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PARENTAL POLYMORPHISM ANALYSIS IN MARKER ASSISTED SELECTION FOR β-CAROTENE RICH MAIZE

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Abstract

Marker assisted selection (MAS) is widely implemented into modern grain breeding programs. Molecular markers are used in foreground selection to control the target gene, as well as in background selection to accelerate the reconstruction of the recurrent parent genotype. The best results have been achieved with the qualitative traits, regulated by the action of a single or several genes and clearly phenotypically defined. One successful example of MAS is the improvement of β-carotene content using *crtRB1* specific molecular marker. Maize Research Institute "Zemun Polje" has a breeding program aimed at conversion of standard maize to β-carotene rich genotypes adapted to temperate regions. The objectives of this study were to test the utility of gene-specific SSR marker in foreground selection and to identify polymorphic markers between parental lines to be used in background selection. Genetic variability between two standard and three high β-carotene parental inbred lines was analyzed with 40 SSRs distributed over the maize genom. Total number of alleles detected with 30 informative markers was 77, average being 2.57. The genetic similarity values calculated on Dice coefficient ranged from 0.49 to 0.66. Parental polymorphism for *crtRB1* showed a 543 bp fragment in donor lines, whereas a distinct 296 bp amplicon and a faint 1221 bp amplicon were generated in the recurrent parents. This marker will be used as foreground selection marker for the *crtRB1* gene in the conversion of standard maize to β-carotene enriched lines for growing in temperate regions.

Keywords: *β-carotene, crtRB1 gene, maize, marker assisted selection, SSR.*

Introduction

Maize is an important source of food and nutritional security for millions of people in the developing world, especially in sub-Saharan Africa, Latin America and in many countries in Asia (Shiferaw et al., 2011). However, most of the maize varieties do not contain enough provitamin A (ProVA), the precursor that leads to the formation of vitamin A. Consequently, diets mainly relying on maize may lead to vitamin A deficiency (VAD), primarily in nutritionally demanding periods of life, such as early childhood and pregnancy (Song et al., 2017). The World Health Organization has classified VAD as a public health problem, the leading cause of preventable childhood blindness, anaemia, and weakened host resistance to infection, which can increase the severity of infectious diseases and risk of death (WHO, 2009). Improvement of nutritional quality in food crops (biofortification) is a promising strategy to fight undernutrition, particularly among the rural poor in developing countries (Shiferaw et al., 2011). One of the most important breeding objective is improving the ProVA content of maize grain. Research under the HarvestPlus, Consultative Group on International Agricultural Research Challenge program designed to use breeding for crop biofortification, has provided significant leads for developing ProVA enriched maize (Harjes et al., 2008; Yan et al., 2010). Two genes have the most significant effect on ProVA concentrations in maize grain. One gene, *lcyE* on chromosome 8, encoding lycopene epsilon cyclase is associated with variation in the ratio of various carotenoids in the α- to β-branches of the carotenoid biosynthesis pathway (Harjes et al., 2008). Another gene, *crtRB1* on chromosome 10, encodes

β-carotene hydroxylase enzyme and it has a significant impact on variation for β-carotene concentration in the endosperm (Fu et al. 2013). Babu et al. (2013) reported that *crtRB1* gene had a much larger effect on ProVA concentration than *lcyE*.

CrtRB1 converts β-carotene into β-cryptoxanthin, whose ProVA activity is theoretically only half that of β-carotene. Natural genetic variation in *crtRB1* gene has been reported by Yan et al. (2010). That one favourable allele (*crtRB1* 3′TE) alone is responsible for effecting 2- to 10-fold increase of β-carotene concentration in maize kernel (Babu et al., 2013). The 3′TE (transposable element) polymorphism of the gene that spans the 6th exon and the 3′-UTR (untranslated region) generates three alleles associated with altering β-carotene accumulation: *allele 1* (543 bp; without TE insertion), *allele 2* (296 bp+875 bp; with 325 bp TE insertion) and *allele 3* (296 bp+1221 bp+1880 bp; with 1250 bp TE insertion). *Allele 1* of the *crtRB1* gene is favourable and increases the level of β-carotene, whereas *allele 2* and *allele 3* cause unfavourable effects (Yan et al. 2010).

