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MARKER-ASSISTED SELECTION FOR THE GENE OF β-CAROTENE HYDROXYLASE IN MAIZE

Aim. Estimation of the allelic status of marker *crt*RB1-3'TE of the β-carotene hydroxylase gene and marker-assisted selection by this marker in Ukrainian maize breeding material. ***Methods***. Field method and polymerase chain reaction. ***Results***. The analysis of the allelic state of β-carotene hydroxylase gene for marker *crt*RB1-3'TE in maize breeding populations $(DK23\times F2)F_2$ and $(DK23\times F2)F_3^{MAS}$ having been obtained after the first and second self-pollinations of single cross DK23×F2 was provided. It was established that the parental inbred lines DK23 and F2 contained respectively 296 bp (unfavorable) and 543 bp (favorable) alleles of marker *crt*RB1-3'TE. The three kinds of genotypes appeared to present at different frequencies in $(DK23\times F2)F_2$ – homozygous for allele 296 bp, homozygous for allele 543 bp and heterozygous with both alleles 296 bp and 543 bp. For further cultivation and self-pollination, only plants with allele 543 bp within $(DK23\times F2)F_2$ were selected. All tested plants in population $(DK23\times F2)F_3^{MAS}$ were homozygous for allele 543 bp. ***Conclusions***. Marker-associated selection in two generations for the β-carotene hydroxylase gene, involved in β-carotene accumulation, allowed to select homozygous plants of maize by favorable *crt*RB1-3'TE allele.

Keywords: *Zea mays* L., molecular genetic markers, carotenoids, breeding populations, allele.

In recent years, there has been a growing need in the world for high quality and safe food. At the First Global Conference in November 2010 in Washington, USA, dedicated to the problem of food quality it was proposed to use actively the selection of high yielding crops for increased nutritional value, ensuring the bioavailability of vitamins and minerals [1].

According to K. P. Jr. West [2], T. J. Safawo et al. [3] and K.V. Pixley et al. [4] 127 million preschool children in the world are deficient in vitamin A, in particular in Ukraine 24% of preschool children have such deficiency [1]. Increasing the content of β-carotene in cereals (biofortification on provitamin A) will help to overcome this problem with the least economic cost [4].

Carotenoids are yellow, orange or red plant pigments which present in the leaf apparatus of photosynthetic plants as well as in fruits of flowering plants. Mutant plants devoid of carotenoids are generally not viable [5-7]. Among the various forms of carotenoids, only β-carotene is the precursors to vitamin A in humans and animals, which makes them particularly valuable.

Increasing the content of carotenoids in the grain of crops, in particular maize, is possible via bio-fortification [8, 9] with the involvement of techniques of marker-associated selection (MAS). The testing by molecular genetic markers allows to select the initial breeding material with favorable alleles [6, 10-13]. To increase significantly the content of carotenoids in maize is also possible with genetic engineering methods [14, 15].

The biosynthesis of carotenoids in maize is carried out in a complex pathway [16]. Geranyl-geranyl pyrophosphate is the precursor of biosynthesis of any carotenoids. From the geranyl-geranyl pyrophosphate, the first coloured substance lycopene, is synthesized in a series of intermediate reactions under the enzymes phytoene synthase, phytoene desaturase, ζ-carotene desaturase, ζ-carotene isomerase and carotenoid isomerase. By lycopene ε-cyclase, the cyclization of lycopene occurs with the formation of cyclic carotenoids. The branching of the carotenoid biosynthesis pathway into ε- and β-branches leads to the formation of β-carotene and its

derivatives as well as α-carotene and its derivatives, respectively [6, 16, 17].

The synthesis of α-carotene begins with the synthesis of δ-carotene by lycopene ε-cyclase, which catalyzes the formation of a ε-ionone ring. Further, lycopene β-cyclase synthesizes α-carotene with a β-ionone ring. Therefore, α-carotene comprises one ε- and one β-ring. Unlike α-carotene, β-carotene has two β-ionone rings, which are formed in two stages. Initially, with the help of the enzyme lycopene β-cyclase, γ-carotene is formed, and then, under the same enzyme, β-carotene is synthesized. Further the process of xanthophylls formation begins with hydroxylation of ε- and β-rings of carotene, respectively, by ε- and β-hydroxylases. In the ε-branch, with the participation of β-hydroxylase, zeinoxanthin is first formed and then lutein. The β-branch under β-carotene hydroxylase forms β-cryptoxanthin, and then zeaxanthin. The final reaction is the conversion of zeaxanthin to abscisic acid, which occurs in low light or in the dark [3, 6, 16-18].

