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The Functional Characterization of CCD Genes in Loquat

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Abstract

Carotenoid cleavage dioxygenase (CCD) is a pivotal enzyme that is widely distributed in plants, catalyzing the cleavage of carotenoids to produce a variety of apocarotenoid volatile compounds. However, the functional characterization of the CCD gene in loquat (*Eriobotrya japonica*) has not been thoroughly investigated. In this study, we compared 3-oxo-ionol glycosides in loquat and speculated that a highly expressed CCD enzyme in the leaves is responsible for carotenoid cleavage. We employed bioinformatics approaches to analyze the CCD gene family in loquat, constructing a phylogenetic tree and an expression heatmap. Based on subfamily classification and expression patterns, we inferred the potential functions of these genes. Three genes with high expression levels were identified, and their expression patterns correlated with the accumulation of carotenoid cleavage derivatives in loquat leaves. To validate their functions, we cloned these genes and successfully obtained two of them. And we confirmed that Eja10G000940.1 exhibits cleavage activity on zeaxanthin. This study lays a foundation for further analysis of the function of CCD genes in loquat.

Keywords

Loquat; CCD; expression pattern; function; zeaxanthin.

1. INTRODUCTION

Loquat (Eriobotrya japonica (Thunb.) Lindl.) is an evergreen tree that belongs to the Rosaceae family. Loquat has a long history of medicinal and edible use in China, recognized for its efficacy in relieving cough and moistening the lungs, etc [1]. Currently, loquat leaves are widely utilized in clinical applications due to its antitussive, bronchodilatory, expectorant, anti-inflammatory, antimicrobial, and antidiabetic properties[2]. To elucidate the pharmacological substances in loquat leaves, various bioactive compounds, including ursolic acid, oleanolic acid, nerolidol and acacia alcohol, were isolated [3]. Additionally, 3-oxo-ionol and its glycosides are abundant in loquat leaves at approximately 1‰ [4][5][6]. These compounds possess distinct aroma profiles, making them essential components in the fragrance of tea, grapes, roses, onions, and fruit wines [7][8].

3-oxo-ionol and its glycosides were presumed to be the degradation product of carotenoids [9]. However, the precise biosynthetic pathway of 3-oxo-α-ionol and its glycosides remained unknown. During the degradation of carotenoids, the cleavage of carotenoids catalyzed by

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carotenoid cleavage dioxygenases (CCDs) was considered pivotal [10]. To date, the CCD genes have been identified and categorized into two distinct families. The first family, designated as the CCD family, comprises CCD1, CCD2, CCD4, CCD7, and CCD8 subfamily [11]. The second family, known as the 9-cis-epoxycarotenoid dioxygenase (NCED) family, includes NCED1, NCED2, NCED3, NCED4, NCED5, NCED6, and NCED9 subfamily [12][13]. Members of the NCED family, along with CCD7 and CCD8 subfamily, are critically involved in the biosynthesis of phytohormones, such as abscisic acid (ABA) and strigolactones (SLs), as well as their derivatives [14]. CCD1, CCD2, and CCD4 subfamily could produce a variety of apocarotenoid volatile compounds with different carbon chain lengths [15].

In this study, we performed a systematic characterization of CCD genes in loquat. A phylogenetic tree was constructed to assign specific subfamily of CCDs. Expression profiling revealed that three CCD genes showed high expression levels in leaf tissue. In vitro enzyme activity assays showed that Eja10G000940.1 exhibit carotenoid cleavage activity to produce 3-hydroxy ionone. These findings provide a robust foundation for the functional roles of CCD genes in loquat.

2. MATERIALS AND METHODS

2.1. Experimental materials

Plant materials: The loquat plant used in this study were cultivated in the Kunming Institute of Botany, Chinese Academy of Sciences.

