improved, undesirable changes being suppressed. The use of unspecific mutagenic agents, like radiations and alkylating agents, will lead to a random variation in all kinds of properties. The success of such breeding is above all dependent on the availability of efficient screening methods and of appropriate design of experiments. Poliploidy character of hexaploid wheat makes it as promissing source of good biological background for induced mutation either by ionizing radiation and chemical mutagens (EHRENBERG et al. 1959., MAC KEY, 1959., BHASHARAN and SWAMINATHAN, 1960., MOUTSCHEN - DAHMEN and EHRENBERG, 1968., ĐOKIĆ et al. 1967., BOROJEVIĆ and BOROJEVIĆ, 1969., KONSTANTINOV, 1968., KONSTANTINOV et al. 1970a., KONSTANTINOV and MOUTSCHEN, 1970b)

In our program on induced mutations several mutants of hexaploid wheat, with changed protein content and quality were obtained by ionizing irradiation, gama rays or fast neutrons and treated with alkylating agents, ethylenimine (EI) and ethylmethane sulphonate (EMS) (DUMANOVIĆ et al. 1969). All applied mutagens induced chromosomal aberations in the first mitotic cycle of meioses (fig 1.). There was no detectable effect of EI, EMS and gama rays on the chromosomes in M5 progeny of chemically treated or irradiated seeds. Fast neutrons induced chromosomal aberrations in M5 progeny which is in agreement with the mutagenic

behaviour of this kind of irradiation. Also there is no evidence for a direct correlation between the changed content of protein in the seeds and the induced chromosomal aberrations (JOVANOVIĆ and KONSTANTINOV, 1973).

Therefore second procedure, i.e. use of existing known mutant genes controlling desired trait must be mostly relied in mutation breeding for improved biochemical characters.

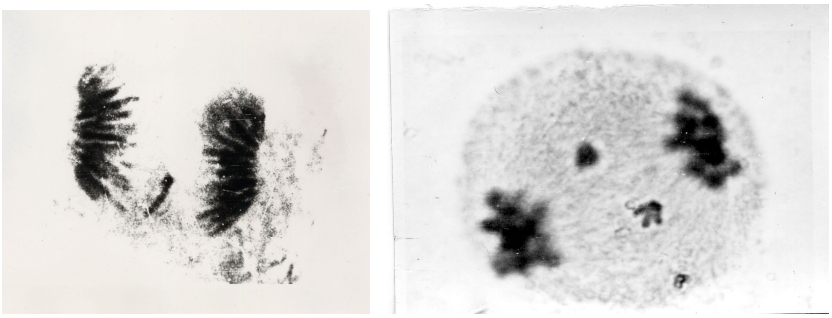

Fig 1. a) EMS inducted chromosomal aberation in hexaploid wheat variety
b)Chromosomal aberation in the first meiotic mitoses of maize induced by iradiation

1.3 Biochemical plant breeding with special reference to existing protein mutants

The first consideration in estimating the quality of any livestock fodder or human food is the content of protein and its composition. The great achievements beneficial to food production can be expected by an increase in the relative proportion of protein, with a corresponding decrease of carbohydrate – fat production. Since the cereal grains are the main source of protein for man and animals, the best way to increase the protein supplies is to increase the protein content in cereals and to modify the proteins, i.e. to increase the biological value of crop protein, which quality is primarily determined by the content of certain essential amino acids, first-hand lysine, tryptophane and methionine (DUMANOVIĆ and EHRENBERG, 1968).

The most striking advancement in these efforts came in 1964 when the high protein quality of the *opaque*-2 mutant was described (MERTZ et al. 1964). At that time primary point of *opaque*-2 mutation, having as genetic effect the changes in the proportion of protein fractions in maize endosperm, has been unidentified. Zeins represent 50% of maize endosperm protein and are strictly developmentally controled, first detected 12 days after pollination and accumulates till maturity at 45-50 days after pollination with very large increase over next 20 days. Also higher ribonuclease (RNase) activity has been reported as characteristic of opaque-2 endosperm (MERTZ et al. 1964). In our experiments we did verifyied that Rnase activity could be used as biochemical marker for opaque-2 gene presence in recesive homozygous triploid endosperm, because difference in the enzyme activity between wild and mutant endosperm follows the patern of zein

accumulation during endosperm development (DENIĆ et al, 1971., DENIĆ, et al., 1973).

