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MARKER-ASSISTED *GA1-S* INCORPORATION INTO WHITE MAIZE HYBRID´S PARENTAL LINES[a)]

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Abstract

Maize Research Institute Zemun Polje has a marker-assisted backcross breeding (MABB) program aimed to incorporate the strong allele of incompatibility gene *Ga1* into parental components of the hybrids with specific traits (white kernel). The main objective of this research was marker-assisted development of the white maize hybrid´s parental lines with complete cross-incompatibility to foreign pollens. *Ga1-S*-tightly-linked molecular markers successfully identified heterozygous plants with a percentage of approximately 50% after all three backcrossings. Regarding the background selection, 69% of progenies had recurrent parent's genome (RPG) above the theoretical value for BC_3 generation. Also, a few individuals even had 99%, the value theoretically achieved in the BC_6 generation, showing that MABB made a genetic gain in RPG recovery. Foreground selection in this generation aimed to identify homozygous dominant individuals. Out of 264 BC_3F_2 plants, 70 (26.5%) were dominant homozygotes, which is in accordance with the expected Mendelian ratio. Progenies of the BC_3F_2 homozygous dominant plants were planted alternatively with yellow-kernel maize of the same maturity to check cross-incompatibility. Unfortunately, none of the dominant progenies had a 100% white kernel, most likely due to the presence of modifier genes that increase/decrease the effectiveness of pollen exclusion or that, alternatively, modifiers are lost during the backcrossing. It could be expected that successfully employed functional markers (developed from the sequence polymorphisms present within a functional gene(s) associated with phenotypic trait variations) would outbalance the noted impediments and enhance MABB efficiency to transfer the desired gene(s) controlling simple or complex trait(s) into cultivated varieties.

Key words: background selection, foreground selection, *Ga1-S,* maize, marker-assisted backcross breeding

Introduction

Three *Gametophyte factors (Ga),* that regulate the receptivity of the pistils to pollen of different strains, have been described in maize (*Zea mays* L.): *Ga1, Ga2*, and *Teosinte crossing barrier1* (*Tcb1*) (Nelson 1994; Evans and Kermicle 2001; Kermicle and Evans 2010; Dresselhaus et al. 2011). Both *Ga1* and *Ga2* were originally identified in domesticated maize genotypes, while strains of teosinte carrying *Tcb1-s* typically grow as weeds in maize fields (Kermicle 2006). Two functions are associated with them: a female function that produces a

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barrier to non-self-type pollen and a male function that enables self-type pollen to overcome that barrier. Plants carrying the *Ga1-strong* (*Ga1-S*) haplotype have both male and female functions. *Ga1-S* females reject *ga1* pollen but accept *Ga1-S* pollen. However, these cross-incompatibility (CI) systems are unilateral; *ga1* females are receptive to *Ga1-S* pollen. (Lu et al., 2014). The rejection of *ga1* pollen by *Ga1-S* silks is associated with the failure of *ga1* pollen tube growth in vivo (Lausser et al. 2010). Because maize is a cross-pollinating crop with a very high cross-fertilization rate, various strategies (e.g. physical barriers, temporal and spatial isolation) have been taken to reduce and avoid cross-fertilization among adjacent maize fields. Specialty types of maize, such as sweet and waxy maize and white kernel maize are therefore enabled to be free from foreign pollen. Also, seed production and non-GM maize fields require foreign- and GM-free pollen to maintain high hybrid purity and avoid contamination. The *Ga1-S* might be a potential alternative to effectively manage pollen flow among maize fields, as a biological reproductive barrier for the containment of gene flows between different types of maize (Liu et al., 2014).

Mangelsdorf and Jones first characterized the *Ga1* cross-incompatibility system on chromosome 4 back in the 1920s, but research was only recently intensified since the contamination of conventional maize from genetically modified varieties is a constant issue in maize production, especially for organic production. Genetic analysis and fine mapping of the *Ga1-S* identified from a Chinese popcorn strain SDGa25 were reported by Zhang et al. (2012). The genetic region mapped between molecular markers SD3 and SD12 was 1.5 cM in length, and the physical distance was 2,056,343 bp on ctg156 based on the *B73 RefGen_v2sequence*. Furthermore, Liu et al. (2014) delineated the locus between markers SD9 and SD12. The genetic region mapped spanning the *Ga1-S* locus was estimated to be 0.089 cM in length, and the physical distance on ctg156 based on the *B73 RefGen_v2sequence* was 100,524 bp. With the assistance of five tightly linked and codominant molecular markers, using SDGa25 as the *Ga1-S* allele donor and JKN2000 parental lines as recurrent parents, JKN2000 parental lines possessing homozygous *Ga1-S* alleles were developed after six generations of backcrossing and one generation of selfing. Finally, the homozygous *Ga1-S*/*Ga1-S* elite white waxy maize hybrid, surrounded by yellow and purple maize, showed full cross-incompatibility.