2.2. RNA extraction and cDNA synthesis

Plant samples were ground into powder using liquid nitrogen, and total RNA was extracted using the FastPure Universal Plant Total RNA Isolation Kit (Vazyme). RNA integrity was assessed by 1% agarose gel electrophoresis. RNA concentration was measured using a nucleic acid-protein analyzer. The RNA was then reverse-transcribed into cDNA using the All-In-One 5X RT MasterMix (abm) and stored at -80°C for subsequent experiments.

2.3. The analysis of candidate gene

Genomic data for loquat were downloaded from the China National Center for Bioinformation (Accession: GWHBOTF00000000) [16]. CCD genes were screened using the CCD HMM (hidden Markov model) model (PF03055; https://www.ebi.ac.uk/interpro/entry/pfam/PF03055) implemented in the TBtools software. Multiple sequence alignments and phylogenetic tree construction were performed using MEGA11. Expression patterns of CCD candidate genes were also visualized using TBtools [17].

2.4. The content analysis of 3-oxo-ionol glycoside in loquat

Fresh tissue samples (2 g) were collected from various parts of Eriobotrya japonica, including leaves, flowers, fruits, stems, and roots. These samples were immediately frozen in liquid nitrogen and ground into a fine powder. The powder was then subjected to ultrasonic extraction using 10 mL of methanol for 1.5 h. After extraction, the samples were centrifuged at 4000 rpm for 20 minutes, and 1 mL of the supernatants were collected. This supernatants were centrifuged at 12000 rpm for 10 minutes before being analyzed by LC-MS.

2.5. Gene cloning and plasmid construction

cDNA was used as a template to amplify CCD candidate genes with primers listed in Table 1. The target MBP-pET28a(+) plasmid was digested with HindⅢ and Xho Ⅰ restriction enzymes (Thermo Scientific, USA), and the PCR products of the candidate genes were assembled using the ClonExpress II One Step Cloning Kit (Vazyme, China). The assembled mixture was transformed into Escherichia coli DH5α competent cells. After incubation at 37°C, the

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transformed cells were plated on LB agar plates containing kanamycin (50 μg/mL) for selection. Positive clones were verified by PCR and sequencing.

Table 1. EjCCD primers used in the experiment

Primer name	Primer sequences
Eja07-MBP-F	agctccgtcgacaagctggcATGGATGCCTTCTCTTCCTCCTTTCTATCCACATTTC
Eja07-MBP-R	tggtggtggtggtgctcgagCTACAACTTGTTGAGATCACTTTCCTTCACAAAGAGTCC
Eja10-MBP-F	agctccgtcgacaagctggcATGGCGGGTGTCGAAGTTAACGACG
Eja10-MBP-R	tggtggtggtggtgctcgaggTTAGAGCTTTGCTTGTTCTTGCAATTGCTCCTCTG
Eja05-MBP-F	agctccgtcgacaagctggcATGGCGGAGGTCGAAGTTAACGACG
Eja05-MBP-R	tggtggtggtggtgctcgaggTTAGAGCTTCGCTTGTTCTTGCAATTGCTCC

2.6. Expression and purification of candidate genes in escherichia coli

The positive plasmids were transformed into E. coli BL21 cells and cultured overnight at 37°C on LB agar plates with kanamycin (50 μg/mL). Target colonies were transferred to LB liquid medium containing kanamycin (50 μg/mL) and incubated at 37°C for 16 hours. 20 mL overnight culture was then inoculated into 2 L of LB medium, supplemented with kanamycin (50 μg/mL), and grown at 37°C with shaking at 200 rpm until the OD600 reached 0.6 – 0.8. The culture was cooled to 16°C, and a final concentration of 0.2 mM IPTG (isopropylthio-β-galactoside) was added to induce protein expression overnight at 16°C. Cells were harvested and resuspended in 35 mL of buffer A (15 mM imidazole, 50 mM Tris-HCl, 300 mM NaCl, 10% glycerol, pH 8.0). The following procedures were carried out on ice or at 4°C. After the cell were lysed by sonication, the lysis solutions were centrifugated at 24,000 rpm for 45 minutes. The supernatants were filtered through a 0.22 μm PES membrane. The filtered solutions were loaded onto a HisTrap FF 5 mL column, and proteins were eluted using a gradient program: 0–12.5 minutes, 100% buffer A; 12.5–25 minutes, 50% buffer A; 25–30 minutes, 100% buffer B; 30–37.5 minutes, 100% buffer A (Buffer B contained 500 mM imidazole, 50 mM Tris-HCl, 300 mM NaCl, and 10% glycerol, pH 8.0). The target proteins were concentrated using an Amicon Ultra-4 centrifugal filter (Ultracel, 10,000 NMWL) at 3,800 rpm and washed with storage buffer (100 mM NaH2PO4, 10% glycerol, pH 7.0) to remove imidazole. Protein concentrations were measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). The protein was aliquoted, rapidly frozen in liquid nitrogen, and stored at -80°C.

