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MARKER-TRAIT ASSOCIATION ANALYSIS OF FUNCTION GENE MARKERS FOR CAROTENOIDS ACROSS DIVERSE MAIZE INBRED LINES

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ABSTRACT

The carotene is a very important nutrient that provides the activity of molecular and biochemical processes in cells. Therefore, the estimation of genetic determinants involved in the management of the carotene synthesis is necessary for the selection of maize. DNA markers allow to select lines with high carotenoids content in grain for breeding purposes. 110 maize lines with a high content of carotenoids in grain using DNA markers *lcyε*-5'TE, *lcyε*-SNP216, *lcyε*-3'INDL and *crtRB1*-3'TE were studied. The dynamic and carotenoid content in simple hybrids and lines were determined by the absorption spectrum of β-carotene. Carotenoid content in grain of the lines under study ranged from 0.7 to 7.15 μg/mg. The highest values of the average content of carotene were obtained in the maize lines containing favourable alleles for the marker combinations *lcyε*-SNP216+*lcyε*-3'INDL and *lcyε*-SNP216+*crtRB1*-3'TE. For the marker *lcyε*-5'TE, no favourable allele was found among the studied genotypes. With the aid of spectrometry, the dynamics of carotenoid accumulation as a function of the grain development stage. Among the lines under study, the highest carotenoid content was recorded on the 15th and 23rd days after pollination (DAP). For the genotypes involved in the study, the total content of carotenoids in hybrids was higher than in their parent components. Therefore, DNA markers *lcyε*-SNP216, *lcyε*-3'INDL and *crtRB1*-3'TE are informative for assessing the maize genotypes for high carotenoid content in grain.

Keywords: maize, carotenoid, DNA markers, function marker

INTRODUCTION

Carotenoids are a widespread class of yellow, orange and red pigments. They are contained in bacteria, fungi, algae, plants and animals. Today, more than 800 types of carotenoids are known (**Koyama and Fujii, 1999; Karnauhov 2000; Berman *et al.*, 2017**). β-carotene, which contains two β-ion fragments, has the highest biological activity. All such substances are hydrocarbons and belong to the group of carotene. β-carotene was first isolated in 1831 from carrots by Wacenroeder. This substance was in the focus of biologists' and physicians' attention due to the obvious property, that is the formal division into two equal parts resulting in two molecules of retinal or retinol (vitamin A) from one molecule (**Gallagher *et al.*, 2004; Joyard*et al.*, 2009; Ilioaia*et al.*, 2011**).

Nowadays, a biological (selective genetic) approach to the natural increase of vitamins and trace elements in plants, which is known as biofortification, has been formulated. The ultimate goal of this strategy is to create plants with a high content of certain nutrients (including those that are not specific to them) or with reduced content or even lack of undesirable compounds (**Graham and Welch., 1996; Burlaka and Sorochynskyi, 2010; Rodriguez-Amaya and Kimura, 2004**). In 2010, the First Global Conference on Biofortification was held in Washington, DC. In the conference, it was concluded that quality food can be provided with the help of breeding high-yielding crops of high quality, bioavailability and efficiency of vitamins and minerals from high-yielding plant lines (varieties) (**Sajilata *et al.*, 2008; Zhukov *et al.*, 2013**). On the background of the general state of 'latent starvation', the shortage of carotenoids (provitamin A) is becoming a one of majority problem.

Maize is one of the most common cereal, fodder and silage crops in the world. According to the State Statistics Service of Ukraine, the sown area of maize over the past five years accounted for 4.5–4.8 million hectares. It is the third crop in a group of cereals after wheat and barley (**Markovskyi*et al.*, 2014, Agriculture of Ukraine, 2018**). Maize contains a large number of nutrients; therefore, it is one of the leaders in consumption among cereals, a source of oil, flour, cereals, starch, ethyl alcohol, dextrin, glucose, vitamin E, ascorbic and glutamic acid and other substances (**Zhukov *et al.* 2013; Tyutyaev *et al.* 2015**). Maize is known for containing a wide range of carotenoids, including precursors of vitamin A (α-carotene, β-carotene and β-cryptoxanthin), however, in low concentrations (on average, 0–1.30, 0.13–2.70 and 0.13–1.90 nmol/g, respectively) (**Tiwari *et al.*, 2012; Xu *et al.*, 2009**).

Biosynthesis of carotenoids in maize occurs during seed maturation. Accumulated carotenoids make endosperm yellow or orange (**Li *et al.*, 2008**). In plant cells, carotenoids are localized in plastids in the form of globules, crystals, protein-carotenoid complexes that are part of membrane structures (**Deynekaet *al.*, 2008; Azmachet *al.*, 2013**). Accumulation of carotenoids in plants in qualitative and quantitative terms depends on both plant species (genus, family) and the variety (**Wang et al., 2013**).