Using the tightly linked molecular markers to select the individuals possessing genomic regions which influence the expression of certain traits of interest is an indirect selection method known as marker-assisted selection (MAS). Maize Research Institute "Zemun Polje" (MRIZP) has a marker-assisted backcross breeding (MABB) program aimed to incorporate the strong allele of incompatibility gene *Ga1* into parental components of the hybrids with specific traits (white kernel). The main objective of this research was marker assisted development of the white maize hybrid´s parental lines with complete cross-incompatibility to foreign pollens.

Material and Methods

Plant material

Three MRIZP commercial inbred lines were selected as the recurrent parents (RP_1, RP_2 and RP_3) for marker-assisted introgression of the favourable allele of the *Ga1-S* gene. These lines are components of the three-way white kernel MRIZP hybrid adapted to the local environmental conditions in Serbia. Two inbred lines (provided by the gene bank in Illinois, USA) were used as the donor parents (D_1 and D_2) of the favourable allele of the *Ga1-S* gene. The conversion process is given in Figure 1. Shortly, F_1, BC_1F_1, BC_2F_1, BC_3F_1 and BC_3F_2 generation were developed. Both BC_1F_1 and BC_2F_1 plants heterozygous for the gene-specific markers were selected for backcrossing. A

two-level selection procedure was carried out in BC_3F_1 generation and heterozygotes with the highest recovery of recurrent parent's genome were selfed to produce BC_3F_2 generation. These plants were subjected to foreground selection to identify homozygous dominant individuals. The ZP inbred lines, homozygous at the *Ga1-S* locus, were surrounded by yellow maize to be evaluated for cross-incompatibility. All materials were planted at the experimental fields in Zemun Polje, Serbia, from 2016 to 2022.

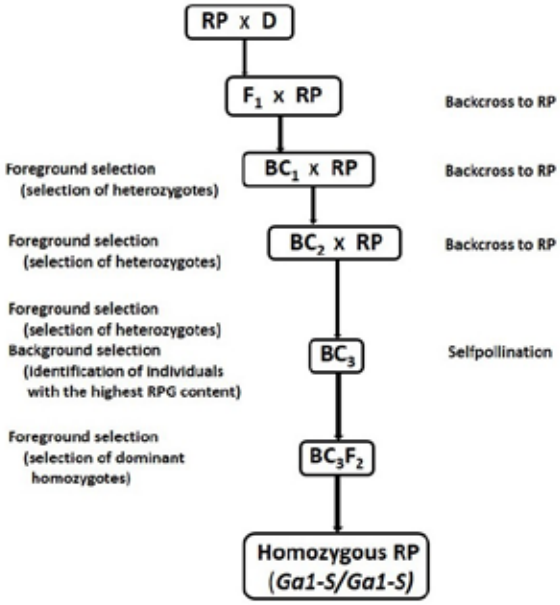

Figure 1. Schematic presentation of marker-assisted incorporation of Ga1-S into white maize hybrids´ parental lines (RP-recurrent parent, D-donor line, RPG-recurrent parent's genome)

Slika 1. Šematski prikaz inkorporacije Ga1-S u roditeljske linije hibrida belog zrna (RP-rekurentni roditelj, D-donor linija, RPG-genom rekurentnog roditelja)

DNA extraction

Genomic DNA was isolated from the four-week-old plants according to the modified Dorokhov and Klocke protocol (1997). The DNA was quantified using a biospectrometer (BioSpetrometer kinetic, Eppendorf) and diluted to a working concentration of 20 ng/μL.

