A Single Amino Acid Insertion in LCYB2 Deflects Carotenoid Biosynthesis in Red Carrot

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Abstract

Carotenoids are phytochemicals that are precursors of vitamin A and effective antioxidants, required for human health. The mechanisms and underlying genetic network responsible for regulating carotenoid production in plants, however, is poorly understood, despite the carotenoid biosynthesis pathway being known. We found that a single amino acid insertion in lycopene β-cyclase2 (LCYB2) caused catalytic failure, possibly due to a flux down of lycopene to the carotenoids which may be the molecular basis for the color of red carrot roots.

Key Message

A single amino acid insertion in LCYB2 was found to cause catalytic failure and a flux of lycopene, which may be the molecular basis for the color of red carrot.

Introduction

Cyclization of lycopene distinguishes branching point of the carotenoid biosynthesis pathway to complete the metabolic processes in higher plants (DellaPenna and Pogson 2006). LCYB catalyzes two β-rings (δ- and γ-lycopene) of lineal lycopene to produce β-carotene, while lycopene ε-cyclase (LCYE) creates one ε-ring to give δ-carotene and together with LCYB they produce α-carotene. The demands of the two branches of the carotenoids depends on the plant species, as expression levels for the two cyclases varies (Harjes, et al. 2008). LCYB gene paralogs have been linked to organ-specific functions, such as flowering and fruiting (Blas, et al. 2010; Ronen, et al. 2000). Carrot harbors two LCYB genes, DcLCYB1 and DcLCYB2, and a functional analysis of DcLCYB1 found that it is crucial for β-carotene biosynthesis as chloroplast-targeted enzymes and the expression level affected plant development and carotenoid abundance (Kössler, et al. 2021). DcLCYB2 is a chromoplast-specific enzyme whose functions and relationship to carrot carotenogenesis are unknown (Fig. S1). This study aimed to provide a molecular explanation for the different red- and orange-colors resulting from carotenoid biosynthesis in natural carrot inbred lines.

Seeds of inbred carrot lines were obtained from NongWoo-Bio (Anseong, Gyeonggi-do, Korea) and grown for 12 weeks in a growth room (16 h light / 8 h dark). There was a distinct difference in the color of the storage roots between the red (D802 and D904) and orange (GD2002 and GD2003) lines (Fig. 1a). As red-colored vegetables have an increased lycopene content (Li, et al. 2018; Zhu, et al. 2008), we measured the carotenoid content in all lines. Notably, the red carrots had an average of 3700 mg/kg DW of lycopene, but it was not detected in the orange carrots, while the β-carotene content of the red carrots was approximately four times less than that of the orange carrots. Furthermore, the α-carotene content of the orange carrots was 2500 mg/kg DW on average but it was not detected in the red carrots (Fig. 1b and 1c). These results led us to assume that the differences in root color may be due to an abnormality in the LCY enzymes that convert lycopene to α- and/or β-carotenes (Zhang, et al. 2020). We aligned the LCY enzyme amino acid sequences to examine the genetic variations between the red and orange lines, and found

nine amino acid differences in DcLCYB1 (Fig. S2), three in DcLCYB2 (Fig. S3), and two in DcLCYE (Fig. S4) (Fig. 1d). Color complementation assays were conducted in E. coli that lacked lycopene cyclase activity (Cunningham, et al. 1994) to determine if the amino acid differences affected the catalytic activity. We used pAC-LYC (Addgene, Watertown MA, USA) to produce lycopene in E. coli and pAtLCYbSK (Addgene) to producing Arabidopsis LCYB as a positive control (Fig. S5a). DcLCYB1, DcLCYB2, and DcLCYE genes from the two-representative orange- (GD2003) and red-colored (D904) inbred lines were tagged with HA and cloned into the same place in the pBluescript SK- vector after the AtLCYB gene was removed (Fig. S5b, Table S1). pAC-LYC was transformed alone as a negative control and co-transformed with lycopene cyclases in E. coli strain TOP10 competent cells. The enzymatic activity of each lycopene cyclase can mediate the conversion of lycopene (red) to β-carotene (yellow) (Zeng, et al. 2015). There were no color differences in the E. coli cells that expressed DcLCYB1 or DcLCYE between GD2003 and D904 along with yellow-colored exchange, indicating catalytic activities. For DcLCYB2, only the GD2003 recombinant protein produced carotene, giving it a yellow color, whereas there was no carotene in D904 due to the lack of color exchange in the negative control and a loss of enzyme activity (Fig. 1e). This suggests that the differences in the LCYB2 amino acid sequences between GD2003 and D904 influenced the enzyme activity, resulting in differences in carotene production.