Muthusamy et al. (2014) reported accelerated development of β-carotene enriched maize, using marker assisted selection (MAS) that holds immense promise as it selects desirable plants precisely and eliminates large scale biochemical estimation in the segregating generations. The introgressed inbreds possessing favourable allele of *crtRB1* can be used in the biofortification program. Moreover, improved hybrids with immense increase of b-carotene (>15 μg/g) can be directly utilised in alleviating VAD worldwide.

Maize Research Institute "Zemun Polje" (MRI) has a breeding program aimed at conversion of standard maize to β-carotene rich genotypes adapted to temperate regions. The main objective of the research presented herein was polymorphism analysis of parental lines used in MAS for β-carotene rich maize. The utility of the SSR marker specific to the *crtRB1* gene in foreground selection was tested. Also, genetic variability between parental lines was analyzed and a set of polymorphic SSR markers was selected to be used in background selection.

Material and Methods

Plant material

Three maize inbred lines provided by the International Maize and Wheat Improvement Center (CIMMYT) were used as the donor parents (DP_1, DP_2 and DP_3) of the favorable allele *crtRB1*-3′TE of the *crtRB1* gene. Two MRI commercial inbred lines were used as the recurrent parents (RP_1 and RP_2). These lines are adapted to the local environmental conditions in Serbia and due to their excellent combining abilities they are components of the leading MRI hybrids.

DNA extraction

Genomic DNA was isolated according to Doyle and Doyle (1987) from the four-weeks-old plants. Bulks were prepared by pooling an equal amount of leaf tissue taken from 20 leaves per line. The DNA was quantified using biospectrometer (BioSpetrometer kinetic, Eppendorf) and diluted to a working concentration of 20 ng/μl. The samples were stored at -20^{0}C until their application in polymerase chain reaction (PCR).

Polymorphism analysis for *crtRB1*

The set of primers used in polymerase chain reaction for the marker specific to the *crtRB1* is presented in Table 1.

Table 1. The set of primers used as a foreground selection marker for the *crtRB1* gene

Primer	Sequence
crtRB1-3′TE-F	5′-ACACCACATGGACAAGTTCG-3′
crtRB1-3′TE-R1	5′-ACACTCTGGCCCATGAACAC-3′
crtRB1-3′TE-R2	5′-ACAGCAATACAGGGGACCAG-3′

Polymerase chain reaction was carried out in 20 µl reaction volume containing: 1×enzyme buffer, 200 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 95ºC/5min, followed by 19 cycles each of denaturation at 95ºC/1min, annealing at 60ºC/1min (-0.5ºC/cycle) and extension at 72ºC/1min; another 19 cycles of 95ºC/1min, 55ºC/1min and 72ºC/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) at 80 V for 1.5 h. After staining with 0.5 µg/µl ethidium bromide, they were visualized under UV transilluminator and documented in gel documentation system (BioDocAnalyze, Biometra). The size of the amplification products was determined comparing with the 100 bp molecular weight ladder.

Genetic similarity analysis

Simple sequence repeat (SSR) analysis was done with 40 primer pairs spanning over the whole genome. PCR 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 95ºC/5min, followed by 15 cycles each of denaturation at 95ºC/30 s, annealing at 63.5ºC/1min (-0.5ºC/cycle) and extension at 72ºC/1min; another 22 cycles of 95ºC/30 s, 56ºC/1min and 72ºC/1min with final elongation at 72°C for four min. The PCR products were separated by electrophoresis on 8% polyacrylamide gel in 1 x TBE buffer, with 100 bp molecular weight ladder as a marker. Gels were run on small format (7.3 x 10 cm) vertical gel system (Mini Protean Tetra-Cell, BioRad), at 80 V for 1.5 h. After staining with 0.5 µg/µl ethidium bromide they were photographed under UV light using 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 - the number of fragments present in both variety *i* and *j* (1.1),

b - the number of fragments present in *i* and absent in *j* (1.0),

c - the number of fragments absent in *i* and present in *j* (0.1).

Marker data analyses were performed using statistical NTSYSpc2 program package (Rohlf, 2000).

