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EXPRESSION OF THE LYCOPENE-ε-CYCLASE *LcyE* GENE CORRELATES WITH THE CONTENT OF β-CAROTENE AND CHLOROPHYLLS IN MAIZE VEGETATIVE TISSUE

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Abstract

Maize (*Zea mays* L.) is an important world crop. One of the valuable traits of this plant is the biosynthesis of vitamin A precursors in kernel (dietary nutrition) and photosynthetic tissue (protection of the plant from stress; silage with increased dietary value). The amount of synthesized provitamin A in kernel depends on the level of expression of the lycopene-ε-cyclase *LcyE* gene, which catalyzes the formation of α-carotene and is involved in the regulation of the ratio of β-β and β-ε fluxes of carotenoid metabolism. The aim of the study was to analyze the correlation between the content of the sum of carotenoids, β-carotene, and chlorophylls a and b with the expression of the *LcyE* gene in the leaves of inbred maize lines of domestic selection. To achieve the goal, four inbred maize lines were used in the study: three white-grained (6097-1, MBK and Shumny's Tetraploid) and one (5580-1) with yellow grain color. Expression of the *LcyE* gene in leaves was determined by quantitative real-time PCR. Quantitative determination of the amount of carotenoids, chlorophylls a and b, and β-carotene in leaves was carried out spectrophotometrically using the Folch method. Correlations between pigment content and *LcyE* gene expression were evaluated using statistical methods. As a result, we assessed a possible correlation between the activity of the *LcyE* gene and the content of carotenoids and chlorophylls in the photosynthetic tissue of four maize lines: three white-grained (6097-1, MBC and Shumnoy Tetraploid) and one (5580-1) with yellow-colored kernel. Quantification of carotenoids revealed the highest content of these pigments in the leaves of the Tetraploid Shumny line. The accessions of the remaining three lines synthesized a smaller amount of carotenoids and were similar to each other in this parameter. At the same time, β-carotene, as well as chlorophylls a and b, were most of all contained in the leaves of line 6097-1 — approximately 2 times more than in other analyzed lines, where the pigment content did not differ significantly. Thus, the absence of associations between the color of the kernel and the content of the β-carotene and sum of carotenoids in maize leaves was confirmed. On the other hand, the obtained data suggest a positive relationship between the amount of β-carotene and chlorophylls (a and b). It is possible to assume an increased rate of photosynthesis in the photosynthetic tissues of line 6097-1 in comparison with other analyzed maize lines. Accordingly, line 6097-1 may have an increased resistance to oxidative stress, as well as be a donor of a trait with an increased content of provitamin A (as a silage crop). The expression of the lycopene-ε-cyclase *LycE* gene was determined in the same leaf tissues. It was shown that the *LycE* gene was expressed ~ 4-5 times higher in the leaves of accessions of lines 5580-1 and Tetraploid Shumny than

in the leaves of accessions of lines MBK and 6097-1. Correlation analysis showed an inverse relationship between the content of β-carotene and chlorophylls (a and b) and the level of *LycE* gene expression. Thus, in this study, for the first time, we assessed a possible correlation between the activity of the *LcyE* gene and the content of carotenoids and chlorophylls in the photosynthetic tissue of white and yellow grain maize lines of domestic selection. No associations were found between grain color and the content of total carotenoids and β-carotene in maize leaves. A positive relationship was found between the amount of β-carotene and chlorophylls a and b. For the first time, an inverse relationship between the content of β-carotene and chlorophylls a and b and the level of *LycE* gene expression was determined. The possibility of using data on the expression of the *LycE* gene in the leaf as an expression molecular marker of the amount of provitamin A synthesized in the leaves, as well as the degree of plant resistance to photooxidative stress, was demonstrated. The data obtained can be used in maize breeding to search for donors of the trait of increased content of provitamin A in the leaves.

Keywords: *Zea mays* L., maize, lycopene-ε-cyclase, LycE, carotenoids, chlorophylls, gene expression

Photosynthesis is accompanied by the formation of reactive oxygen species (ROS) which have a pronounced reactivity [1]. The action of ROS is aimed at the metabolic modification of proteins, nucleic acids, and lipids; however, an excess of ROS causes increased oxidative degradation of chemical compounds in cells.

Protection against oxidative stress is achieved by regulating the amount of ROS and leveling the damage they cause, including through chlorophylls and carotenoids [2]. Carotenoids absorb light energy and carry out singlet-singlet transfer of excitation energy to chlorophyll molecules. In turn, chlorophylls give excess energy to carotenoids through triplet-triplet transfer. The return of carotenoids from the triplet state to its original state occurs due to the dissipation of energy in the form of heat [1].