To identify the key residue for LCYB2 enzyme activity, three-point mutations of LCYB2 in GD2003 that differed from D904: GD2003 L14S, 140insS, and V344I were conducted. With a serine insertion at the 140th GD2003 LCYB2 amino acid, the color of the E. coli complementation changed from orange to red like D904, but there was no change with the L14S or V344I mutagenesis (Fig. 1f). HPLC analysis also showed that the negative control and pAC-LYC + DcLCYB2 of D904 exhibited only single peaks of lycopene, however, pAC-LYC + DcLCYB2 of GD2003, GD2003L14S, and GD2003V244I had double peaks with higher beta-carotene contents than the lycopene, whereas pAC + DcLCYB2 of GD2003ins140S was distinctly different, displaying single lycopene peaks without β-carotene (Fig. 1f and g). In addition, immunoblotting results showed that the protein expression of the LCYB2s clones from GD2003, GD2003-mutated constructs, and D904 became uniform, confirming that the difference in color was not due to protein expression but to differences in enzyme activity (Fig. 1h). Consequently, we have revealed that the differences in root color between GD2003 and D904 are due to the 140 serine of LCYB2, which affects enzyme activity during E. coli complementation.

To conclude, red-colored carrot was found to have a natural serine insertion mutation at the 140th amino acid resulting in the loss of LCYB2 enzyme activity. This could be due to a modified protein structure, resulting in the accumulation of lycopene by inhibiting its conversion to carotenes (Fig. 1i). These results reveal the molecular basis for the red carrot root color, although other mechanisms may be involved. The results support the notion that DcLCYB2 is required for the formation of α-carotene despite the existence of DcLCYB1 and DcLCYE.

Author contributions: H.C. and Y.K. conceived the project and designed the research. S.J. and H.P. performed the majority of experiments and analyzed the data. A.L., H.J., J.H., and M.J. assisted with the

molecular and physiological analyses. S.M., H.L., and H.K. discussed the data. S.J., H.P., Y.K., and H.C. wrote the manuscript.

Declarations

Author contributions:

H.C. and Y.K. conceived the project and designed the research. S.J. and H.P. performed the majority of experiments and analyzed the data. A.L., H.J., J.H., and M.J. assisted with the molecular and physiological analyses. S.M., H.L., and H.K. discussed the data. S.J., H.P., Y.K., and H.C. wrote the manuscript.