2.7. In vitro enzyme assay for candidate genes

Purified protein (100 μg) was incubated in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM sodium ascorbate, 4% (w/v) n-octyl-β-D-glucopyranoside, 0.05 mM Fe2+, 0.1% Triton X-100, and 1 mM zeaxanthin at 30°C for 4 hours. After the reaction, acetonitrile was added to quench the reaction, and the mixtures were centrifuged at 12,000 rpm for 10 minutes. The supernatants were analyzed by LC-MS.

Chromatography was performed using a YMC-Triart C18 column (4.6 mm × 250 mm) with water (solvent A) and acetonitrile (solvent B) as mobile phases. The gradient was 10% to 100% B over 20 minutes, followed by 25 minutes of isocratic elution with 100% B, and a final 4-minute wash with 10% B. The flow rate was 1.0 mL/min, and MS data were collected in positive ion mode (mass range: 50–800 m/z).

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3. RESULTS

3.1. Metabolomic analysis of 3-oxo-ionol glycosides in loquat

Specific tissue in plants exhibit a high content of natural products, which is typically correlated with the expression levels of their biosynthetic genes in this specific tissue. To further determine the biosynthetic tissue of 3-oxo-ionol glycosides, we compared the content of (6R,9S)-3-oxo-α-ionol glucoside across leaf, stem, root, flower and fruit tissues of loquat. Consequently, loquat leaves accumulated the highest concentrations of (6R,9S)-3-oxo-α-ionol glucoside (Figure 1). Additionally, an isomer of (6R,9S)-3-oxo-α-ionol glucoside may be enriched in loquat leaves. The high abundance of 3-oxo-ionol glycosides in loquat leaves indicated that the genes involved in their biosynthesis showed high expression pattern in leaf tissue.