To date, in general, biochemical pathways of the synthesis of carotenoids in cells have been revealed. This is a complex multi-stage process under the control of several genetic systems of the plant genome. Researchers have identified the major enzymes involved in maize carotenogenesis, their genetic determinants, and also established their localization on chromosomes (**Kreuzet *al.*, 1982; Ytterbergt *al.*, 2006; Joyardet *al.*, 2009; Baiet *al.*, 2009; Wang et al., 2013**).

The main genes related to the accumulation of carotenoids in maize grain include the lycopene ε-cyclase (*lcyε*) and β-carotene hydroxylase (*crtRB1*) (**Li *et al.*, 2008**). The gene of lycopene ε-cyclase is located on a chromosome of 8 bin 5; it has a length of 3631 bp and consists of 10 exons. Three polymorphic sites have been identified in this gene: an indel at the 3'-end (marker *lcyε*-3'INDL), single nucleotide polymorphism (marker *lcyε*-SNP216) and an indel at the 5'-end (marker *lcyε*-5'TE) (**Harjeset *al.*, 2008; Fu *et al.*, 2013; Muthusamyet *al.*, 2015; Zunjare et al., 2018**).

The gene of β-carotene hydroxylase 1 is located on a chromosome of 10 bin 5; it has a length of 3007 bp and consists of 8 exons. The polymorphism of *crtRB1* gene is determined with the *crtRB1*-3'TE marker. Three alleles with varying length of amplification fragments have been identified for the marker *crtRB1*-3'TE: 543 bp (allele 1), 296+875 bp (allele 2) and 296 bp (allele 3) (**Sagareet *al.* 2015; Orlovskayaet *al.*, 2016; Sagareet *al.*, 2018**). Allele 1 is known to be favourable for a high content of β-carotene (**Robert, 1999; Zhou *et al.* 2012; Liu *et al.*, 2015**). The purpose of the study was the evaluation the allele state of carotenogenesis genes and determintion the total content of carotenoids in grain of Ukrainian maize lines depending on the combination of favourable alleles and the dynamics of carotenoid accumulation.

MATERIAL AND METHODS

The study involved 110 maize inbred lines and 4 maize simple hybrids from the Institute of Grain Crops of the National Academy of Agricultural Sciences (Dnipro, Ukraine). 56 lines are pure heterotic groups: 16 lines are belonged to

Iodent, 14 – to EF, 10 – to Lancaster, 10 – to Raid, 6 – Lacaune and 54 lines are represented by mix of heterotic groups. Accessions included 108 lines with yellow and 2 lines with white grain of the 2015 harvest year. The studied hybrids were obtained by cross the following lines: DK296×DK253; DK272×DK633/266; DK231×DK366; DKD3264×RNR60. The study was carried out in 2016–2018 at the Biotechnology Laboratory of the Institute of Grain Crops and in the Department of Laboratory Research on the Qualification Examination of Plant Varieties (Centre for Certification Tests) of the Ukrainian Institute for Plant Varieties Examination (Kyiv, Ukraine).

DNA isolation and PCR

DNA isolation was performed according to **Velikov (2013)**, using CTAB (cetyltrimethylammonium bromide). This method is included the follow stages of DNA extraction: the lysis of cell wall and membranes with CTAB, purification DNA solution with chloroform-isoamyl alcohol and ethanol solution (70%), dissolving DNA in TE buffer (buffer solution which is contained Tris and EDTA). For DNA isolation, 100 mg of 5-day maize sprouts were used. The primers sequences to functional markers (three markers for the *l*cyε gene **(Harjes*et al.*, 2008; Muthusamy*et al.*, 2014**) and one for *crtRB1* gene (**Muthusamy *et al.*, 2015**) are shown in Table 1.

Table 1 Sequences of primers to functional markers

Marker name	Thenucleotidesequence of primers 5'→3`	Expected amplicones size (bp)
*l*cyε-5'TE	F aagcatccgaccaaaataacag R gagagggagacgacgagacac	650* 250
*l*cyε-SNP216	F gcggcagtgggcgtggat R tgaagtacggctgcaggacaacg	234+395 234*
lcyε-3'INDL	F1 gtacgtcgttcatctccgtaccc R1 cttggtgaacgcatttctgttgg F2 ggaccggaacagccaactg R2 ggcgaaatgggtacggcc	144+502* 399+502
crtRB1-3'TE	F acaccacatggacaagttcg R1 acactctggcccatgaacac R2 acagcaatacaggggaccag	543* 296+875 296

Note: * Favourable allele

To identify the single nucleotide polymorphism by the marker *lcyε*-SNP216, internal control of the reaction, i.e. a pair of primers to the alcohol dehydrogenase (adh1, can be detected in the electropherograms a 234-bp fragment) was used. Thus, in the case of detecting a favourable allele that corresponds to the presence of a marker of nucleotide G on the site, only one 234 bp allele was identified in the electropherogram.