For β-carotene accumulation it is very important that lycopene would participate in symmetric cyclization, that is, follow the β-branch. This requires that the expression of genes involved in α-carotene biosynthesis would be inhibited but genes responsible for β- and γ-carotene synthesis would be activated. For maize, such a key gene is the gene of lycopene ε-cyclase (*lcy*ε), allelic variants of which have been studied in details in [16, 19-22].

The β-carotene hydroxylase gene (*ctr*RB1) is also crucial for β-carotene accumulation, as it controls the conversion of β-carotene to β-cryptoxanthin. Three polymorphic regions of this gene were found to influence the quantitative values of β-carotene accumulation in maize: 5'TE, InDel4 and 3'TE. These regions are used as molecular genetic markers for the selection of plants with a favorable allelic status of the *crt*RB1 gene. Polymorphism at the 3'TE region is formed by the action of a transposon, which leads to the formation of three possible allelic states: allele 1 (543 bp), allele 2 (296 bp + 875 bp) and allele 3 (296 bp). The favorable for the accumulation of β-carotene in the mature maize grain is the allele 1 (543 bp). In its presence, the content of β-carotene in maize endosperm can be doubled, so identification of this allele among maize breeding materials has great prospects [11, 17, 20, 21].

Three primers were used to determine the allelic state of the maize β-carotene hydroxylase gene by the marker *crt*RB1-3′TE: F, R1, and R2. According to J. Yan et al. [17], primers F and R2 identify the intact *crt*RB1-3′TE region and form a single amplicon (allele 543 bp). Primer R1 recognizes the insertion of the 3′TE transposon within the *crt*RB1 gene and generates more than one fragment – alleles 296 bp + 875 bp and 296 bp [17].

As polymorphism of genes of carotene pathway has not been sufficiently investigated in maize, the purpose of this work was to evaluate the allelic status of marker *crt*RB1-3'TE of the β-carotene hydroxylase gene and to carry out marker-assosiated selection by this marker in Ukrainian maize breeding material.

Materials and methods

As the material for the investigation the perspective inbreds of *Zea mays* L. DK23 and F2 were used as well as populations $(DK23\times F2)F_2$ and $(DK23\times F2)F_3^{MAS}$. The population $(DK23\times F2)F_2$ was obtained by self-pollination of single cross $(DK23\times F2)F_1$. After MAS in $(DK23\times F2)F_2$ only plants with favorable allele status of marker *crt*RB1-3'TE of the carotene hydroxylase gene were self-pollinated. The seeds after this second self-pollination were collected and they formed the population $(DK23\times F2)F_3^{MAS}$.

Maize plants were grown in the field under standard techniques of field experiments [24]. Germination of the seeds to obtain seedlings was carried out by the laboratory method on filter paper at a temperature of 26°C for 5 days.

The determination of the allelic status of the marker *crt*RB1-3'TE was performed by the polymerase chain reaction method.

For isolation of genomic DNA of inbreds DK23 and F2 5-7-day seedlings were involved. For isolation of genomic DNA and MAS within the population $(DK23\times F2)F_2$ leaves of adult field plants were used. Isolation of genomic DNA and MAS in the population $(DK23\times F2)F_3^{MAS}$ was made with 5-7-day seedlings. Individual seedlings of $(DK23\times F2)F_3^{MAS}$ with favorable allele of marker *crt*RB1-3'TE were then planted into the soil for further field cultivation and self-pollination.

Determination of the allelic state of the *crt*RB1 gene by the marker *crt*RB1-3'TE was performed for inbreds DK23 and F2 in a mixture of DNA from five plants but for populations $(DK23\times F2)F_2$ and $(DK23\times F2)F_3^{MAS}$ – in DNA of 17 and 20 individual plants respectively.

DNA extraction was performed by a modified CTAB method [23]. To identify the allelic state of the *crt*RB1-3'TE marker, the direct primer F: 5'ACACCACATG GACAAGTTCG3 ', the

reverse primers R1: 5'ACACTCTGGCCCATGAA-CAC3' and R2: 5'ACAGCAATACAGGGGA-CCAG3' were used [5, 17, 22].

The PCR-reaction was carried out in 20 µl of a reaction mixture containing: 2.0 µl of DNA of the tested plants, 1.0 µl of each primer, 2.0 µl mix of dNTP, 2.0 µl of Green Taq buffer, 0.15 µl of Taq polymerase and 10.85 µl of deionized water.