After *opaque*-2 gene incorporation in different valuable commercial inbred lines by the 4 -5 backcrossing and 2 – 3 generation of selfpollination (DUMANOVIĆ et al. 1974) variation of endosperm texture has been obtained, varying from standard soft *o*-2 to nearly normal dent types. Interaction of *o*2 gene with host genome was quite different in different genetic background. Changes in protein content and composition of endosperm in different categories of „normalized" endosperm and changes in RNase activity at different degrees of endosperm vitreosity have been investigated. As the rule, all converted *o*2 versions kept in each genetic background protein quality (aminoacid composition of endosperm proteins) simmilar to original *o*2 type. It was concluded that, in general, the amount of protein is slightly increased in „normalized" endosperm compared to standard *o-2* soft floury endosperm. A tendency was indicated for RNase activity to be decreased with increasing proportion of modified („normalized") endosperm.

It has been sugested that variation of endosperm texture is consequence of polygenic character of this trait (VASAL, 1972., POPOVIĆ et al. 1974). Nowdays, more probable explanation is presence of different transposon elements families in inbred lines used in the experiments for the conversion to *opauue*-2 phenotype. Genetic instability at the *opaque* -2 locus in maize was first reported for *o2-m(r*) (SALAMINI, 1980), an allele responding to the autonomous *Bg* element (HARTINGS et al. 1991). MOTTO et al. (1986, 1988) reported *Ds*-induced alleles phenotypically indistinguishable from wild – type in the absence of *Ac*, but mutable with opaque sectors when *Ac* is present.

Ribonuclease activity and pattern of modification of endosperm texture have been used as genetic markers of *opaque*-2 versions of converted inbred lines. Several of them were later used as parental lines in the process of several *opaque*-2 hybrid creation. Results on nutritive quality and energy yield of *opaque*-2 maize hybrids created at Maize Research Institute, as well as high oil and waxy maize hybrids are published by EGGUM et al.(1985).

Among many approaches to analyse diference between wild and *opaque*-2 gene expression (DENIĆ, 1970., DENIĆ, 1971) interesting example are results on difference of protein bodies, which contain mostly or exclusively zein in homogenous matrix (BURR and BURR, 1976, DENIĆ and MILIVOJEVIĆ, 1978), fig2.

In order to study the mode of gene action on storage protein synthesis, during early of seventeens attempts have been made to isolate chromatin from the endosperm of developing seeds (KONSTANTINOV, 1975). After modification of method adopted for isolation of crude chromatin from plant tissue (BONNER et al. 1968) we found that it was possible to isolate chromatin from maize endosperm tissue (KONSTANTINOV and DENIĆ, 1975). Obtained results confered that chromatin, isolated by modifyied method is in native, transcriptionally active form (fig 3). To approve whether the genetic activity is preserved in isolated chromatin, a chromatin dependent protein synthetizing system was set up in the coupled cell-

free protein synthesizing system from wheat embryo (ALLENDE and BRAVO, 1966). From the obtained results it has been obvious that *de novo* proteins are synthesized in response to messenger - RNA synthesized on the chromatin as a template (KONSTANTINOV, 1975, 1978, POPOVIĆ et al. 1981., POPOVIĆ, 1986). It was established in the system with chromatin, isolated from an *opaque*-2 mutant endosperm 25 days after pollination as template, relativelly higher incorporation of lysine in comparison with the chromatin of wild genotype.

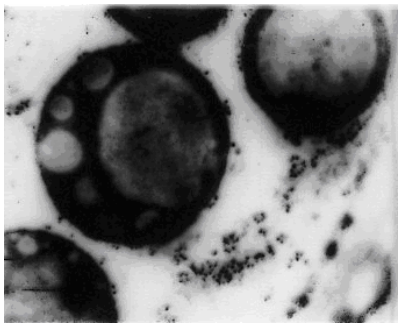

Fig.2 Protein granules from maize endosperm 26 days after pollination

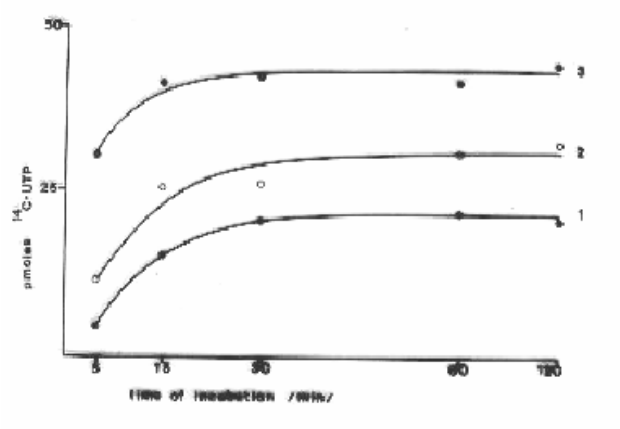

Fig 3. Transcription product of endogenous RNA polymerase activity of chroamtin: 30µg chromatin-DNA (60º) plus 1.5U of E.coli polymerase (curve 1); 30µg thymus DNA plus 1.5U of E.coli polymerase (curve 2); 30µg chromatin-DNA (0º) p lus 1.5U of E.coli polymerase (curve 1)