A reaction solution of 20 μL contained: 1×DreamTaqTMGreen buffer, 1 U of DreamTaqTM polymerase (ThermoScientific), 200 μM of each dNTP, 30 ng of DNA sample and 0.2 μM of the primer under study. Polymerase chain reaction (PCR) was performed according to the following protocol: Stage 1 (initial denaturation): 94 °C for 5 min; Stage 2 (obtaining specific reaction products), 30 cycles of: 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (elongation); Stage 3: 72°C for 5 min (final elongation). To visualize the amplification products, electrophoresis was performed in 2% agarose gel, and the results were recorded using visualization device GelDocTM EZ (UK). The size of the obtained alleles was determined and their frequency was calculated (**Sivolap*et al.*, 1998**).

Determination of carotenoid content by spectrophotometry

The determination of carotenoid content was carried out in different number of DAP (days after pollination). Two maize lines with yellow endosperm colour (DK411 and DK772) were used in the study. Total carotenoid content was determined by the absorption spectrum of β-carotene. Carotenoid content of maize grain (stored for one year under darkroom conditions and 14% humidity at the time of the analysis) was measured in chloroform spectra according to **Rodriguez-Amaya and Kimura (2004)**. The optical density was measured at a wavelength of 450 nm on spectrophotometer SF 2000 (Russian Federation) using chloroform as a control. The research was carried out at the Biotechnology Laboratory of the Institute of Grain Crops of the National Academy of Agricultural Sciences in 2016. To determine the best combination of alleles that would characterize genotypes with high carotenoid content in grain, a dispersion analysis was applied with the evaluation of the least significant difference ($LSD_{0.5}$) at the significance level of 0.5 % with STATISTICA 10.0 (trial version) (**Drozdov, 2010; Fortin *et al.*, 2014**).

RESULTS AND DISCUSSION

Allele state of carotenogenesis markers

Both genes of lycopene ε-cyclase (*lcyε*) and β-carotene hydroxylase 1 (*crtRB1*) are polymorphic and their allele variants can be considered as genetic markers for characterizing gene pools or individual plant genotypes (**Harjes, 2008; Muthusamy*et al.*, 2015, Yan *et al.*, 2010**). The genetic analysis in this study revealed polymorphism in most of the studied sites.

For the marker *lcyε*-5'TE, it is possible to obtain two allele variants, favourable (650 bp) and unfavourable (250 bp) (**Muthusamy*et al.*, 2015**). No polymorphism was detected for the marker *lcyε*-5'TE (Fig. 1).

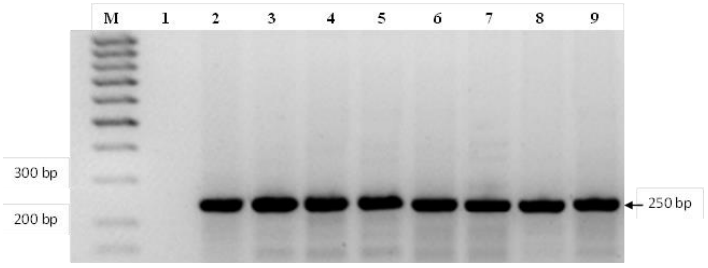

Figure 1 Molecular characterization of maize inbred lines using primer *lcyε*-5'TE: *M* molecular weight marker Thermo Scientific O'RangeRuler 100 bp DNA Ladder; *1* control; *2–9* products of DNA amplification of maize lines ISK17, ISK18, ISK19, ISK20, ISK21, ISK22, ISK23 and ISK24.

Electrophoretic distribution of the amplicons showed that 250 bp allele was identified in lines ISK17, ISK18, ISK19, ISK20, ISK21, ISK22, ISK23, ISK24 (Fig. 1) and in all other analysed maize lines under examination. Consequently, in view of the lack of polymorphism, no favourable allele of 650 bp was found in this study.

For the marker *lcyε*-SNP216 and primers for internal control of the reaction (gene *adh*), alleles of 234 and 395 bp were detected. The results of the electrophoretic distribution of amplicons for lines DK411, DK257, DK742, DK325, DK744, DK680, DK296, DK267, DK633 and DK366are shown in Fig. 2.