The amplification products were separated via electrophoresis in 1% agarose under 120V in 1 hour. DNA ladder of 100 bp was used to determine amplicon sizes. The analysis of electrophoresis results was performed with GelDoc (BioRad) and its special software.

The frequency of plants with specific allele status of marker *crt*RB1-3'TE of β-carotene hydroxylase gene was calculated as the percentage of a number of plants with specific allele status to the total number of tested plants.

Results and discussion

Maize parental inbred line DK23 contained an allele of 296 bp but parental inbred line F2 contained an allele of 543 bp of molecular genetic marker *crt*RB1-3′TE.

The results of PCR analysis of the allelic status of β-carotene hydroxylase gene by the *crt*RB1-3'TE marker for maize breeding populations $(DK23\times F2)F_2$ and $(DK23\times F2)F_3^{MAS}$ are presented in figures 1, 2 and table.

Polymorphism at the marker *crt*RB1-3'TE was observed among 17 plants of population $(DK23\times F2)F_2$ obtained after self-pollination of cross DK23xF2. Two versions of the allelic state of this marker were identified in $(DK23\times F2)F_2$ and respectively the frequencies of three genotypes were calculated: plants homozygous for allele 543 bp (favorable) were met with frequency of 47%; plants homozygous for allele 296 bp (unfavorable) – with frequency of 41%; heterozygous plants with 543 bp + 296 bp were detected at a frequency of 12%. Plants with allele 296 bp + 875bp were not found.

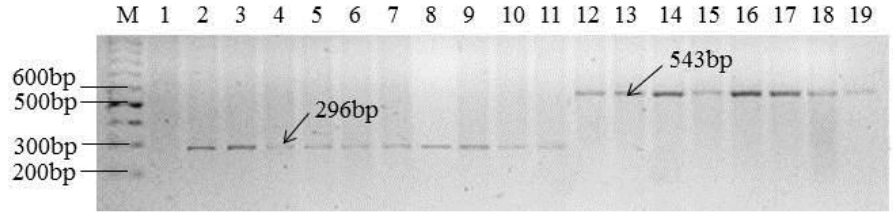

Fig. 1. Allele status of individual plants in maize population. $(DK2\times F2)F_2$ at marker *crt*RB1-3'TE of gene of **β**-carotene hydroxylase: M – molecular weight marker, 100 bp; 1 – control without DNA, 2-3 – plant 1, 4-5 – plant 2, 6-7 – plant 3, 8-9 – plant 4, 10-11 – plant 5, 12-13 – plant 6, 14- 15 – plant 7, 16-17 – plant 8, 18-19 – plant 9.

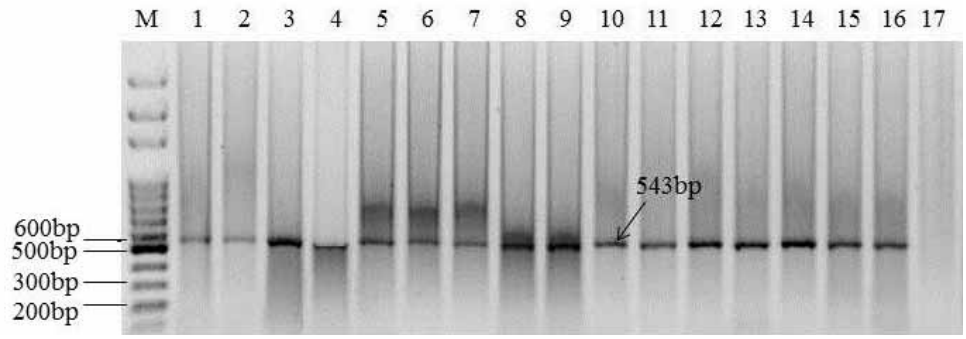

Fig. 2. Allele status of individual plants in maize population. $(DK2\times F2)F_3^{MAS}$ at marker *crt*RB1-3'TE of gene of **β**-carotene hydroxylase: M – molecular weight marker, 100 bp; 1-2 – plant 1, 3-4 – plant 2, 5-6 – plant 3, 7-8 – plant 4, 9-10 – plant 5, 11-12 – plant 6, 13-14 – plant 7, 15 -16 – plant 8, 17 – control without DNA.