Based on the results obtained after analysis at the molecular level, reviewed in Mutants of Maize (NEUFER et al. 1997) o*paque*2 (o2) plays an important regulatory role in the expression of zein seed storage protein genes (AUKERMAN and SCHMIDT, 1994). This gene encodes a 48-kD protein belonging to the basic leucine zipper (bZIP) class of eukaryotic transcriptional activators (HARTINGS et al., 1991., SCHMIDT et al., 1990). The gene was cloned by SCHMIDT et al. (1987) and MOTTO et al. (1988) and sequenced by MADDALONI et al. (1989).

There are very short upstream open reading frames that reduce the efficiency of *o2* translation (LOHMER et al.1993). Recesive, *o2* expression appears to be endosperm – specific (SCHMIDT et al. 1987., MOTTO et al., 1988). The protein is localized in the nuclei of developing endosperm cells (VARAGONA et al. 1991) and found to contain two nuclear localization signals (VARAGONA et al. 1992). The O2 protein is a transcriptional activator that binds to the promoter of 22 – kD zein genes (SCHMIDT et al. 1990, 1992) and to the promoter of the *rip*1 (LOHMER et al. 1991). In recent years (PIRONA et al., 2007), cDNA microarray is in use to investigate the transcription profiles and different gene expression of maize endosperm frrom two different opaque mutants (o2 and o7) and in double mutant combination.

After recombinant DNA technology and different technologies for plant transformation have been developed we did maize transformation by selective marker – *NPT II* gene integration in maize genome (MLADENOVIĆ, 1990**,** KONSTANTINOV et al.1997). It has been proved that genome of transformed plants contained the selective marker gene sequence in an active form. Besides foreign gene integration and expression in maize plant, foreign integrated DNA induced several heritable changes - mutations, changing host genome function (MLADENOVIĆ et al. 1991., KONSTANTINOV et al. 1997).

It is important to stress that mutation breeding must be applied along with, and partly combined with hybridization – selection methods.

2. MOLECULAR BREEDING

Determination and measuring of genetic variation is the most important step in any breeding program and data obtained by the methods of molecular genetics could be best interpreted by correlation with the procedure already well developed in conventional genetics. Maize is extremely diverse genus, having morphological and biological differences. Despite the nearly unlimited diversity of germplasm, the main problem is the creation of suitable new crosses, arises from divergent parental lines. To find the best methode, which provides discrimination according to the purpose of selection, will remain a chalenge both for classical and molecular geneticists.

Information on the genetic diversity and relationships of lines or populations is useful for choice of parents, crossing, and classification of germplasm into heterotic groups, prediction of heterosis and plant variety protection. Maize breeders are mainly concerned with the genetic diversity among

and within breeding population and elite germplasm, because it largely determines the future prospects of success in breeding programs. Compressive studies of genetic diversity based on molecular markers has been reported in maize (MESSMER et al., 1992; MELCHINEGER et al., 1991; LIVINI et al., 1992; AJMONE MARSAN et al., 1998; DUBREUIL et al., 1996, SRDIĆ et al., 2006).

Standard methods in maize breeding imply numerous crosses of inbred lines to different testers so as to gain information on genetic similarity, i.e. diversity of these inbreeds. According to this, inbreeds are allocated into specific heterotic groups. Just these extensive field studies are the most expensive and the most time consuming part of contemporary maize breeding and selection, and at the same time this procedure is very restrictive due to the fact that only a few inbreeds can be crossed and estimated.

An alternative could be an allocation of inbred lines in heterotic groups based on molecular markers. As this approach would provide the use of a greater number of inbreeds it would significantly accelerate the process of development of superior hybrids and decrease cots that burned the maize breeding programme.

