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APPLICATION OF MOLECULAR MARKER crtRB1-3′TE IN MAIZE SELECTION

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Abstract: Biosynthesis of β-carotene in maize is influenced by three key genes: psy, lcyE and, at most, crtRB1. The gene of β-carotene hydroxylase 1 (crtRB1) on marker crtRB1-3′TE has three allelic states: 296 bp, 296 + 875 bp and 543 bp. The aim of the study was to identify perspective inbreds of Ukrainian selection with a favourable for the accumulation of β-carotene allele of marker crtRB1-3′TE (543 bp). The study revealed DK315MV and DK267MV inbreds, which had the 543 bp allele in the homozygous state and are recommended as parental forms for breeding programs for increased β-carotene content in mature grain.

Keywords: Zea mays L., molecular marker, β-carotene hydroxylase 1, MAS

INTRODUCTION

Insufficient micronutrients in food lead to serious human health problems. This mainly applies to zinc, iron, vitamin C and vitamin A *(Frano et al, 2014; Muthusamy et al, 2014)*. By involving methods of biofortification of major crops using integrated approaches to plant breeding and genomics, it is possible to solve the problem of vitamin deficiency, in particular, provitamin A *(Ashokkumar et al,2020)*.

More than three million children in developing countries are affected by xerophthalmia, and 250,000 to 500,000 people become blind each year due to vitamin A deficiency (VAD) *(Food and Agriculture Organization, 2017)*.

Vitamin A manifests itself in the human body as a multifunctional compound. It is involved in a number of important physiological processes such as: visual acuity, cell growth and differentiation, embryogenesis and immune response. Vitamin A in the human body forms a number of structurally similar substances: retinol, dehydroretinol, retinal, retinoic acid, esters of these substances and their spatial isomers. Directly retinoic acid is actively involved in the regulation of the transcription process *(Klyuchnikov et al, 2007; Shamitova and Viktorovich, 2019)*.

Maize is one of the most important cereals, which is able to accumulate a significant amount of carotenoids in the endosperm. Thus, improving the balance of micronutrients in maize grain through biofortification is an economically and socially reasonable way to overcome vitamin and micronutrient deficiencies, including VAD *(Yan et al., 2010; Pixley et al., 2012)*.

Currently, about 750 carotenoids have been found from natural sources. Depending on the presence or absence of oxygen in their structure, carotenoids are divided into oxygen-containing xanthophylls and oxygen-free carotenes *(Nimishi et al., 2016)*. Carotenoids are rather unstable compounds that degrade under the action of high temperatures, light and oxygen *(Boon et al., 2010; Stephen et al., 2016)*. Some carotenes, such as α-carotene, β-carotene and β-cryptoxanthin, can be converted into vitamin A. However, only β-carotene is able to form 2 molecules of vitamin A per 1 original molecule, while α-carotene and β-cryptoxanthin only one *(Berman et al., 2017; Harjes et al., 2008)*.

In addition to vitamin activity, carotenoids in plant cells play an important role as auxiliary pigments for photosynthesis, promote protection against photooxidation, attract pollinating insects, etc. *(Sagare et al., 2018)*.

The synthesis of β-carotene in plants begins with the starting substance – geranylgeranyl-pyrophosphate -GGPP (Figure 1).

With the participation of phytoene synthase encoded by gene *psy*1 (*y*1) two GGPP molecules condense into one phytoene molecule. Plants containing *psy*lgene produce carotenoids in both endosperm and leaves. The allelic construction of *psy*1 gene significantly affects the colour of maize grain, with a corresponding accumulation of carotenoids in it. Genotypes *Y1Y1* and *Y1y1* produce yellow kernels as a result of carotenoid accumulation, while genotype *y1y1* forms white grains that do not contain carotenoids *(Fu et al., 2013; Sagare et al., 2018)*.