Foreground selection

Among 12 *Ga1-S*-tightly-linked markers tested on parental lines, two markers showed distinct polymorphism for *Ga1-S* (Kostadinović et al., 2019). These two markers were used as foreground selection markers for the incorporation of *Ga1-S* gene (Table 1). Polymerase chain reaction (PCR) was carried out in 25 μL reaction volume containing: 1×enzyme buffer, 100 μM dNTP, 0.5 μM primers, 1U *Taq* polymerase and 20 ng DNA template. Amplifications were performed in thermocycler Biometra TProfessional Standard 96 with the following program: an initial denaturation at 95 °C/5min, followed by 35 cycles each of denaturation at 95 °C/1min, annealing at 55 °C/30s and extension at 72 °C/1min with final elongation at 72 °C for 10 min. The amplified fragments were resolved by 8% polyacrylamide gel electrophoresis (Mini Protean Tetra-Cell, BioRad). After staining with ethidium bromide, they were visualized under a UV transilluminator and documented in a gel documentation system (BioDocAnalyze, Biometra). The amplification products were determined based

on the positions of the bands relative to the donor (dominant allele) and recipient (recessive allele) parental lines used as controls.

Table 1. Primers used in foreground selection for the Ga1-S gene
Tabela 1. Prajmeri korišćeni u foreground selekciji za Ga1-S gen

Primer	Sequence
PR_1 F	5´-CAAATTGAGCCCATTACC-3´
PR_1 R	5´-TTCATTCTATTGCGGGTC-3´
PR_2 F	5´-GAGAGCTACGCACGACTTAT-3´
PR_2 R	5´-CAAGACTTGCACAATCGAGG-3´

Background selection

The set of 30 SSR markers polymorphic between parental lines were previously chosen for the background selection (Kostadinović et al., 2019). Polymerase chain reaction was carried out in 25 μL reaction volume containing: 1×enzyme buffer, 200 μM dNTP, 0.5 μM primers, 1U *Taq* polymerase and 20 ng DNA template. The following touch-down program (thermocycler Biometra TProfessional Standard 96) was performed: an initial denaturation at 95 °C/5min, followed by 15 cycles each of denaturation at 95 °C/30 s, annealing at 63.5 °C/1min (-0.5 °C/cycle) and extension at 72 °C/1min; another 22 cycles of 95 °C/30 s, 56 °C/1min and 72 °C/1min with final elongation at 72 °C for four min. The PCR products were separated by electrophoresis on 8% polyacrylamide gel, with a 20 bp molecular weight ladder as a marker. After staining with ethidium bromide, they were photographed under UV light using the Biometra BioDocAnalyze gel documentation system. SSR profiles were converted into a binary matrix based on the presence (1) or the absence (0) of a specific allele. Genetic similarity (GS) was calculated in accordance with Dice (1945): $GS_{ij} = 2a/2a+b+c$; where: a is the number of fragments present in both variety *i* and *j* (1,1), b is the number of fragments present in *i* and absent in *j* (1,0), c is the number of fragments absent in *i* and present in *j* (0,1). Marker data analyses were performed using the statistical NTSYSpc2 program package (Rohlf, 2000).

Results and Discussion

The initial step towards the *Ga1-S* incorporation into parental components of the white kernel hybrid was the identification of molecular markers for foreground and background selection. Twelve gene-specific SSR markers were previously tested on parental lines and two markers showed distinct polymorphism for *Ga1-S* (Kostadinović et al., 2019). Hence, they were used as foreground selection markers for the incorporation of *Ga1-S* gene.

Heterozygous individuals were distinguishable in BC_1, BC_2 and BC_3 generations with a percentage of approximately 50%, which follows the expected 1*GaGa*:1*Gaga* Mendelian ratio. Out of 90 BC_1 plants, PCR amplification with the specific markers identified 44 heterozygous individuals (48.9%) that were used for backcrossing and developing BC_2 generation. In BC_2, the same markers identified 56 out of 120 plants (46.7%) as heterozygous, while 57 out of 120 plants (47.5%) in BC_3 generation. Out of these BC_3 heterozygous individuals, 19 originated from RP_1, 20 from RP_2 and 18 from RP_3.

Once the heterozygotes were selected, our second goal was to identify individuals among them with the highest recurrent parent's genome (RPG) content with SSR markers distributed over the maize genome. Based on previously shown polymorphism between parental lines (Kostadinovic et al., 2019), 30 SSR markers were chosen for the background selection. Monomorphic markers were discarded due to the ina-

bility to distinguish the two parental genotypes and therefore they bear no value in selection work (Miah et al., 2015). The total number of alleles detected with these informative markers was 39 for RP_1, 49 for RP_2 and 40 for RP_3, average being 1.3, 1.63 and 1.33, respectively. Genetic similarity values between three analysed parental lines and their corresponding BC_3 progenies, calculated using the Dice coefficient, ranged from 0.87 to 0.99 (87-99% RPG). For each line separately, RPG values were 94-99% (RP_1), 87-99% (RP_2) and 95-99% (RP_3). Average values for the RPG content ranged from 92 to 97%.