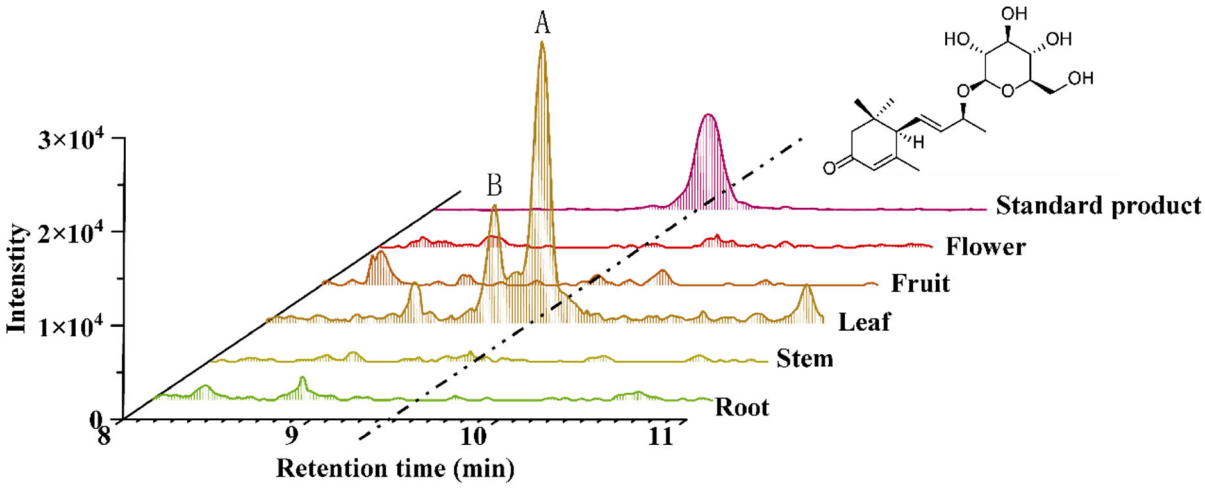

Figure 1. Comparison of carotenoid cleavage products in loquat. Peak B is an analogue of peak A

3.2. Phylogenetic analysis of the CCD protein family members

19 CCD candidate genes were identified from the genome of loquat by HMM model screening and selecting based on the size of CCD genes. Subsequently, we performed a protein sequence alignment between the 19 CCD candidate protein sequences and 28 CCD sequences publicly available on NCBI. A comprehensive phylogenetic tree was constructed using the Maximum Likelihood Tree (ML) method with 1000 bootstrap replicates to categorize these candidate proteins into different subfamilies (Figure 2). Based on the tree's branching and functional classifications, the 47 CCD protein sequences were divided into six groups. Within this group, CCD4 consisted of six members from loquat, and CCD1 consisted of two members from loquat. These enzymes could be capable of cleaving a variety of carotenoids at different sites and were considered as key enzymes in carotenoid cleavage. Six EjCCD protein sequences from loquat were assigned to the NCEDs subfamily, which might function in the biosynthesis of plant hormones such as abscisic acid (ABA) and strigolactones (SLs) [19][20]. CCD7 and CCD8, which contained three members, might play a critical role in the cleavage of 9-cis-epoxycarotenoids to generate precursors for the plant hormones strigolactones [20].