Through a series of intermediate reactions from GGPP, lycopene is formed - the first coloured substance. At this point, the path of carotenoid biosynthesis branches into α-branch and β-branch (fig. 1). In symmetrical cyclization, lycopene forms a molecule with two β-rings (producing first γ-carotene and then β-carotene) under the action of lycopene β-cyclase (LCYB) at both ends of the linear lycopene. In asymmetric cyclization under the action of the enzyme lycopene ε-cyclase (LCYE), lycopene is converted into δ-carotene – a precursor of α-carotene, zeinoxanthin and lutein. Decreasing level of LCYE reduces the formation

of substances from the branch of α-carotene in favour of substances of the branch of β-carotene *(Bai et al., 2009; Harjes et al., 2008; Pixley et al., 2012)*.

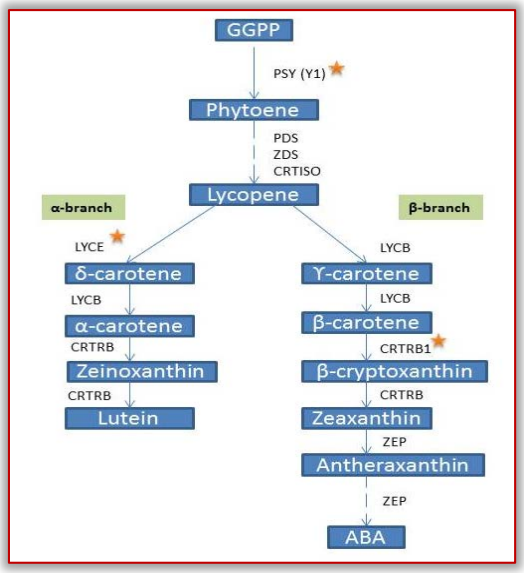

Figure 1. Simplified carotenoid biosynthetic pathway in plants (Berman et al., 2017; Harjes et al., 2008; Thanh, 2019; with modifications)
Enzymatic reactions are represented by arrows, dashed lines – multiple enzymatic steps. Compounds: GGPP – Geranylgeranylpyrophosphate; ABA – Abscisic acid. Enzymes: PSY – phytoene synthase; PDS – phytoene desaturase; ZDS – zetacarotene desaturase; CRTISO – carotene isomerase; LCYE – lycopene ε-cyclase; LCYB – lycopene β-cyclase; CRTRB – carotene hydroxylase enzymes, which include ε-carotene hydroxylase and β-carotene hydroxylases; CRTRB1 – β-carotene hydroxylase 1; ZEP – zeaxanthin epoxidase; Major genes for carotenoid biosynthesis pathway

Another key gene in carotenoid synthesis is gene of β-carotene hydroxylase 1 (*crt*RB1; also known as HYD), which causes hydroxylation of α-carotene and β-carotene into lutein and zeaxanthin, respectively. Hydroxylation of carotenes reduces the content of carotenoids with the properties of provitamin A, thereby increasing the content of non-provitamin xanthophylls *(Sagare et al., 2018)*. As a result, maize endosperm accumulates a significant amount of other substances, primarily zeaxanthin through two hydroxylation reactions of β-carotene. Blocking these hydroxylation reactions can increase the content of β-carotene in the endosperm of mature maize grain *(Berman et al., 2017; Muthusamy et al., 2015)*. Thus, mutations in the β-carotene hydroxylase gene (*crt*RB1) in maize lead to a slowing of the transition of β-carotene into β-cryptoxanthin during grain ripening and, consequently, to an increase in the content of β-carotene in mature grain.

Berman et al. (2017) used RNA interference to silence genes *Zmcrt*RB1 and *Zmcrt*RB3 genes encoding two β-carotene hydroxylases on both branches. *Zmcrt*RB1 regulates the transition of β-carotene to β-cryptoxanthin while *Zmcrt*RB3 mostly hydroxylates β-ring of α-carotene. The content of β-carotene in the endosperm increased significantly in all hybrids in which *Zmcrt*RB1 was silenced, regardless of whether *Zmcrt*RB3 was silenced.