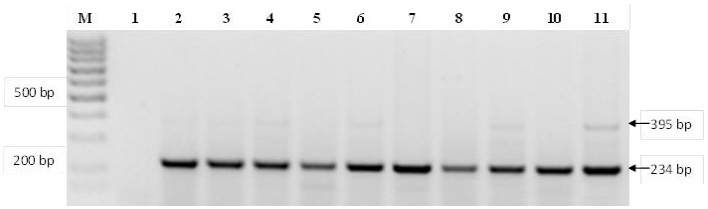

Figure 2 Molecular characterization of maize inbred lines using primer *lcyε*-SNP216: *M* molecular weight marker Thermo Scientific O'RangeRuler 100 bp DNA Ladder; *1* control; *2–11* products of DNA amplification of maize lines DK411, DK257, DK742, DK325, DK744, DK680, DK296, DK267, DK633 and DK366.

The presence of a 234 bp allele (internal control of the reaction) indicates the efficiency of PCR of studied DNA samples of maize lines. Obtained in the amplification reaction 395 bp allele indicates the presence of a single-nucleotide polymorphism of thymine (T). The results on tracks 2, 4, 6, 9 and 11 show the presence of amplicons of the internal control gene and an allele that is not related to the characteristic of high carotenoid content (lines DK411, DK742, DK744, DK267 and DK366). The presence of an internal control amplicon and the absence of a 395 bp allele indicate that the lines which amplification products are represented on tracks 5, 7, 8 and 10 contain a single-nucleotide polymorphism of guanine (G). The presence of this polymorphism indicates the ability of lines DK325, DK680, DK296 and DK633 to increase the carotenoids synthesis in grain. Consequently, the favourable allele G (absence of 395 bp amplicon) was detected in 30 (27.8%) studied maize lines.

The marker *lcyε*-3'INDL is identified by PCR products of 144+502 bp and 399+502 bp. The results of the electrophoretic distribution of the amplicons obtained from DNA of maize lines DK247, ISK22, ISK25, ISK25, DK129-4, DKF2, DK744 and DK959 are presented in Fig. 3.

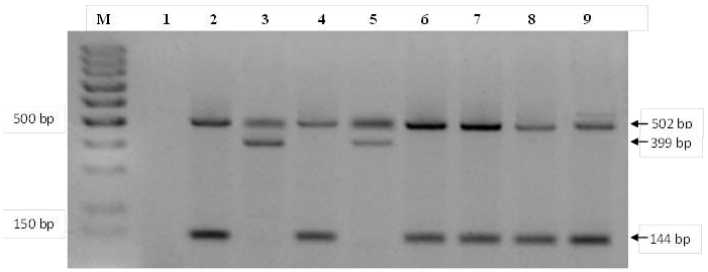

Figure 3 Molecular characterization of maize inbred lines using primer *lcyε*-3'INDL: *M* molecular weight marker Thermo Scientific O'RangeRuler 100 bp DNA Ladder; *1* control; *2–9* products of DNA amplification of maize lines DK247, ISK22, ISK25, ISK25, DK129-4, DKF2, DK744 and DK959

It is known that the allele in maize genotypes represented with 144 and 502 bp amplicons is associated with the accumulation of carotenoids. Thus, maize lines DK247, ISK25, DK129-4, DKF2, DK744 and DK959, which amplicons are presented on tracks 2, 4, 6–9, contain the favourable allele that means the ability of a genotype to gain high carotenoid content. Another allele of this marker is unfavourable for the selection of maize with high carotenoid content. This allele is visualized on the electropherogram as amplifications of 399+502 bp. In Fig. 4, 399+502 bp amplicons are detected on tracks 3 and 5 which correspond to maize lines ISK22 and ISK25.

The favourable allele for the marker *lcyε*-3'INDL was identified in 97 maize lines, that is, in 89.9% of the studied lines in this study. For the *crtRB1*-3'TE marker, all three possible allele variants were identified among the studied lines. Due to the specific design of primers of the marker (one forward and two revers), polymerase chain reaction may be obtained the 543, 296 and 296+875 bp amplicons (Fig. 4).

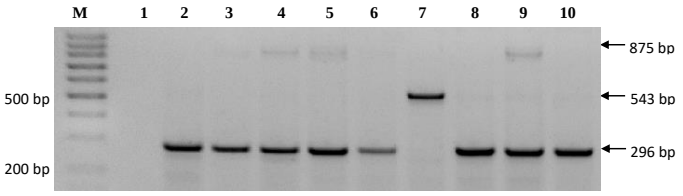

Figure 4 Molecular characterization of maize inbred lines using primer *crtRB1*-3'TE: *M* molecular weight marker Thermo Scientific O'RangeRuler 100 bp DNA Ladder; *1* control; *2–10* products of DNA amplification of maize lines DK23, DK3573, DK4538, DK541, DK673, DK200, DK4173, DK50 and DK744.

