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CLONING AND EXPRESSION ANALYSIS OF *OFCCD1* AND *OFCCD4* GENES IN SWEET OSMANTHUS

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Abstract

Sweet osmanthus is an extraordinary aromatic flower, an excellent greening tree species in gardens, and a raw material for processing spice of high economic value. More than 60 main aroma components have been identified in it. According to their chemical structures, the components can be divided into terpenes, aldehydes, esters, ketones, alcohols, and other compounds. Carotenoid cleavage dioxygenase is a key enzyme in the degradation of carotenoids, which catalyzes the cleavage of carotenoids to produce a variety of products, including the floral substances α-ionone and β-ionone. In order to explore the critical enzyme genes of sweet osmanthus, two *CCD* genes named *OfCCD1* (GenBank No. OM256439) and *OfCCD4* (GenBank No. OM145981) were cloned by RACE and RT-PCR and analyzed by bioinformatics and their expression pattern. The results showed that the total length of the cDNA of *OfCCD1* and *OfCCD4* genes were 1811 bp and 2076 bp, respectively. They both contained a complete open reading frame, 1632 bp and 1833 bp, encoding 543 aa and 610 aa, respectively. Analysis showed that both OfCCD1 and *OfCCD4* were stable hydrophilic proteins with an RPE65 domain and without signal peptide cleavage sites and transmembrane regions. Phylogenetic tree results showed that *OfCCD1* and *OfCCD4* were closely related to the evolution of the homologous proteins of *Olea europaea*. Expression analysis showed that the expression of *OfCCD1* was greatest in leaves at the full flowering stage, while the expression of *OfCCD4* was greatest in inflorescences at the bud-eye stage; *OfCCD4* had obvious tissue specificity. In conclusion, the cloning and correlation analysis of *OfCCD1* and *OfCCD4* genes can provide a further basis to study sweet osmanthus's floral aroma metabolism.

Key words: Sweet osmanthus, *OfCCD1*, *OfCCD4*, Gene clone, Expression analysis.

Introduction

Carotenoid cleavage dioxygenases (CCDs) belong to a family of non-heme iron-dependent enzymes. They are the critical enzymes for the degradation of carotenoids and catalyze the production of various apocarotenoids from carotenoids (Harrison *et al*., 2014). In plants, CCD1 and CCD4 are involved in the formation of flower color and fruit flavor and form small molecular volatile substances, such as β-ionone and geranylacetone. CCD7 and CCD8 can oxidize carotene to produce strigolactone precursors, which are involved in biological processes such as plant growth and stress resistance (Wei *et al*., 2022). In *Arabidopsis*, four CCDs have been identified, namely AtCCD1, AtCCD4, AtCCD7, and AtCCD8 (Tan *et al*., 2003). CCD1 and CCD4 enzymes can cleave substrates at the 9,10 (9', 10') double-bond position, but CCD4 enzymes can only cleave cyclic nonpolar carotenoids and apocarotenoids (Auldridge *et al*., 2006). In *Escherichia coli* cells, the expression of two chili pepper, CaCCD1, resulted in the production of α-ionone from β- and ε-carotene (Cheng *et al*., 2021). *In vitro* experiments show that although *Arabidopsis* AtCCD4 has little effect on C40 β-carotene, it can convert β-apo-8'-carotene in C30 apocarotenoids into fragrant β-ionone (Huang *et al*., 2009). The CCD1 enzyme is a cytoplasmic enzyme, whereas the CCD4 enzyme has access to carotenoids in the plastid (Ahrazem *et al*., 2010).

Sweet osmanthus blooms in autumn with a strong and pleasant aroma. It is a famous ornamental tree species (Wang *et al*., 2022) widely distributed in Asian countries such as China, South Korea, and Japan. After long-term artificial cultivation and breeding, Sweet osmanthus comprises four major cultivar groups with 154 cultivars, namely, the Siji, Albus, Luteus, and Aurantiacus Groups (Xin *et al*., 2013; Zang *et al*., 2004). Sweet osmanthus can be added to food as a spice to improve its quality and also provides the raw material for making scented tea, extract, essence, perfume, and other products (Wu *et al*., 2009). The aromatic substances in sweet osmanthus are plant secondary metabolites of significant research value (Li *et al*., 2020). In sweet osmanthus petals, linalool, linalool oxide, β-ionone, dihydro-β-ionone, n-hexanal, leaf aldehyde, etc. constitute the main aromatic components (Cai *et al*., 2014). The genome and transcriptome sequencing of sweet osmanthus has been published, laying the foundation for further research on its aroma-related genes (Chen *et al*., 2021). In this paper, sweet osmanthus inflorescence was used as material to clone *OfCCD1* and *OfCCD4* genes and conduct bioinformatics analysis to further detect the expression patterns in different tissues and different flowering conditions. The study of gene expression patterns and bioinformatics analysis provides a basis for the next step to verify the function of the genes.

Materials and Methods

Material: Sweet osmanthus (*Osmanthus fragrans*) was selected as the test material. Its leaf margins are wavy, and its floral fragrance is strong. The collected materials were quick-frozen in liquid nitrogen and stored in a -80°C refrigerator for future use. Different tissues (roots, stems, leaves, and inflorescences) from different flowering stages, initial flowering (IF), early flowering (EF), full flowering (FF), and last flowering (LF), were selected for expression analysis.