Therefore, carotenoids play the role of antioxidants associated with the quenching of triplet chlorophyll and singlet oxygen [1]. We are talking about carotenoids of the xanthophyll cycle [3] which is mainly involved in the regulation of the redistribution of light energy between violaxanthin, zeaxanthin and chlorophyll a. In response to light stress, violaxanthin is converted to zeaxanthin via antheraxanthin, which acts as a lipid-protective antioxidant and stimulates non-photochemical quenching in the light-harvesting chlorophyll a/b-protein complex [3]. In low light, violoxanthin acts as a light-harvesting compound, serving as an energy donor for chlorophyll. In addition to the main one, higher plants have an additional second type of xanthophyll cycle, the lutein-5,6-epoxide type which is based on the reversible transformation of lutein into lutein-5,6-epoxide [3, 4].

The biosynthesis of carotenoids begins with the formation of the precursor of all carotenoids, phytoin, under the action of PSY phytoin synthase. In subsequent reactions, lycopene is synthesized. Further, the metabolic pathway is divided into branches β-β and β-ε leading to the formation of xanthophylls of the main (zeaxanthin, antheraxanthin, violaxanthin) and additional (lutein) types of the xanthophyll cycle. In the cycle of the first type, xanthophylls are derivatives of the β-β branch of the carotenoid biosynthesis metabolic pathway, when, under the action of lycopene-β-cyclase (LcyB), β-ionone rings (β-carotene; hydroxylation products of β-carotene zeaxanthin, antheraxanthin and violaxanthin) appear [5, 6]. In the second type of cycle, xanthophylls are derivatives of the β-ε branch of the carotenoid biosynthesis pathway. Their synthesis begins with α-carotene which is a molecule with a β-ionone ring at one end and a ε-ionone ring at the other end of the isoprenoid chain, resulting from the combined action of LcyB and lycopene-ε-cyclase (LcyE) (the end product of the branch β-ε is lutein) [5, 6].

Interestingly, β-cryptoxanthin (xanthophyll of the β-β branch), like α- and β-carotenes, not only performs a photoprotective function in the host plant, but also serves as a precursor of deficient vitamin A. For this, β-carotene is the most significant, since its structure has two β-ionone rings, as a result, the oxidative

cleavage of β-carotene leads to the formation of two vitamin A molecules [7-9].

Maize *(Zea mays* L.) plays an increasing role in the economy of the Russian Federation as a source of food and technical raw materials, as well as a silage crop. In connection with the wide use of corn plants, it is important that vitamin A precursors be contained in an increased amount not only in grain (dietary nutrition), but also in photosynthetic tissue (plant protection from stress, silage with increased fodder value).

Silage includes the above-ground part of the plant (cobs, leaves, stems) and in the feeding of farm animals provides about 50% of the dry matter of the main feed [10-12]. The use of photosynthetic tissue (especially leaves) of maize plants in animal husbandry may be more economically advantageous compared to grain. In the grain of traditional maize varieties and lines, carotenoids make up only 0.5-2.5 μg/g ww (13-15), while xanthophylls in leaves are about 200 rg/g ww, which is about 100 times more (16).

In maize, both lycopene cyclases, LycE and LycB, have been identified and characterized, including the expression of genes encoding them in grain [17-19]. However, there are no data on the activity of LycE and LycB in maize photosynthetic tissue. Polymorphisms in the sequence of the *LycE* gene make it one of the molecular markers that determine the amount of provitamin A in the tissue [13, 15, 20, 21]. Donors of mutant *lcyE* alleles are actively used in breeding maize lines that produce grain enriched with provitamin A [14, 15, 22]. There is an inverse relationship between thr *LycE* gene expression and provitamin A content [13]. This correlation is conservative in higher plants, as demonstrated by the example of the leaves of the model species *Arabidopsis thaliana* L. (23).

This research, for the first time, showed the absence of associations between grain color and the total carotenoids and β-carotene levels in corn leaves, a positive relationship between the amount of β-carotene and chlorophylls a and b, an inverse relationship of the contents of β-carotene and chlorophylls a and b with the *LycE* gene expression. The *LycE* gene expression in leaves can serve as a molecular marker associated with provitamin A synthesis in leaves and the degree of plant resistance to photooxidative stress.