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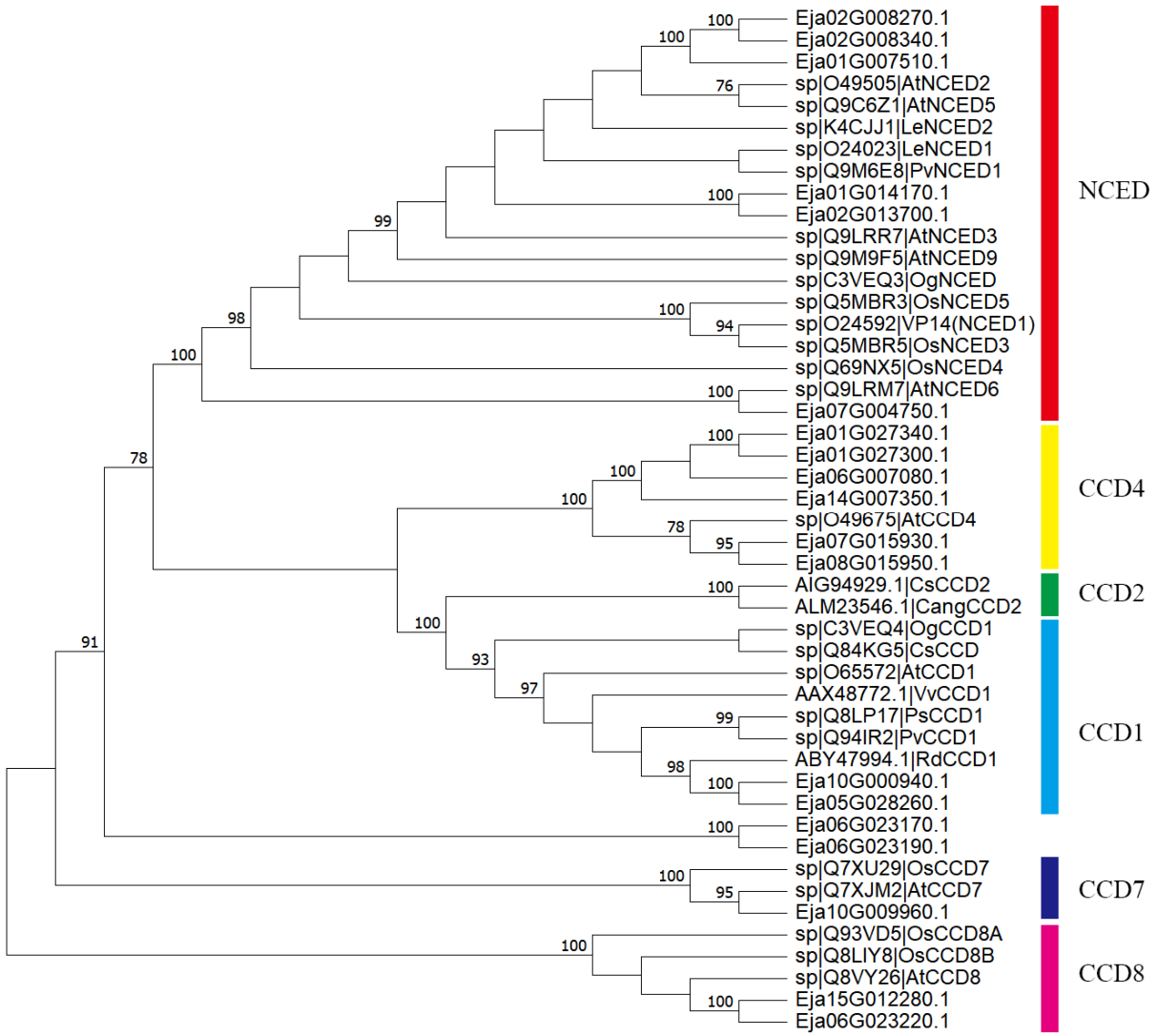

Figure 2. Phylogenetic Tree of CCD Proteins

3.3. Expression pattern analysis of CCD protein family members

The expression levels of loquat CCD genes were visualized using a heatmap generated by TBtools software (Figure 3). As shown in the Figure 2, the genes sets were classified into five groups based on phylogenetic analysis. Among them, genes in CCD1 and CCD4 groups showed high expression levels in loquat leaves. Eja07G015930.1, which belongs to the CCD4 subfamily, and Eja05G028260.1, belonging to the CCD1 subfamily, showed leaf-specific expression patterns. Eja10G000940.1, which belongs to the CCD1 subfamily, exhibited high expression levels in all tissues except for root tissue. These three leaf-specific expression CCD genes were speculated to be candidate genes involved in the biosynthesis of 3-oxo-ionol glycosides, consistent with the high content of 3-oxo-ionol glycosides in loquat leaves. Genes of NCED, CCD7 and CCD8 subfamilies showed relative low expression in loquat, which was consistent with the low content of ABA and SLs phytohormones.