It is known that the allele of 543 bp (allele 1) is formed by attaching one forward and one revers primer, indicating no insertion near the 3'-end of *crtRB1* gene. Allele of 296+875 bp (allele 2) is formed by hybridization one forward and two revers primers and identifies an insert of 325 bp. Allele of 296 bp (allele 3) is formed by hybridization a forward and a revers primer and identifies an insert of 1250 bp at the 3' end of the gene (in the area of the sixth exon and 3'-UTR) (**Yan *et al.*, 2010**).

According to our results, allele 2 was identified on tracks 3–5 and 9, which represent amplification products of lines DK3573, DK4538, DK541 and DK50. Allele 3 was found on tracks 2, 6, 8 and 10, as evidenced by 296-bp amplicons. This allele is characteristic of lines DK23, DK673, DK4173 and DK744 that correspond to the mentioned tracks.

A favourable allele of 543 bp (allele 1) for the marker *crtRB1*-3'TE was detected in 30 (27.8%) of the studied yellow-grained maize lines. Shown in Fig. 4 is a 543-bp allele identified in line DK200.

Carotenoid content in grain of maize lines

The content of carotenoids among 108 yellow-grained maize lines ranged from 0.7 (ISK 31) to 7.15 μg/mg (DK2732) line and averaged 1.95 μg/mg. Two white-grained lines had expectedly significantly lower carotenoid content (about 0.47 μg/mg). Among the lines of yellow-grained maize, six genotypes with high carotenoid content were found: ISK43, DK367, DK206A, DK2323, DK6356 and DK2734. The content of carotenoids in these lines ranged between 3.58 and 7.15 μg/mg, which several times exceeded the average total carotenoid content of the studied lines.

Accumulation of carotenoids in grain of maize lines

According to some researchers, the content of carotene in maize grain after harvest is not constant and changes under the influence of drying and storage conditions and storage term (**Quackenbush et al., 1961; Quackenbush 1963; Weber 1987; Burt *et al*. 2010**). It is known that the accumulation of carotenoids in maize grain occurs during the development of endosperm (**Bartley and Scolnik, 1995**). It starts on the 10–15 day after pollination (DAP) and reaches its maximum on the 20–25 DAP in different varieties.

The dynamics of carotenoid accumulation in maize grain during the period of maturation was analysed in this study. Determination of carotenoid content in maize grain of DK411 was carried out on the 15, 20, 47 and 60 DAP. The dynamics of carotenoid accumulation is presented in Fig. 5.

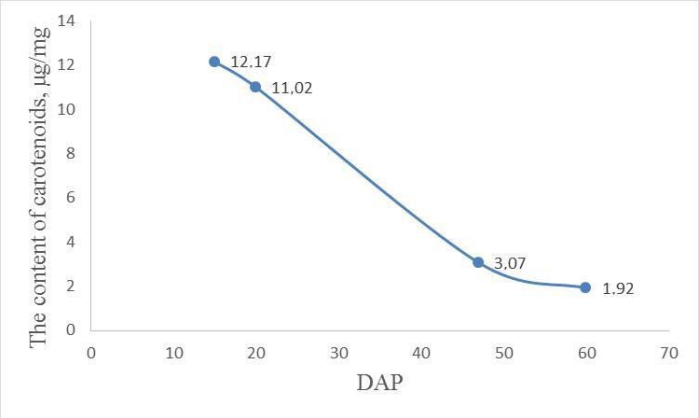

Figure 5 The content of carotenoids in maize grain of line DK411 on the 15, 20, 47 and 60 DAP

As can be seen from the figure 5, the maximum value of carotenoid content in maize grain of DK411 line (12.17 μg/mg) was determined on the 15 DAP. The content of carotenoids was decreasing after pollination, with the value of 11.02 μg/mg on the 20 DAP, 3.07 μg/mg on the 47 DAP and 1.92 μg/mg on the 60 DAP. In the line DK772, carotenoid content determined on the 15, 23, 30 and 60 DAP (Fig. 6).

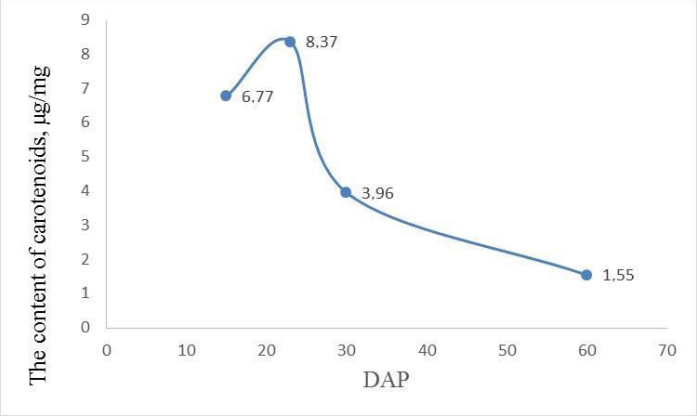

Figure 6 The content of carotenoids in maize kernels of line DK772 on the 15, 23, 30 and 60 DAP

In the grain of DK772 line, the peak carotenoids accumulations fell to 23 DAP and amounted to 8.37 μg/mg. The content of carotenoids varied unevenly. To illustrate on figure 6, it was 6.77 μg/mg on the 15 DAP and increased towards the 23 DAP. From the 23 to the 60 DAP, carotenoid content was constantly decreasing. On the 30 DAP, it was 3.96 and decreased to 1.55 μg/mg on the 60 DAP which corresponds to complete maturity.