The aim of the work was to analyze the correlation between the content of the sum of carotenoids, β-carotene, and chlorophylls a and b with the *LcyE* gene expression in the leaves of Russian inbred maize lines.

Materials and methods. Four inbred maize lines were used in the work: three white-grained (6097-1, MBK, Shumnoy's tetraploid) and one (5580-1) with yellow grain color. The seed material was kindly provided by the Institute of Agriculture, a branch of the Kabardino-Balkarian Scientific Center RAS. The grains were germinated in moist soil at 23/25 °C and 16/8 h (day/night) under the conditions of the EUIK artificial climate experimental facility (Federal Research Center of Biotechnology RAS) until the 4th true leaf appeared.

Total RNA was extracted from 50-100 mg of leaf tissue (RNeasy Plant Mini Kit, QIAGEN, Germany), purified from DNA impurities (RNase-free DNasy set, QIAGEN, Germany) and used for cDNA synthesis (GoScript™ Reverse Transcription System, Promega, USA) according to manufacturer's protocols. The quality of RNA was checked by electrophoresis in 1.5% agarose gel. RNA and cDNA concentrations were determined on a Qubit 4 fluorimeter (Thermo Fisher Scientific, USA) using appropriate reagents (Qubit RNA HS Assay Kit and Qubit DS DNA HS Assay Kit, Invitrogen, USA).

Expression of the *LcyE* gene in leaves was determined by quantitative real-time PCR (RT-PCR) with normalization using the reference gene *Zea mays polyubiquitin* (NM_001329666.1; primers ZmUBI-rtF: 5'-ATCGTGGTTGTGG-CTTCGTTG-3', ZmUBI-rtR: 5' -GCTGCAGAAGAGTTTTGGGTACA-3′).

3 ng cDNA template, cDNA-specific primers (ZmLcyE-F: 5'-TTTACGTG-CAAATGCAGTCAA-3', ZmLcyE-R: 5'-TGACTCTGAAGCTAGAGAAAG-3'), kit Reaction mixture for RT-PCR in presence of SYBR GreenI and ROX (OOO Synthol, Russia) and thermal cycler CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Reactions were carried out in three technical and two biological repetitions. The program for RT-PCR was as follows: 5 min at 95 °C (initial denaturation); 15 s at 95 °C (denaturation), 40 s at 60 °C (annealing and alongation) (40 cycles).

Quantification (mg/g fresh weight) of the total carotenoids, chlorophylls a and b, and β-carotene in leaves was carried out in three technical and two biological replicates [24-26]. Leaves (0.2 g) were homogenized in Folch solution (chloroform:methanol, 2:1 v/v) with trace amount of Mg_2CO_3, allowed for 1 h at 4 °C in an ice-water bath, and centrifuged for 10 min at 4000 rpm and 4 °C (Eppendorf 5418 R centrifuge, Eppendorf, Germany). In the chloroform phase, lycopene, β-carotene, total carotenoids, chlorophylls a and b were quantified. Absorption spectra were recorded on Eppendorf BioSpectrometer® basic (Eppendorf, Germany) and Cary 50 (Agilent Technology, USA) spectrophotometers. The amount of pigments was calculated [24, 25].

The results were processed using GraphPad Prism v.8 (GraphPad Software Inc., USA; https://www.graphpad.com/scientific-software/prism/). Data were expressed as means (*M*) with standard deviations (±SD). Unequal variance Welch's *t*-test was used to assess the significance of differences in gene expression or pigment content between maize lines (at $p < 0.05$, the differences are statistically significant). The correlations between pigment content and *LcyE* gene expression in the leaves of maize lines were also evaluated using GraphPad Prism v.8. The correlation was unambiguously present at $R^2 > 0.7$, highly probable at $R^2 = 0.4$-0.7, and absent at $R^2 < 0.4$.

Results. Different shades of corn grain color (from yellow to orange) depend on the composition and quantitative ratio of carotenoids [27]. Therefore, the white-grain lines 6097-1, MBK, and Shumny's tetraploid were taken as samples with presumably impaired synthesis of lycopene, carotenes, and xanthophylls. The yellow grain line 5580-1 with preserved biosynthesis of colored carotenoids served as a control. We also investigated presumed correspondences between the peculiarities of carotenoid biosynthesis in grain and in photosynthetic tissue. The dark green color of the leaves in all four analyzed lines indicated successful photosynthesis and photoprotection, i.e. xanthophylls were biosynthesised in the leaves [3].