Table. Allelic variation of the gene of β-carotene hydroxylase at the marker *crt*RB1-3'TE in maize breeding material

Genotype / Trait	DK23	F2	$(DK23\times F2)F_2$	$(DK23\times F2)F_3^{MAS}$
Number of tested plants, pcs.	5	5	17	20
Frequency of homozygous plants for allele 543 bp (favorable),%	0	100	47	100
Frequency of homozygous plants for allele 296 bp (unfavorable),%	100	0	41	0
Frequency of heterozygous plants (543 bp, 296 bp),%	0	0	12	0

Note. Plants with allele 296 bp + 875p were not found.

Only those plants within $(DK23\times F2)F_2$ which contained favorable allele 543 bp in homozygous state were selected for further cultivation and self-pollination. The grain obtained from self-pollinated plants of $(DK23\times F2)F_2$ was harvested in the fall of 2018. In the spring of 2019, these seeds were germinated on moistened filter paper at a temperature of 26°C for 5 days and the seedlings of $(DK23\times F2)F_3$ were obtained. About 600 mg of young leaves of each seedling $(DK23\times F2)F_3$ were taken for PCR identification of the allelic state of the marker *crtRB1*-3′TE but each remainder seedling with caryopsis were allowed to continue to grow on moistened filter paper. All tested twenty plants of $(DK23\times F2)F_3$ appeared to have allele 543 bp and the frequency of plants homozygous for allele 543 bp (favorable) amounted to 100% (fig. 2, table). So this population of $(DK23\times F2)F_3$ was indicated as $(DK23\times F2)F_3^{MAS}$.

All plants of $(DK23\times F2)F_3^{MAS}$ were field cultivated and self-pollinated in summer 2019. In autumn 2019 the seeds from this self-pollination were harvested. Embryos in these seeds have genotype $(DK23\times F2)F_4^{MAS}$. They will be used in further cycle of MAS at the stage of seedlings and the propagation of homozygous (543 bp) plants via self-pollination to obtain finally a maize inbred line with homozygous favorable allele state of β-carotene hydroxylase gene at the marker *crt*RB1-3′TE.

Conclusions

The study showed the possibility and effectiveness of marker-assisted selection for screening genotypes with the favorable allele of the β-carotene hydroxylase gene at molecular marker *crt*RB1-3′TE in selection for increased β-carotene content in maize. The allelic state of the *crt*RB1 gene at the marker *crt*RB1-3′TE and the frequency of occurrence of homozygous and heterozygous plants in maize populations $(DK23xF2)F_2$ and $(DK23xF2)F_3^{MAS}$ were evaluated. As a result of marker-assisted selection within the population $(DK23xF2)F_2$ at the favorable allele of the marker *crt*RB1-3'TE individual plants were selected and self-pollinated They gave rise to the population $(DK23xF2)F_3^{MAS}$ with a frequency of homozygous plants for a favorable allele of 100%.

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МАРКЕР-АСОЦІЙОВАНА СЕЛЕКЦІЯ ЗА ГЕНОМ β-КАРОТИНГІДРОКСИЛАЗИ У КУКУРУДЗИ

Мета. Оцінка алельного стану маркера *crt*RB1-3'TE гена β-каротингідроксилази та маркер-асоційована селекція за цим маркером у вітчизняному селекційному матеріалі кукурудзи. ***Методи***. Польовий, полімеразна ланцюгова реакція. ***Результати.*** Проведено аналіз алельного стану гена β-каротингідроксилази за маркером *crt*RB1-3'TE у популяціях кукурудзи (ДК23xF2)F_2 та (ДК23xF2)F_3^{MAS}, отриманих після першого та другого самозапилення гібрида ДК23xF2. Встановлено, що батьківські інбредні лінії ДК23 і F2 містять алелі маркера *crt*RB1-3'TE відповідно 296 п.н. (несприятливий) і 543 п.н. (сприятливий). Три варіанти генотипів були присутні з різною частотою в популяції (ДК23xF2)F_2 – гомозиготні за алелем 296 п.н., гомозиготні за алелем 543 п.н. та гетерозиготні, з обома алелями – 296 п.н. та 543 п.н. Для подальшого культивування та самозапилення серед (ДК23xF2)F_2 були відібрані тільки рослини з алелем 543 п.н. Усі протестовані рослини в популяції (ДК23xF2)F_3^{MAS} були гомозиготні за алелем 543 п.н. ***Висновки.*** Маркер-асоційована селекція протягом двох поколінь за геном β-каротингідроксилази, задіяному в накопиченні β-каротину, дозволила відібрати гомозиготні рослини кукурудзи за сприятливим алелем маркера *crt*RB1-3'TE.

Ключові слова: *Zea mays* L., молекулярно-генетичні маркери, каротиноїди, селекційні популяції, алель.