The RPG recovery rates calculated using molecular markers provided realistic estimations rather than just the theoretical ratio of the recurrent parent percentage. Theoretically, the proportion of the RPG after n generations of backcrossing is given by $(2^{n+1} - 1)/2^{n+1}$ (Collard et al., 2005), but these percentages are usually lower with smaller population sizes that are typically used in actual plant breeding programs. In this study, 69% of progenies had RPG above this theoretical value and they were self-pollinated for allele fixation. Furthermore, there are progenies within all three lines with 99% RPG, which is the value theoretically achieved in the BC_6 generation, showing that marker-assisted backcrossing made a genetic gain in RPG recovery. In contrast, 31% of individuals had lower RPG content, which can be explained by the linkage between a target gene and nearby genes from the donor parent and/or chance (stochastic or nonrandom positions of chiasmata) (Semagn, 2006). In the research by Liu et al. (2014), *Ga1-S* individuals in the backcross segregating population were checked by crossing them as males onto *Ga1-S/Ga1-S* silks for seed-setting evaluation. Marker-assisted selection enabled eliminating this step and those individuals that do not carry the *Ga1-S* allele and thus greatly improved backcrossing efficiency. In our study, the RPG results confirmed the successful background selection. This represents another marker benefit that makes the conventional breeding program more efficient (Wu et al., 2022).

Plants with the highest RPG were self-pollinated to produce BC_3F_2 generation which was subjected to the foreground selection before flowering to identify homozygous dominant individuals. Out of 264 BC_3F_2 plants, 70 (26.5%) were dominant homozygotes, which is following the expected Mendelian ratio (1*Ga-*

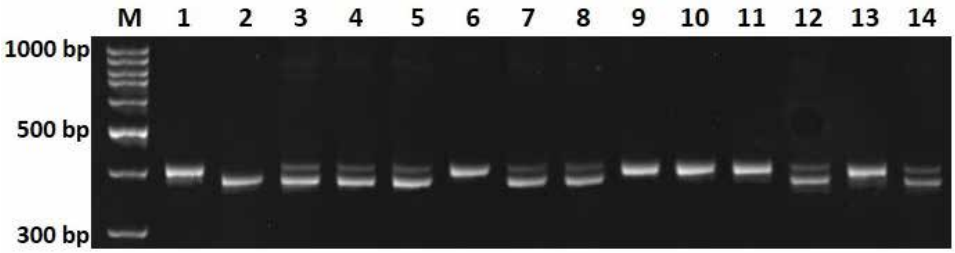

Figure 2. SSR profile of BC₃F₂ individual plants detected with the Ga1-S-specific marker. M: 100bp DNA ladder, 1: donor parent, 2: recurrent parent, 3-14: BC₃F₂ individuals (6, 9, 10, 11 and 13 - dominant homozygotes).
Slika 2. SSR profil BC₃F₂ pojedinačnih biljaka pomoću Ga1-S-specifičnog markera. M: 100 bp DNK marker, 1: donor linija, 2: rekurentni roditelj, 3-14: BC₃F₂ (6, 9, 10, 11 i 13 - dominantni homozigoti)

Ga:2*Gaga*:1*gaga*). For each RP line individually, the percentage of dominant homozygotes was also approximately 25% (27% in RP_1, 26.1% in RP_2, and 26.4% in RP_3). The co-dominant nature of the *Ga1-S*-specific marker is presented in Figure 2 where the homozygous dominant individuals (lanes 6, 9, 10, 11 and 13) were clearly distinguished. Homozygous dominant plants were self-pollinated to produce BC_3F_3 kernels for the cross-incompatibility evaluation.