The highest content of carotenoids in leaves was characteristic of the line Shumnoy's tetraploid (Fig. 1, A). The rest lines synthesized a smaller amount of carotenoids and were similar to each other in this trait. In the leaves of line 6097-1, there was approximately 2 times more β-carotene than in other analyzed samples (see Fig. 1, B). For chlorophylls a and b, a quantitative profile was similar to that of β-carotene (see Fig. 1, C, D).

Therefore, the absence of associations between grain color and the content of total carotenoids and β-carotene in maize leaves was confirmed. We suggest that this may be due to the activity of other genes in the carotenoid biosynthetic pathway, for example, the *PSY* gene for phytoin synthase which catalyzes the synthesis of phytoin, the precursor of all carotenoids. The maize genome contains three *PSY* paralogs. *PSY1* triggers carotenoid synthesis in grain endosperm, while carotenogenesis in leaves depends primarily on *PSY2* activity [16].

The analysis data also suggest a positive relationship between the amount of β-carotene and chlorophylls a and b. Since β-carotene is a precursor of xanthophylls in the main xanthophyll cycle of plant photoprotection [3], one can

speak of an increased rate of photosynthesis in the photosynthetic tissues of line 6097-1 compared to other lines. Accordingly, line 6097-1 may have an increased resistance to oxidative stress, as well as be a donor of the trait of an increased content of provitamin A as a silage crop.

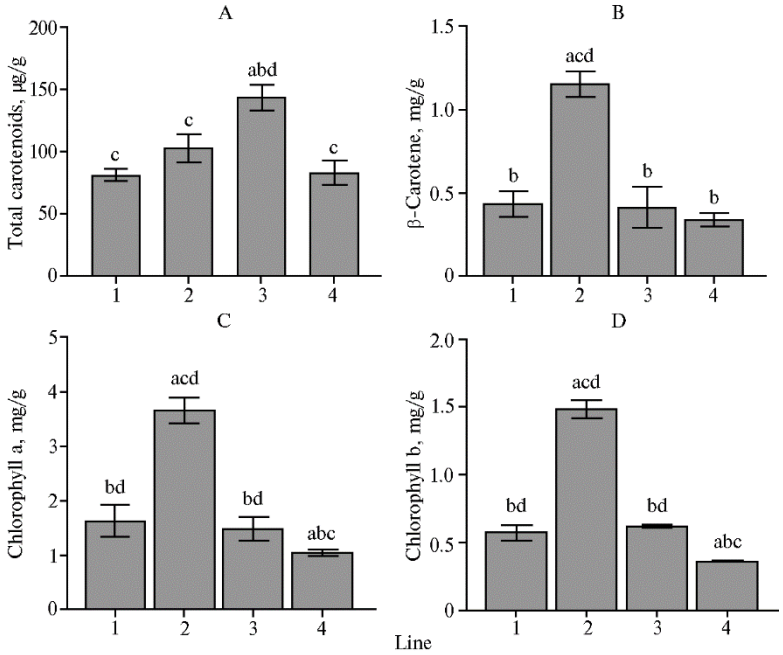

Fig. 1. Total carotenoids (А)**, β-carotene** (Б)**, chlorophyll a** (В) **and chlorophyll b** (Г) **accumulation in leaves of inbred corn (*Zea mays* L.) lines:** 1 — MBK, 2 — 6097-1, 3 — Shumnoy's tetraploid, 4 — 5580-1 (lab test, $n = 3$, $N = 2$).
[a, b, c, d] Differences between samples are stistically significant at $p < 0.001$.

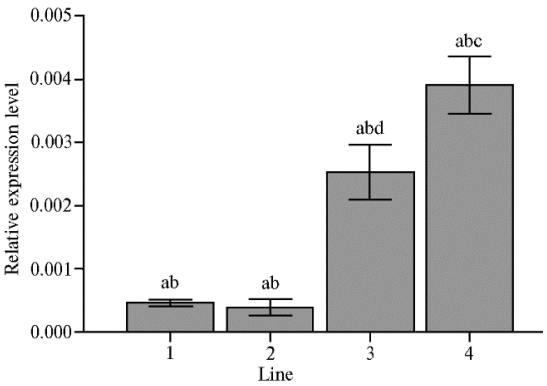

Fig. 2. Relative expression of the gene *LcyE* in leaves of inbred corn (*Zea mays* L.) lines: 1 — MBK, 2 — 6097-1, 3 — Shumnoy's tetraploid, 4 — 5580-1 (lab test, $n = 3$, $N = 2$).
[a, b, c, d] Differences between samples are stistically significant at $p < 0.001$.