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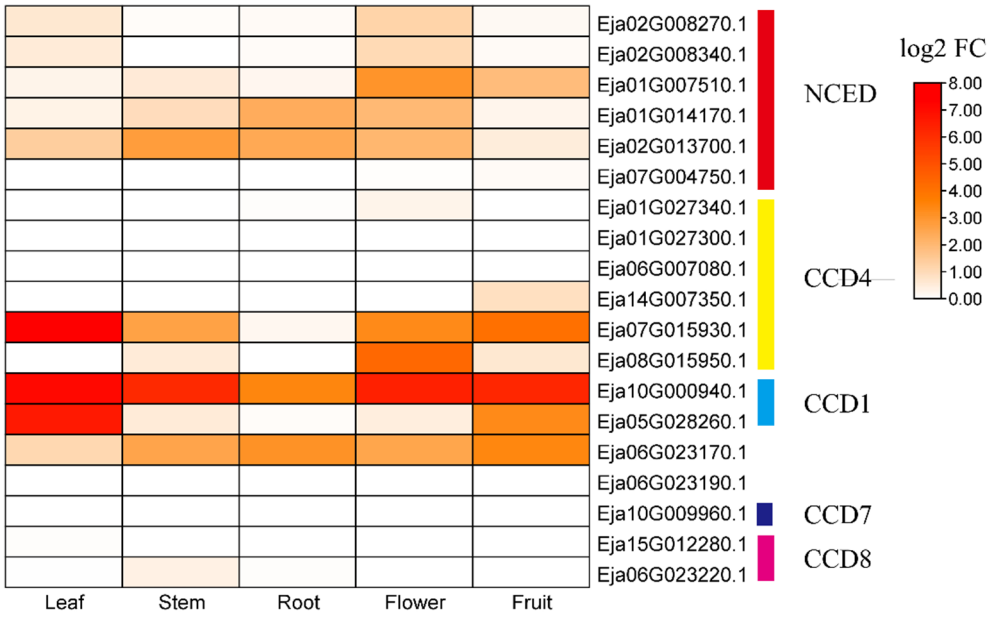