Results and Discussion

The results presented here are a part of the MRI project on conversion of standard maize to β-carotene enriched lines for growing in temperate regions trough marker assisted backcross breeding. Being the carriers of the favorable allele *crtRB1*-3′TE, three maize inbred lines provided by CIMMYT were used as the donor parents (DP_1, DP_2 and DP_3). As the recurrent

parents, to be the recipients of favourable allele and to improve the β-carotene content, two comercial lines were selected (RP_1 and RP_2).
The first goal of this study was to test the utility of SSR marker specific to the *crtRB1*gene in foreground selection. In any particular cross, it is necessary to run the markers first on the two parents, to confirm the polymorphisms (size differences) for the marker alleles. Similar to Muthusamy et al. (2014), distinct marker polymorphism was observed between the donor and recurrent parents for *crtRB1* gene. The donor parents carried the favorable allele with a 543 bp band (*allele 1*), while the recurrent parents exhibited the unfavorable allele with a 296 bp+1221 bp band (*allele 3*). An illustration of the amplification with this gene specific SSR marker is given in Figure 1.

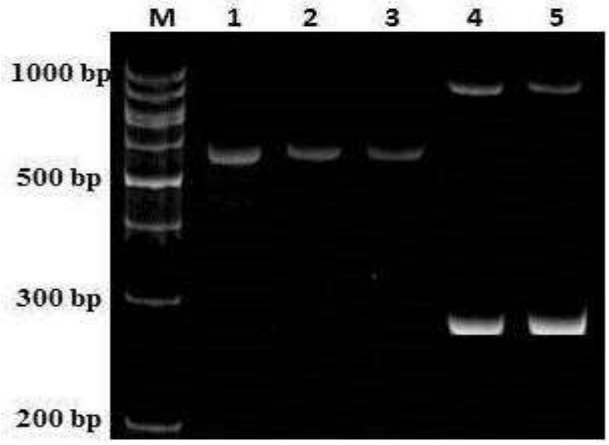

Figure 1. Parental polimorphism with the *crtRB1*-specific SSR marker. M: 100 bp DNA ladder, 1-3: donor parents DP_1, DP_2 and DP_3, 4-5: recurrent parents RP_1 and RP_2.

Being the codominant marker, *crtRB1*-3′TE can detect homozygous dominant, heterozygous and homozygous recessive plants separately. Therefore, it will be used in marker assisted selection for the *crtRB1* gene. Heterozygous plants (*allele 1*/*allele 3*) will be selected in the BC_1 and BC_2 generations, while homozygotes (*allele 1*/*allele 1*) will be selected in the BC_2F_2 generation. Plants without favourable allele will be discarded prior to pollination, hence the size of the breeding population will be reduced. As stated in Choudhary et al. (2016), assessment of genetic relationships among the inbreds is key to success of hybrid breeding programme. Genetic diversity analyses based on molecular markers proved to be very effective in this assessment. This can be extremely helpful for identifying the most appropriate parental lines to be crossed (Ribaut and Hoisington, 1998). Consequently, another goal of this paper was to estimate genetic similarity between parental lines selected for MAS for β-carotene rich maize. Donor and recurrent parent lines were analyzed using 40 SSRs distributed throughout the maize genome. Total number of alleles detected with 30 informative markers was 77, average being 2.57, which is similar to those previously reported in maize inbreds (Maphosa et al., 2011; Bante and Prasanna, 2003; Choudhary et al., 2016). These polymorphic SSR markers will be employed for identification of the genotypes with the highest proportion of recurrent parent genome in BC_2 generation. The pairwise genetic similarity values calculated on Dice coefficient ranged from 0.49 (D_2/RP_2) to 0.66 (D_2/RP_1) (Table 2). These results enable identification of potential high β-carotene hybrid combinations for the biofortification programme. As concluded in Choudhary (2016), genetically diverse and contrasting inbred lines can be used in developing segregating mapping populations for identifying modifier genetic loci influencing β-carotene accumulation.

Table 2. The pairwise genetic similarity values calculated on Dice coefficient between donor (DP_1, DP_2 and DP_3) and recurrent parent lines (RP_1 and RP_2).

	D_1	**D_2**	**D_3**
RP_1	0,62	0,66	0,65
RP_2	0,57	0,49	0,65

Conclusions

The *crtRB1*-3′TE marker showed codominant polymorphism between the donor and recurrent parents. Therefore, it will be used in foreground selection for the *crtRB1* gene. Selected polymorphic SSR markers will be employed in background selection to recover the recurrent parent genome. The results of this analysis constitute the framework for marker assisted introgression of the favourable allele of *crtRB1* into the local maize genotype.

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