A significant increase in β-carotene in maize grain was achieved using genetic engineering technologies *(Aluru et al., 2008; Naqvi et al., 2009; Simkin, 2019; Zhu et al., 2009)*. Most gene modifications concerned key carotenogenesis genes: *psy*1, *pds*, *zds*, *lcy*B, *lcy*E, *crt*RB1 *(Thanh, 2019)*. Transgenic maize plants with the high-value carotenoid astaxanthin in grain endosperm were obtained by combining overexpression of the *psy* gene for enhanced carotenoid production and silencing the *lcy*E gene to direct more precursors to the β-branch *(Farré et al., 2016)*. *Zhu et al. (2008)* used the construction containing five genes of carotenoid biosynthesis for the genetic transformation of maize genome: maize gene of phytoene syntase 1, gene of phytoene desaturase from *Pantoea ananatis*, genes of lycopene cyclase and carotene hydroxylase from *Gentiana lutea*, and the carotene ketolase gene of *Paracoccus*.The authors generated transgenic maize plants with extraordinary levels of β-carotene (57.35 μg / g DW) and other carotenoids, including complex mixtures of hydroxycarotenes and ketocarotenes.

Among the considered mechanisms of influence on the accumulation of β-carotene in maize grain, marker-assisted selection (MAS) on the allelic state of key carotenoid biosynthesis genes is important for practical use. *Yan et al. (2010)* found that provitamin A content was 5.2-fold higher with favourable alleles of markers *crt*RB1-5′TE and *crt*RB1-3′TE in gene *crt*RB 1. According to *Babu et al. (2013)* and *Muthusamy et al. (2014)* the presence of a favourable *crt*RB1-3′TE allele increases the concentration of β-carotene by 2–10 times regardless of the genetic constitution of gene *lcy*E.

According to *Muthusamy et al. (2014)* polymorphism of gene *crt*RB1 due to transposon insertion in exon 6 led to the appearance of three alleles of this gene in maize, namely allele 1 (543 bp; without TE insert), allele 2 (296 bp + 875 bp; with an insert TE - 325 bp) and allele 3 (296 bp + 1221 bp + 1880 bp; with an insert TE - 1250 bp), which were associated with changes in the accumulation of β-carotene *(Muthusamy et al., 2014)*. The presence of allele 1 of the *crt*RB1 gene (hereinafter allele 1) is favourable and increases the level of β-carotene in the grain, while alleles 2 and 3 are unfavourable for the accumulation of this substance.

The aim of our study was to determine the allelic status of the gene of β-carotene hydroxylase 1 by the molecular marker *crt*RB1-3'TE in maize inbreds of Ukrainian and world selection.

MATERIAL AND METHOD

The materials for the study were perspective inbreds of maize (*Zea mays* L.) of Ukrainian selection DK3044, DK267MV and DK315MV and well-known inbreds of world selection P354 and A188. DNA was isolated from seedlings by CTAB method *(Murray and Thompson, 1980)*. For each inbred, the DNA of one seedling and DNA of a mixture of 5 seedlings picked over the average sample were analyzed. Evaluation of the β-carotene hydroxylase 1 gene by the allelic

state of the marker *crt*RB1-3′TE was performed by PCR with primers according to *Yan et al. (2010)*:

F: ACACCACATGGACAAGTTCG, R1: ACACTCTGGCCCATGAA CAC and R2: ACAGCAATACAGGGGACCAG.

The reaction mixture of 20 µl contained: 2.0 µl of DNA of the test lines, 1.0 µl of each primer, 2.0 µl of a mixture of deoxyribonucleotides (dNTP), 2.0 µl of Green Taq Buffer, 0.15 µl of Taq polymerase and 10.85 µl deionized water.