Figure 3. Expression heat map of loquat CCD gene family

3.4. Protein expression and in vitro functional validation of candidate genes

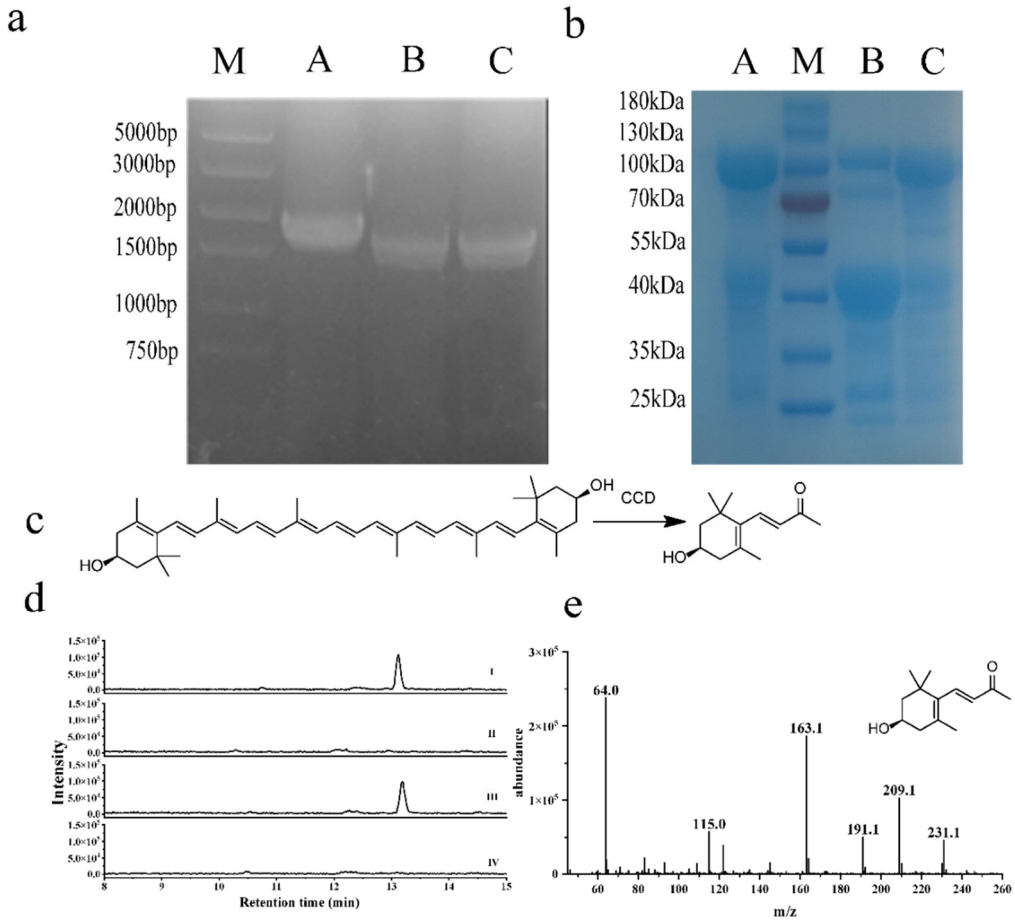

Figure 4. CCD functional verification. (a)Gel electropherogram of the EjCCD gene. (b)10%SDS-PAGE gel. Lane A:FaCCD1; Lane M: Maker; Lane B: Eja07G015930.1; Lane C: Eja10G000941.1(c) The cleavage of zeaxanthin catalyzed by CCD enzymes. (d) In vitro enzyme reaction results of CCDs, with the extracted ion at m/z 209. Protein I: FACCD1; Protein II: Eja07G015930.1; Protein III: Eja10G000940.1; IV: Negative control.(e) Mass spectrometry data at 13.2 minutes.

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To validate the functions of these three CCD candidate genes, the candidate genes were amplified from the cDNA of loquat leaves and the amplified products were analyzed by 1% agarose gel electrophoresis, resulting in three distinct bands within the 1500–2000 bp range (Figure 4a). The sequencing results showed that lane A corresponded to the amplification product of Eja07G015930.1, as well as lane B and C corresponded to Eja10G000940.1. The failure to amplify Eja05G028260.1 was due to the high nucleic acid consistency between Eja07G015930.1 and Eja10G000940.1. Thus, Eja07G015930.1 and Eja10G000940.1 genes were used for further functional analysis. The open reading frame (ORF) of Eja07G015930.1 is 1743 bp in length, encoding 601 amino acids, and the ORF of Eja10G000940.1 is 1647 bp, encoding 569 amino acids. Additionally, a positive gene FaCCD1 (GenBank: EU314719.1)[18], which can catalyze zeaxanthin into 3-hydroxy-β-ionone, was synthesized and its encoding protein was expressed. After the purification of the target MBP-CCD fusion proteins, the fusion proteins of Eja07G015930.1, Eja10G000940.1 and FaCCD1 exhibited a size of approximately 100 kDa (Figure 4b).

In vitro experiments of CCD enzymes, the product of Eja10G000940.1 was identical to that of the positive FaCCD1 (Figure 4). They both catalyzed the conversion of zeaxanthin into 3-hydroxy-β-ionone. During this catalytic process, the cofactor n-octyl-β-D-glucopyranoside might function as a mediator, facilitating the interaction between the hydrophobic zeaxanthin and the hydrophilic CCD enzyme, thereby promoting the reaction process. Based on the enzymatic assays, the Eja10G000940.1 gene is speculated to be involved in the biosynthesis of 3-oxo-α-ionol glucoside in loquat.

4. CONCLUSION

In this study, 19 CCD family members in loquat were identified through HMM model screening. After the phylogenetic tree analysis and expression pattern analysis of CCD genes, as well as the metabolic analysis of 3-oxo-ionol glycosides, three leaf-specific expressed CCD genes were speculated to be involved in the biosynthesis. The in vitro experiments showed Eja10G000940.1 catalyzed conversion of zeaxanthin into 3-hydroxy-β-ionone. This finding provides valuable insights for the in-depth analysis of the biosynthetic pathway of 3-oxo-ionol glycosides in loquat, and establishes a solid foundation for future research endeavors.

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REFERENCES

[1] Dhiman A, Suhag R, Thakur D, et al. Current status of Loquat (Eriobotrya japonica Lindl.): Bioactive functions, preservation approaches, and processed products. Food Reviews International, 38 sup1 (2022), 286-316.

[2] Zhu X, Wang L, Zhao T, et al. Traditional uses, phytochemistry, pharmacology, and toxicity of Eriobotrya japonica leaves: A summary. Journal of ethnopharmacology, 298 (2022), 115566.