Progenies of the BC_3F_2 homozygous dominant plants were planted in the next generation alternatively with a yellow-kernel inbred line of the same maturity. The material was left in free pollination, to check cross-incompatibility. Unfortunately, none of the dominant progenies

had a 100% white kernel. The quantity of yellow kernels on white lines was around 5%, so the process of MABB in this case was unsuccessful. The most likely explanation of our results, consistent with Lauter et al. (2022), could be the presence of modifier genes that increase or decrease the effectiveness of pollen exclusion or that, alternatively, modifiers were lost during the backcrossing. These authors also concluded that converting dent corn varieties to *Ga1-S* requires careful testing for their ability to exclude pollen. This could be achieved by confirmation of complete exclusion by several *ga1* lines until genetic markers for modifiers are identified.

Molecular markers used in our study are derived from random genomic regions that are closely linked to the gene of interest. They were developed by Liu et al. (2014), and Zhang et al. (2012) and proved to be very efficient in their backcrossing program, resulting in the complete cross-incompatibility. The main drawback of these markers is that their predictive value depends mainly on the known linkage phase between the marker and target locus (Lübberstedt et al., 1998). On the other hand, functional molecular markers (FMMs) are developed from the sequence polymorphisms present within a functional gene(s) which are associated with phenotypic trait variations. They are the diagnostics of desired traits and associated alleles that make them markers of choice for plant breeders who employ targeted marker-assisted selections for crop improvement (Kage et al., 2015). FMMs are not subjected to recombination, the polymorphism occurs within the target gene and fixes the favourable allele in the breeding population, and thus FMMs can also be utilized in the selection of complex traits (Salgotra and Stewart, 2020). It could be expected that successfully employed functional markers would outbalance the noted impediments and enhance MABB efficiency to transfer the desired gene(s) controlling simple or complex trait(s) into cultivated varieties.

Conclusion

Our study confirmed the efficiency of molecular markers in reducing the time and resources involved in the selection process. Using polymorphic SSR markers in background selection, heterozygotes with the highest percentage of recurrent parent's genome were successfully identified. Few individuals even had 99% RPG, the value theoretically achieved in the BC_6 generation, showing that MABB made a genetic gain in RPG recovery. Foreground selection markers were efficient in determining of the success rate of genomic region transfer, although complete cross-incompatibility to foreign pollens was not achieved. Therefore, functional markers developed from the sequence polymorphisms present within the target gene associated with phenotypic trait variations would enhance MABB efficiency to transfer the desired gene.

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UGRADNJA *GA1-S* GENA U RODITELJSKE LINIJE HIBRIDA KUKURUZA BELOG ZRNA POMOĆU MOLEKULARNIH MARKERA

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Sažetak

U Institutu za kukuruz "Zemun Polje" postoji program selekcije pomoću molekularnih markera (MAS) čiji je cilj ugradnja gena za inkompatibilnost *Ga1-S* u linije kukuruza koje su roditeljske komponente hibrida belog zrna. Glavni cilj ovog rada je bio stvaranje linija kukuruza belog zrna sa potpunom unakrsnom inkompatibilnošću sa stranim polenom pomoću molekularnih makera. Molekularnim markerima koji su blisko vezani za *Ga1-S* gen uspešno su identifikovane heterozigotne biljke (50%) nakon sva tri povratna ukrštanja. Rezultati utvrđivanja procenta genoma rekurentnog roditelja (RPG) u BC_3 generaciji pokazali su vrednost iznad očekivane kod 69% potomstva, dok je kod određenog broja RPG iznosio 99%, što predstavlja vrednost koja se teoretski dostiže u BC_6 generaciji. Od 264 BC_3F_2 pojedinačnih biljaka, 70 (26,5%) je bilo dominantno homozigotno, što je u skladu sa očekivanim, prema pravilima Mendelovog nasleđivanja. Potomstva BC_3F_2 dominantno homozigotnih biljaka posejana su sa kukuruzom žutog zrna iste grupe zrenja radi provere unakrsne inkompatibilnosti. Nijedno od dobijenih potomstava nije imalo 100% belo zrno, najverovatnije usled prisustva gena modifikatora koji menjaju efikasnost isključivanja polena, ili je došlo do gubljenja ovih gena prilikom povratnog ukrštanja. Moglo bi se očekivati da će uspešna primena funkcionalnih markera (stvorenih na osnovu polimorfizama sekvenci unutar samog gena vezanog za fenotipsku osobinu) prevazići uočene prepreke i poboljšati efikasnost MAS za prenos željenih gena koji kontrolišu prostu ili složenu osobinu u gajene kulture.

***Ključne reči*:** kukuruz, selekcija pomoću molekularnih markera, *Ga1-S*

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