The results of this study are concordant with **Wurtzel*et al*. (2004), Smolikova and Medvedev (2015)**: carotenoid content in maize grain changes over the stages of seed development. Moreover, the dynamics of the changes is uneven and may vary in each individual genotype.

Carotenoid content in the seeds of simple hybrids and their parent components

For the purpose of efficient breeding for obtaining lines and hybrids with high carotenoid content in grain, it is necessary to study inheritance patterns of genes that are responsible for the increased synthesis of carotenoids in parent components and hybrids produced with them. Determination of carotenoid content was performed in three yellow-grained maize hybrids (DK296×DK253, DK272×DK633/266 and DK231×DK366) and their parent components, and white-grained maize hybrid (DKD3264×RNR60) and its parent components. The content of carotenoids in the investigated grain is shown in Fig. 7.

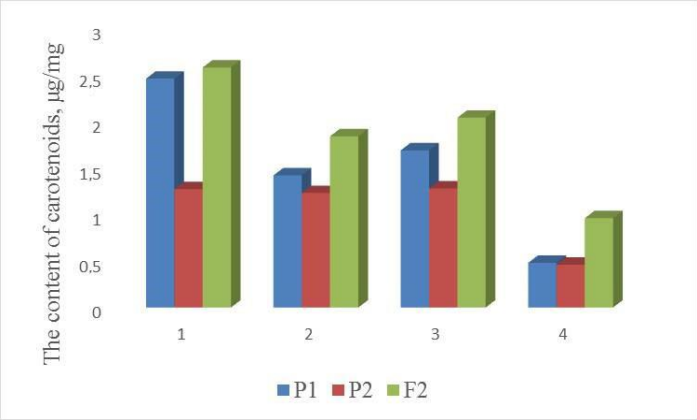

Figure7 Carotenoid content in grain of simple hybrids (F2) and their parent components (P1 and P2): *1* DK296×DK253; *2* DK272×DK633/266; *3* DK231×DK366; *4* DKD3264×RNR60 ($LSD_{0,5}$ 0.12)

The carotenoid content in the parent components of DK296 and DK253 was 2.65 and 1.38 µg/mg respectively; in DK272 and DK633/266 1.53 and 1.33 µg/mg respectively; in DK231 and DK366 1.83 and 1.57 µg/mg respectively; and in DKD3264 and RNR60 0.53 and 0.50 µg/mg respectively. It was determined that all the obtained simple hybrids had a higher content of carotenoids in comparison with their parent components. The carotenoid content in grain of simple hybrid 1 was 2.78 µg/mg, of simple hybrid 2 was 1.98 µg/mg, in simple hybrid was 2.21 µg/mg and in simple hybrid 4 was1.04 µg/mg.

Noticeably, that the largest difference between the average carotenoid content in parent components and the resulting hybrid was noted for white-grained maize (0.53 µg/m). Besides, the heterozygous effect was observed in the hybrid produced with parent components DK296 and DK253(0.77 µg/mg). In general, all the hybrids had higher carotenoid content of grain compared to their parent components.

Dependence of carotene content on the allele state of the studied markers

According to the evaluation of maize lines by DNA markers of carotenogenesis, the lines were distributed based on the availability of favourable alleles of the examined markers. The distribution results are presented in Table 2.

Table 2 Carotene content in maize lines possessing a favourable allele for DNA-markers

DNA-markers	The number of analysed maize lines	Average carotene content (µg/mg)
No allele was found	4	1.71
lcyε-SNP216	30	2.09
lcyε-3'INDL	97	1.99
crtRB1-3'TE	30	1.85
lcyε-SNP216+*lcyε*-3'INDL	29	2.20
lcyε-3'INDL+*crtRB1*-3'TE	26	1.86
lcyε-SNP216+*crtRB1*-3'TE	10	2.13
lcyε-SNP216+*lcyε*-3'INDL+*crtRB1*-3'TE	8	2.22
$LSD_{0,5}$		0.25

As results of the study, there is a significant difference between the carotene content in the lines, in which no favourable allele has been found and the lines in which the alleles have been detected for at least one DNA marker. The absence of favourable alleles was detected in four maize lines (ISK22, ISK28, ISK33 and ISK37), with the average carotene content of 1.71 µg/mg.