PCR was performed according to *Safawo et al. (2010)* in two repetitions under the following conditions: initial denaturation - at a temperature of 94 ° C for 5 minutes; then 40 cycles, which included stages such as denaturation (at 94 ° C for 1 min.), annealing of primers (at 60 ° C for 1 min.) and elongation (at 72 ° C for 1 min.). The final elongation was performed at 72 ° C for 5 minutes, the cooling phase was carried out at + 10 ° C. Visualization of the amplification products after electrophoretic separation in 1% agarose gel was performed with ethidium bromide (0.5 µg / ml) in a tris-borate buffer system at 120 V for 60 min, using a GelDocTM instrument (BioRad). For the *crt*RB1-3′TE marker, bands of 543 bp (favourable allele), 296 bp and 296 + 875 bp (unfavourable alleles) were expected. As reference samples in the determination of allelic state of *crt*RB1-3′TE marker we used DNA of inbreds A619 and B73 which had been tested with the same primers earlier and were regarded in our laboratory for identification of allele 1 (A619 – 543 bp) – and allele 2 (B73 – 296 bp).

RESULTS

The investigated maize inbreds differed in grain colour at full ripeness (fig.2), from white (A188) to yellow of different intensity (DK3044, DK267MV, DK315MV) and even reddish-brown (P354). The given variation may be connected with the different content of colouring substances from the group of carotenoids and xanthophylls.

Figure 2. The grain of maize investigated inbreds DK3044, DK267MV, DK315MV, P354 and A188 varies in colour

The results of PCR-analysis testified that the reference DNA sample of inbred A619 proved the presence of a band in 543 bp that was the instance of allele 1 of *crt*RB1-3′TE marker (fig. 3). The reference DNA sample of inbred B73 demonstrated the allelic status of the same marker as 296 bp that was the evidence of allele 2.

All maize investigated inbreeds revealed only one variant of the *crt*RB1-3′TE marker, both in DNA samples from one plant and in DNA samples from mixture of five plants. This fact indicates the homozygosity of the studied inbreeds on this marker. In DK267MV and DK315MV allele 1 of *crt*RB1-3′TE marker (543 bp) was identified, while in P354, DK3044 and A188 – allele 2 (296 bp). Allele 3 (296 + 875 bp) among the studied samples were not detected.

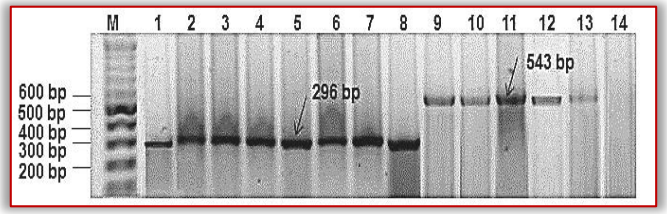

Figure 3. Electrophoreogram of maize DNA amplification products by molecular marker crtRB1-3′TE of the gene of β-carotene hydroxylase 1

M - molecular weight marker with a step of 100 bp; 1 - inbred P354 (DNA of one plant); 2 - inbred P354 (DNA mixture of five plants); 3 - inbred DK3044 (DNA of one plant); 4 - inbred DK3044 (DNA mixture of five plants); 5 - inbred A188 (DNA from one plant); 6, 7 - inbred A188 (DNA mixture of five plants), 8 - control for allele 2 (296 bp) – inbred B73; 9 – inbred DK267MV (DNA of one plant); 10 - inbred DK267MV (mixture of DNA of five plants); 11 - inbred DK315MV (DNA of one plant); 12 - inbred DK315MV (DNA mixture of five plants); 13 - control for allele 1 (543 bp) – inbred A619; 14 - control without DNA

CONCLUSIONS

Among the five investigated maize lines DK3044, DK267MB, DK315MB, P354 and A188, a polymorphism was detected by the *crt*RB1-3′TE marker in the gene of β-carotene hydroxylase 1. This polymorphism is occurred due to the presence of two alleles, which after PCR with appropriate primers ensure the appearance of two bands in 543 bp and 296 bp. All studied inbreds were homozygous for this marker. Favourable allele of the marker *crt*RB1-3′TE 543 bp has been identified in the inbreds of Ukrainian selection DK267MB and DK315MB. So these in breds are recommended for use as parental forms for the creation of maize hybrids with increased content of β-carotene in mature grain.

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Technological Platform", in Bucharest, ROMANIA, 30 October, 2020.

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