Commercial maize germplasm is divided into 12 groups based on patterns of heterotic behavior. Members of partially heterotic groups should be detectable by their genetic relatedness. We have examined 125 maize inbred lines from different heterotic groups and miscellaneous origin using different molecular markers (protein, RAPD). Genetic distances between the genotypes were determined from the molecular marker data, and cluster analysis was used to find groups of related genotypes. The clusters found with molecular markers closely resemble the heterotic groups to which the 125 genotypes belonged. Three main groups were distinguishable: a group of BSSS lines, a group of Lancaster lines, and a set of lines with European background. Similar groups were detected despite differences in marker type and genetic distance method used (DRINIĆ et al.., 2000, RADOJČIĆ 2006, DRINIĆ 2005, SRDIĆ, 2006).

Information on genetic diversity of commercially grown maize hybrids is very important for germplasm enhancement, hybrid breeding and in preventing environmental damage. Genetic uniformity implies risks of genetic vulnerability to stress factors, which may be reduced by use of unrelated single cross hybrids. It is important to choose among hybrids the one that will give highest yield and answer to environmental stress due to their existing genetic diversity (TROYER et al., 1983). Three methods of determining genetic diversity among maize hybrids are being used: a method that is based on molecular markers (MELCHINEGER et al., 1991; NAGY and MARTON, 2006), a method that is based on the genetics of inbreeding and heterosis for grain yield (TROYER et al., 1988, WILLIAMS and HALAUER, 2000) and a method based on pedigree data (SMITH et al, 2004).

Hybrid maize breeding programs in Serbia as well as at Maize Research Institute were started in the 1950s. Five periods, each characterized by introduction of the new potentially higher yielding ZP hybrids with other agronomic characteristics improved, have determined maize breeding program at the Maize Research Institute “Zemun Polje” (DRINIĆ et al., 2006).

ZP hybrids from different periods have been study by RAPD markers (ERIĆ, 2003, BAUER et al. 2005, BAUER et al., 2007). Cluster analysis showed distinctive grouping of hybrids from each period. Changes in genetic background of parental genotypes during the last 50 years have a major impact on genetic diversity among ZP maize hybrids.

Heterozis background investigation by molecular markers

Comparing to other crop species maize has probably the highest level of genetic polymorphism. The most significant practical consequence of the huge genetic diversity between maize genotypes is the phenomenon of hybrid vigor or positive heterosis. Maize breeders have always been interested in choosing the parental lines which would result in positive heterotic combination without necessarily making all possible crosses among the potential parental combination. The various methods are in use to predict heterosis and can be grouped into (i) per se performance, (ii) combining ability and (iii) genetic diversity as determined through geographic origin, morphological and agronomic traits as well as molecular markers. The experimental data indicate that heterosis is a function of heterozygosity in a higher number of loci and that the increase of the heterozygous loci number by crosses to genetically distant lines or populations increases the level of heterosis in the crosses. Based on this hypothesis HALLAUER et al. (1988) assumed that the magnitude of heterosis could be predicted on the basis of inbred lines differences obtained after use of molecular markers.

Different classes of molecular markers have been used to analyze the genetic relationships among maize inbred lines and to examine the relationship between marker-based GD and heterosis in maize (LEE et al. 1989; SMITH et al., 1990; BOPPENMAR et al.., 1992; MELCHINGER et al., 1993). Correlation level varies depending on the analysed material and various types of gene effects, pointing to the complexity of the genetic background of heterosis. The general conclusion of studies based on the results of molecular markers application is that heterosis is significantly related to heterozygosity of marker loci.

In our studies on the possibility to correlate heterosis and to make prediction of heterosis by the use of molecular marker data we did obtain high and significant correlation between GD from analyses of inbred lines with protein markers (KONSTANTINOV et al., 1996, DRINIC et al, 2006), RAPD (SRDIĆ et al., 2006; DRINIĆ et al., 2006, RADOJČIĆ et al., 2006, DRINIĆ et al., 2007), SSR and ALFP (DRINIĆ et al., 2002). The results indicating that GD based on molecular markers at the level of gene expression – polypeptides pattern in dry seed, is correlated with heterosis and that markers could be used for prediction of heterotic effect.

Germplasm characterization

The development of genetics and molecular biology has opened a new chapter in the field of describing agronomical important genotypes and providing their much more detailed characterization, not only in terms of how distant their

germplasms are from those of the existing one but also in the sense of monitoring the uniformity and stability of their characteristics relative to each other. Combining morphological, biochemical and molecular aspects in identification and description of agronomical important genotypes, it is possible to reveal their unique genetic profiles e.g. fingerprints. The most useful markers for maize germplasm characterization are proteins markers, RFLP, SSR, RAPD, and AFLP.