It is known that the type of carotenes and xanthophylls is determined by the ratio of the β-ε and β-β branches of the carotenoid biosynthesis pathway, which depends on the expression levels of the *LcyE* and lycopene-β-cyclase *LcyB* genes. In addition, the accumulation of β-carotene is affected by the activity of the β-carotene hydroxylase 1 gene (*β-CH*, or *crtRB1*) [28].

In the same leaf tissues, we determined the expression of the lycopene-ε-cyclase *LycE* gene (Fig. 2). The expression was the highest in the leaves of line 5580-1 and slightly lower in the line Shumny's Tetraploid. In the lines MBK and 6097-1, the gene transcription in leaves was ~ 4-5 times lower (see Fig. 2). This intersample profile is consistent with the previously shown inverse relationship between *LycE* expression and provitamin A production [13, 22]. High *LycE* expression presumably means a shift in carotenoid biosynthesis towards the β-ε branch with the production of α-carotene and xanthophylls of the minor (second) type of the xanthophyll cycle with the formation of lutein and its derivatives.

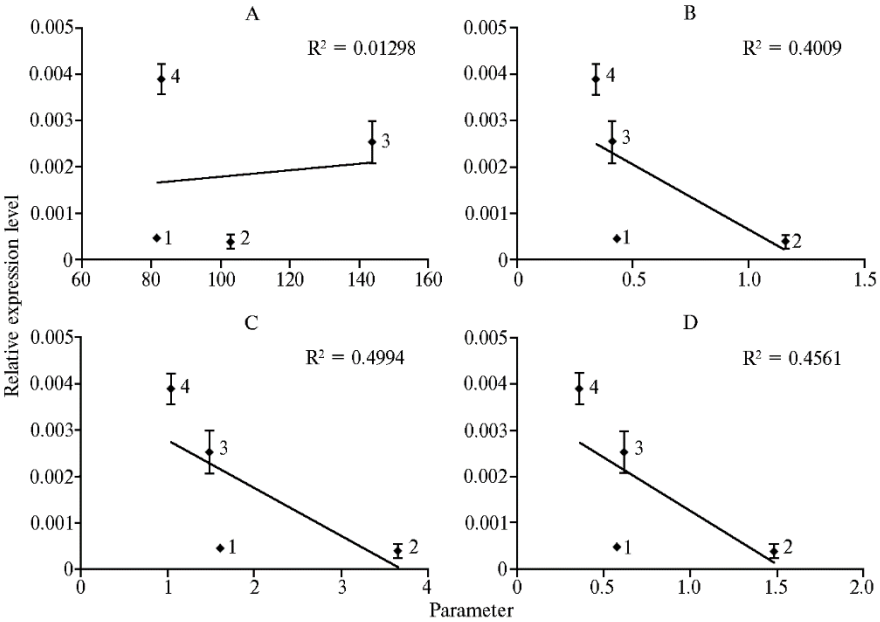

Fig. 3. Correlations between the level of relative expression of the gene *LcyE* and total carotenoids (μg/g) (A)**, β-carotene (mg/g)** (B)**, chlorophyll a (mg/g)** (C) **and chlorophyll b (mg/g)** (D) **concentrations in leaves of inbred corn (*Zea mays* L.) lines:** 1 — MBK, 2 — 6097-1, 3 — Shumnoy's tetraploid, 4 — 5580-1 (lab test, $n = 3$, $N = 2$).

Correlation analysis confirmed our assumptions. While there was no relationship between the *LycE* gene expression and the total carotenoids (Fig. 3, A), an inverse correlation between the *LycE* gene expression and the amount of β-carotene, chlorophylls a and b (Fig. 3, B-D) was predicted with high probability. This is consistent with previously obtained data for the photosynthetic tissue of *A. thaliana* [23].

Thus, the color of the maize grain does not correlate with the sum of carotenoids and β-carotene in the leaves. There is a positive relationship in the leaves between the concentration of β-carotene and chlorophylls a and b. In addition, we revealed an inverse relationship between the content of β-carotene and chlorophylls a and b and the level of *LycE* gene expression. Based on the findings, we suggest that the *LycE* gene expression level can be an expression molecular marker for provitamin A synthesized in maize leaves and also in assessment of plant resistance to photooxidative stress.

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