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Figures

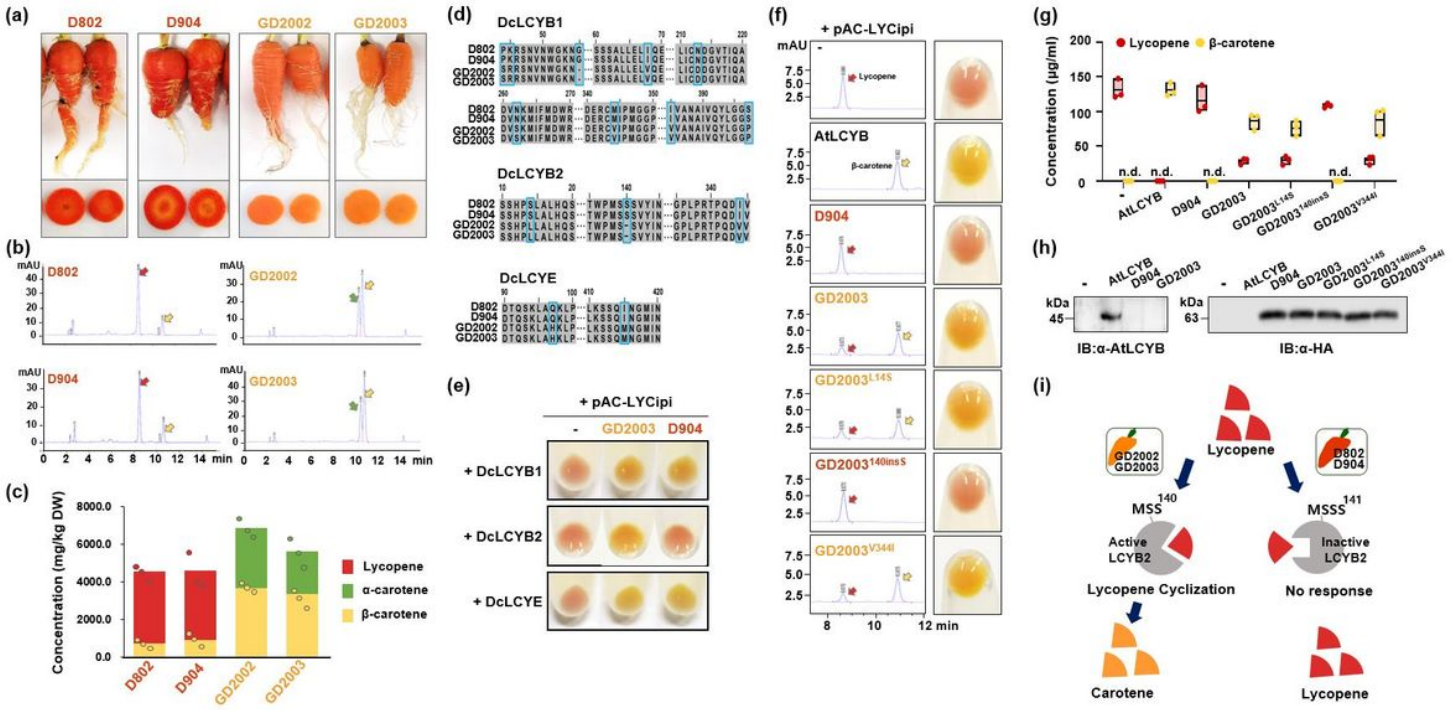

Figure 1

a. Root colors of four inbred lines (D802, D904, GD2002, and GD2003). The plants were grown for 12 weeks in a plant growth room (16 h light / 8 h dark condition). Storage roots and transverse cross sections are presented for the different lines. b-c. HPLC analysis for the roots of the four inbred lines. Carotenoids were extracted from freeze-dried root samples of 12-week-old carrots. The red arrows represent the lycopene peaks. The green and yellow arrows represent the α-carotene and β-carotene, respectively. HPLC analysis was performed for three biological repeats. Each specific value was marked with a circle in C. d. DcLCYB1, DcLCYB2, and DcLCYE amino acid sequence alignments between the red-colored (D802 and D904) and orange-colored (GD2002 and GD2003) inbred lines. The blue boxes indicate the differences between the red and orange-colored inbred lines. e. The color shift assay of E. coli cells to determine the catalytic activities of the three lycopene cyclase enzymes, DcLCYB1, DcLCYB2, and DcLCYE between the red-colored (D802 and D904) and orange-colored (GD2002 and GD2003) lines. pAC-

LYC with LCYB1, LCYB2, or LCYBE of the red- and orange-colored inbred lines were co-transformed into a Top10 competent cell strain. The construct that expressed the pAC-LYC vector alone was used as a negative control. The cells were extracted after culture for 3 days at room temperature. f. The color shift assay and HPLC analysis of the carotenoids in E. coli cells. Carotenoids were extracted from E. coli Top10 cells that co-expressed with pAC-LYC and AtLCYB, D904, GD2003, or point mutated constructs (L14S, 140insS, and V344I) of GD2003. AtLCYB was used as a positive control. The red arrow indicates the lycopene peak, and the yellow arrow indicates the beta-carotene peak. g. The concentration measurements of the lycopene and β-carotene in the E. coli cells using HPLC. The three replicates were conducted and n.d. indicates non-detection. h. The protein expression of LCYB2 proteins in E. coli cells. The AtLCYB protein was detected using α-AtLCYB antibody and the lycopene cyclase B2 proteins of carrot inbred lines were detected using α-HA antibody. i. The mechanistic carotenogenesis according to the LCYB2 of orange- and red-colored carrot inbred lines.

Supplementary Files

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- SupplementaryFigures.pptx