[3] Xiao S, Wang W, Liu Y. Research Progress on Extraction and Separation of Active Components from Loquat Leaves. Separations, 10.2 (2023), 126.

[4] Ito H, Kobayashi E, Li S H, et al. Antitumor activity of compounds isolated from leaves of Eriobotrya japonica. Journal of agricultural and food chemistry, 50.8 (2002), 2400-2403.

[5] Ito H, Kobayashi E, Li S H, et al. Megastigmane Glycosides and an Acylated Triterpenoid from Eriobotrya j aponica. Journal of natural products, 64.6 (2001), 737-740.

ISSN: 2472-3703 DOI: 10.6911/WSRJ.202505_11(5).0002

[6] Wu Q L, Wang M, Simon J E, et al. *Studies on the chemical constituents of loquat leaves (Eriobotrya japonica)*. (2003).292-306

[7] UENOJO M, PASTORE G M. β-carotene biotransformation to obtain aroma compounds. Ciência E Tecnologia de Alimentos, 30.3 (2010), 822-827.

[8] YAHYAA M, BAR E, DUBEY N K, et al. Formation of norisoprenoid flavor compounds in carrot (Daucus carota L.) roots: characterization of a cyclic-specific carotenoid cleavage dioxygenase 1 gene. Journal of Agricultural and Food Chemistry, 61.50 (2013), 12244-12252.

[9] Wang R X.*Tobacco Chemistry*.Beijing:ChinaAgriculture Press (2003), 197-198.

[10] Daruwalla A, Kiser P D. Structural and mechanistic aspects of carotenoid cleavage dioxygenases (CCDs). Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1865.11(2013), 158590.

[11] Tan BC, Joseph LM, Deng WT, et al. Molecular characterization of the Arabidopsis 9-cis epoxycarotenoid dioxygenase gene family. Plant Journal, 2003, 35(1): 44- 56.

[12] Awasthi P, Khan S, Lakhani H, et al. Transgene-free genome editing supports CCD4 role as a negative regulator of β-carotene in banana[J]. Journal of Experimental Botany, 21.2 (2022), 21-27.

[13] Schwartz SH, Qin X, Zeevaart JA. Characterization of a novel carotenoid cleavage dioxygenase from plants. Journal of Biological Chemistry , 276.27 (2001), 25208- 25211.

[14] Jia KP, Baz L, Al-Babili S. From carotenoids to strigolactones. Journal of Experimental Botany, 69.9 (2018), 2189-2204.

[15] Rodríguez-Bustamante E, Sánchez S. Microbial production of C13-norisoprenoids and other aroma compounds via carotenoid cleavage. Critical Reviews In Microbiology, 33.3 (2007), 211-230.

[16] Jing D, Liu X, He Q, et al. Genome assembly of wild loquat (Eriobotrya japonica) and resequencing provide new insights into the genomic evolution and fruit domestication in loquat. Horticulture Research, 10.2 (2023), uhac265.

[17] Chen C, Chen H, Zhang Y, et al. TBtools: an integrative toolkit developed for interactive analyses of big biological data. Molecular plant, 13.8 (2020), 1194-1202.

[18] García-Limones C, Schnäbele K, Blanco-Portales R, et al. Functional characterization of FaCCD1: a carotenoid cleavage dioxygenase from strawberry involved in lutein degradation during fruit ripening. Journal of Agricultural and Food Chemistry, 56.19 (2008), 9277-9285.

[19] Wu W, Cao S, Shi L, et al. Abscisic acid biosynthesis, metabolism and signaling in ripening fruit. Frontiers in Plant Science, 14 (2023), 1279031.

[20] Chen GT, Wang JY, Jamil M, Braguy J, Al-Babili S. 9-cis-β-Apo-10'-carotenal is the precursor of strigolactones in planta. Planta. Nov,256.5 (2022),88.