However, there was no significant difference between the values of carotene content in the lines, in which favourable alleles were detected for each of the markers in this study. Thus, in 30 maize lines, grain of which contained favourable alleles for the marker *lcyε*-SNP216, the average carotene content was 2.09 µg/mg, in 97 lines containing favourable alleles for the marker *lcyε-3*'INDL it was 1.99 µg/mg, and in 30 lines containing favourable alleles for the *crtRB1*-3'TE marker, 1.85 µg/mg.

Eight lines (DK206A, DK212, DK1855, DK9066, IKS2, IKS3, IKS12 and IKS21) had favourable alleles for all of the studied markers. The average carotene content in grain of maize containing favourable alleles for the marker combination *lcyε*-SNP216+*lcyε*-3'INDL+*crtRB1*-3'TE was 2.22 µg/mg; for the marker combination *lcyε*-SNP216+*lcyε*-3'INDL (29 lines) 2.20 µg/mg; and for the marker combination *lcyε*-SNP216+crtRB1-3'TE (10 lines) 2.13 µg/mg. The indicated values significantly differed from the ones of the lines in which favourable alleles were identified for marker *crtRB1*-3'TE (1.85 µg/mg) and marker combinations *lcyε*-3'INDL+*crtRB1*-3'TE (1.86 µg/mg). According to the results of the one-factor analysis, in which the factor was the presence of favourable alleles for different DNA markers, LSD among the mean values of carotene content in grain of studied lines was 0.25 (Table 2). This indicates that the presence of favourable alleles significantly affects carotene content in grain.

To sum up, the highest values of the average carotene content were recorded in the maize lines contained favourable alleles for the marker combinations *lcyε*-SNP216+*lcyε*-3'INDL and *lcyε*-SNP216+*crtRB1*-3'TE. **Harjes*et al*. (2008)** obtained results for high carotene content in maize lines containing favourable alleles for two markers at the same time: *lcyε*-SNP216 and *lcyε*-3'INDL. This indicates the direction of the biosynthesis of carotenoids in lines with the favourable allele for the markers of the gene *lcyε* - towards the formation of the β-ring.

In this study, no favourable allele was found in studied lines for the *lcyε*-5'TE locus. **Muthusamy*et al*. (2015)** studied the polymorphism of 385 maize lines. They identified two alleles of 250 and 650 bp for marker *lcyε*-5'TE and three alleles of 296, 543 and 875 bp for marker *crtRB1*-3'TE (**Muthusamy*et al*., 2015**). Among the analysed maize lines, a favourable allele of 650 bp for the marker *lcyε-5*'TE was found at a frequency of 3.38 % and for the *crtRB1*-3'TE locus at a frequency of 3.9 %. It should be noted that the frequency of the favourable allele for the marker *lcyε*-5'TE is quite low. In the study of 130 tropically adapted yellow-grained maize lines (**Azmach*et al*., 2013**), the frequency of favourable allele of 650 bp was 12%. Thus, the absence the favourable allele in this study for the *lcyε*-5'TE locus can be explained the smaller number of accessions.

For the other markers of high carotenoid content in maize, the frequency of favourable alleles can reached 83%. For the marker *lcyε*-SNP216, scientists identified the largest number of lines with a favourable allele (**Harjes*et al*., 2008); Muthusamy*et al*.,2015**). In this study, the frequency of genotypes with a favourable allele did not exceed 28%. **Azmach*et al*. (2013)** identified favourable alleles for the marker *lcyε*-3'INDL in 38% of the total number of genotypes. The frequency of favourable alleles for this marker was the highest and accounted for 89.9% in this study. Consequently, the study conducted by **Azmach*et al*. (2013)** to determine the total carotenoid content found that the combination of favourable alleles for two genes, *lcyε* and *crtRB1*, explains 38 and 89% of the total variation in the carotenoid content. Thus, that is confirmed this study.