The SDS-PAGE method of protein profile sample mixture is included in ISTA international rules as a technique for distinguishing among and identifying commercial genotypes of different plant species. This method was used for genetic characterization of maize inbred lines as well as for distinguish sister lines at the Maize Research Institute, and for first screening of genetic purity of hybrid seed (DRINIĆ et al., 2000., ERIĆ, et al., 2003., DRINIĆ et al., 2006). All analyzed genotypes have unique protein pattern and unique code - combination of numbers and letters - have been assigned to them.

Most polypeptides identified from the electrophoretic or chromatographic profiles are mostly products of the genes expression that are unevenly distributed in the genome and expressed at the developmental stage of the tissue, the sample has been taken from. This prevents providing a sample that would cover the entire genome (GALOVIĆ et al, 2006). The complete coverage of a genome can be achieved by the use of molecular markers for the variability identification at the level of DNA - DNA polymorphism. Genetic characterization of maize inbred lines with standard kernel type as well as popcorn and sweet corn inbreeds from MRI collection was done by RFLP (fig 4) (KONSTANTINOV and DENIĆ., 1985., KONSTANTINOV et al., 1985., KIDRIČ et al., 1987., KONSTANTINOV et al., 1988) RAPD (fig 5), SSR and AFLP markers (DRINIĆ et al 2000, DRINIĆ et al. 2004., ERIĆ, 2003).

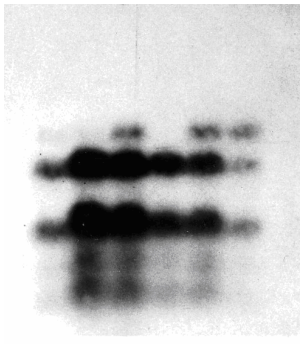

Fig. 4. Hydrolyze DNA of different maize mutants hybridize with same cDNA probe (unpublished data)

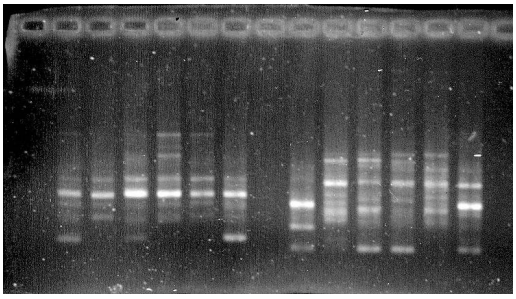

Fig. 5. RAPD of six popcorn inbred lines (unpublished data)

General conclusion, based on the results reported by different research teams, including our, is that plant breeding program demands the multidisciplinary approaches independent on plant species.

The authors feel honored to dedicate this paper to the memory of Prof. Janko Dumanovic, who gave an exceptional contribution to the development and promotion of genetics in Serbia.

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MOLEKULARNA GENETIKA – POSTEPENO UVOĐENJE U OPLEMENJIVANJE KUKURUZA

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Izvod

Efikasnost procesa oplemenjivanja je primarno determinisana mogućnostima utvrđivanja genetičkog polimorfizma, produktivnosti i stabilnosti prinosa početnog materijala. Poznavanjem biohemijske osnove genetičke kontrole željene osobine moguće je izvršiti poželjnu promenu ili modifikaciju ili indukovanjem mutacija ili unošenjem već postojećih poznatih gena nosioca mutacija klasičnim procesom ukrštanja sa različitim genotipovima. U radu je dat parcijalni pregled indukovanja mutacija kod pšenice i kukuruza, dobijenih korišćenjem različitih vrsta jonizujućeg zračenja (brzi neutroni, γ - zraci) ili hemijskih mutagena (EI, iPMS, EMS, ENU).

U drugom delu su prikazani neki od rezultata unošenja *opaque*-2 gena u različite genetičke osnove kukuruza primenom klasičnih metoda oplemenjivanja. Istovremeno su navedeni rezultati istraživanja biohemijske osnove *opaque*2 mutacije, uključujući korišćenje ukopčanog bezćelijskog sistema za sintezu RNK i proteina u prisustvu različitih ćelijskih komponenata (hromatin, RNK polimeraza, mikrozomalna frakcija, proteinska telašca) izolovanih iz endosperma normalnog genotipa i *opaque* 2 mutanta kukuruza.

Deo rada pretstavlja pregled rezultata istraživanja u oblasti molekularnog oplemenjivanja – korišćenja različitih klasa molekularnih markera (RFLP, AFLP, RAPD, SSR) u izučavanju genetičkog polimorfizma kukuruza. Dat je i prikaz rezultata ispitivanja molekularne osnove heterozisa kod kukuruza korišćenjem molekularnih markera.

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