As results of this study data indicate the uneven character of the accumulation of carotenoids and their maximum content on the 15 and 23 DAP. This is confirmed by **Cordoso *et al.* (2015).** They conducted a study on the effect of different drying modes at different grain moisture (19 % and 22 %). The authors found that the content of carotenoids and xanthophylls in yellow-grained maize was higher at harvest moisture of 22 % compared to harvest moisture of 19 %. This shows non-linear nature of their accumulation in grain as affected by the timing of harvesting, moisture and other factors. Different studies on the accumulation of carotenoids in maize grain showed that significant accumulation occurs during the development of endosperm, which starts on the 10–15 DAP and reaches a maximum on the 20–25 DAP in different varieties (**Wurtzel*et al*., 2004**). It can be explained that biosynthesis of carotenoids proceeds at the time of physiological maturation, after which the concentration of carotenoids changes due to water loss (**Bartley and Scolnik, 1995; Cardoso *et al*., 1999; Wurtzel, 2014; Wurtzel*et al*., 2004**). Studies by **Wurtzel*et al*. (2004)** and **Smolikova and Medvedev (2015)** show that the accumulation of carotenoids in endosperm correlates with the expression of *PSY1* gene (phytoene synthase 1) and does not depend on the expression of the *PSY2* gene (phytoene synthase 2). It is possible that the inability of the *PSY2* enzyme to function in the endosperm is due to the specificity of the membrane structure of the storage plastids. The viviparous 9 (vp 9) maize mutants with white-grained endosperm had impaired synthesis of the ZDS enzyme responsible for the production of ζ-carotene (**Smolikova and Medvedev, 2015**). The authors report that the bulk of carotenoids in mature seeds are apparently localized in the form of ethers of fatty acids in plastids. However, there are chlorembryophytes, in which chlorophylls completely degrade after maturation and therefore they are present in residual amounts.

In this study, the lowest total carotenoid content in maize grain was in white-grained hybrid (1.04 µg/mg). The highest carotenoid content was recorded among 108 yellow-grained lines and amounted to 7.15 µg/mg. The same researches were carried out by **Das and Singh (2012)**. They conducted studies on the use of SSR markers to evaluate maize genotypes for carotenoid content. The authors analysed the total content of carotenoids in grain of 25 genotypes. The content of carotene varied from 0.94 µg/g (white-grained maize) to 38.25 µg/g in (dark-yellow-grained maize). Of the 25 studied lines, 11 had a high content of provitamin A and total carotenoids (**Das and Singh, 2012**). Therefore, obtained results in this study confirms the differences between carotenoids content in white and yellow maize grain.

The significant difference between the different combinations of favourable alleles and the total carotenoid content was observed in this study for the marker combinations *lcyε*-SNP216+*lcyε*-3'INDL and *lcyε*-SNP216+*crtRB1*-3'TE, which had favourable alleles at the same time. According to the results obtained by **Yan *et al.* (2010)** who investigated the rare genetic variation of *crtRB1* gene in maize, which increases the synthesis of β-carotene, it was found that there is no significant correlation between the colour of grain and any of the carotenoids. Maize lines with favourable alleles of the *lsyε* and *crtRB1* genes are valuable genetic resources for biofortification breeding programs.

Yan *et al.* (2010) concluded that some genetic variations occur quite rare, and therefore the most favourable genotypes for *crtRB1* and *lcyε* genes cannot be found

in nature in one genotype at the same time. The low frequency of some of favourable alleles for *crtRB1* and *lcyε* genes in this study are confirmed by **Yan's *et al.* (2010)** results. A study by **Harjes*et al*. (2008)** reported that the genotype, which is characterized by high content of β-carotenoids, includes favourable alleles for markers *lcyε*-SNP216 and *lcyε*-3'INDL. According to the marker panel tested by the authors, such genotype was found in 5% of temperate climate inbred lines and in 16% of tropical inbred lines. Thus, the combination of these genotypes characterises the effectiveness of experiments in breeding programs. The presence of favourable alleles for several of the studied markers in maize lines leads to an increase in the content of carotene in maize grain. Therefore, the selected breeding material and the system for assessing maize genotypes for key markers of carotenogenesis are effective and implemented at the Research Institute for Agricultural Business in order to obtain high-quality hybrids.

CONCLUSION

The molecular genetic polymorphism of 110 lines of yellow-grained and white-grained maize was investigated using four DNA markers of carotenogenesis. The most successful combination of favourable alleles for the examined markers based on the concentration of carotenoids was determined and the dynamics of the accumulation of carotenoids in maize grain at different stages of development was evaluated.

It was found that three out of four studied DNA markers appeared polymorphic: *lcyε*-SNP216, *lcyε*-3'INDL and *crtRB1*-3'TE. For the marker *lcyε*-5'TE, no allele was detected in any of the genotypes under study. Based on carotenoid content values, it was determined that the highest carotenoid content of grain had the lines with favourable alleles for the following combination of DNA markers: *lcyε*-SNP216+*lcyε-3*'INDL and *lcyε*-SNP216+*crtRB1*-3'TE. Based on these markers, 39 maize lines were selected to be involved in the breeding program aimed at obtaining hybrids with high carotenoid content in grain. Experimentally confirmed that the dynamics of carotenoid accumulation in maize grain depends on individual genotype and stage of seed development. Among the studied lines, the highest content of carotenoids was determined on the 15 and 23 DAP. The total carotenoid content in grain of hybrids increases significantly compared to their parent components. To conclude, this study resulted in the development of practical approaches to the evaluation of breeding material using DNA markers in order to produce hybrids of improved quality characteristics.

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