GC-MS analysis: One g of petals from the same flowering stage was inserted into a 15 mL headspace bottle and equilibrated at 25°C for 30 min. The pre-aging SPME extraction head (SUPELCO) was inserted into a sampling bottle heated to 50°C and placed 1 cm above the flower.

Headspace adsorption: After 40 min: the GC injection port was inserted, and the sample was analyzed for 5 min; the floral aroma components were determined and analyzed by the gas chromatography-mass spectrometer *Trace DSQ* (Thermo Finnigan).

Chromatographic conditions: TTR-5MS (30 m×0.25 mm×0.25 μm) elastic quartz fiber capillary column; The carrier gas was high-purity helium (99.999%); flow rate, 1mL/min; split ratio, 10:1, and injection port temperature, 250°C.

Heating program: the initial temperature of 40°C was maintained for 2 min, increased to 60°C at 2°C/min, increased to 100°C at 5°C/min, increased to 250°C at 10°C/min, and held for 5 min.

Mass spectrometry conditions: interface temperature 250°C, ionization mode EI, ionization energy 70 eV, mass scanning range 50-450 amu. The detected components were characterized by the MS database NIST14 and retention time, and the relative content of each component in the sample was calculated by the peak area normalization method (Wang *et al*., 2009).

Gene clone: The Total RNA Extraction Kit (Tiangen) was used to extract the sweet osmanthus inflorescence's total RNA. The concentration and OD value of the extracted RNA were detected by the 2000c Thermo Scientific's NanoDrop Spectrophotometer, and the integrity of the RNA was detected by agarose gel electrophoresis. Using the extracted total RNA as the template, the first-strand cDNA was synthesized according to the instructions of the M-MuLV reverse transcriptase (Thermo Scientific). The 3'-end cDNA and 5'-end cDNA were synthesized using the 3'-full RACE Core Set and 5'-full RACE with TAP Kit (Takara Bio).

Using the transcriptome data for sweet osmanthus, the EST (expressed sequence tag) sequence of the CCD4 genes was queried, and the primers of the intermediate fragment were designed using Oligo Software. The first-strand cDNA was used as the template to amplify the intermediate fragment, and the pEASY-T1 Cloning Kit (TransGen Biotech) was used to perform TA clones; positive clones were sequenced. The obtained median fragment sequence was used as a reference. The 3'-end and 5'-end primers were designed, and the sequence results of both ends were obtained by nested PCR (polymerase chain reaction), and the full-length cDNA sequence was obtained after splicing (Table 1).

Bioinformatics analysis: Bioinformatics analysis was performed on *OfCCD1* and *OfCCD4*, including the complete ORF (open reading frame), encoded amino acid sequence, basic physical and chemical properties, protein domains, etc. The homologous sequences were compared using the BLASTP tool in the NCBI database, the more similar sequences were selected, and the Neighbor-joining algorithm in MEGA Software was used to construct a phylogenetic tree to predict the relationship between *OfCCD1* and *OfCCD4*.

Gene expression analysis: The materials collected from the different tissues and different flowering stages to extract RNA and cDNA were synthesized using M-MuLV reverse transcriptase (Thermo Scientific), and real-time quantitative PCR was performed using the SYBR Premix Ex Taq (Tli RNaseH Plus). *OfRAN* and *OfRPB2* were used as internal reference genes to detect the expression of target genes. The reaction system and reaction conditions were derived from the methods of previous experiments (Mu *et al*., 2017).

Table 1. Primer sequences.

Primer_ID	Primer sequence (5' to 3')	Usage
OfCCD1-F	ATTGTTGCTGTAAAGCCG	Intermediate fragment
OfCCD1-R	CCTTTGCCTGTTCTTGAA	
OfCCD4-F	CATTTTCTCTCCAATCCCC	
OfCCD4-R	TTTCACTCACAAAGAGCCC	
OfCCD1-3'Outer	ATCCTAAGGTTGACCCATTTACC	3'RACE
OfCCD1-3'Inner	CAGTTTTGATGCTGCCCAGAAAGCTCGT	
OfCCD4-3'Outer	TATGGTCCAATGCGTCCGTTCTT	
OfCCD4-3'Inner	ATCGTTGTGACTGCATCGTTGCGAGTAG	
OfCCD1-5'Outer	GCCTGATACTGTTATTGGAACGG	5'RACE
OfCCD1-5'Inner	GTAAATGGGTCAACCTTAGGATGAGCAG	
OfCCD4-5'Outer	GATTGAACTGTCCAGCGACCATT	
OfCCD4-5'Inner	AGGGACGAAGAGGAGGGTCAACAAAAGT	
OfCCD1-OF	ATGGGGATGCAAGGAGA	Full-length cDNA
OfCCD1-OR	TCAGACCTTTGCCTGTTC	
OfCCD4-OF	ATGGTCACACTCTCTTCC	
OfCCD4-OR	TCATAGCTTGTTGAGGTC	
OfCCD1-qF	GTAAAGCCGAAACCCAGTCAA	qPCR
OfCCD1-qR	GTTAGGACCAACCCTCACAAAT	
OfCCD4-qF	GAATGGTCGCTGGACAGTT	
OfCCD4-qR	CTTTAGGATGTGCCGTCAT	

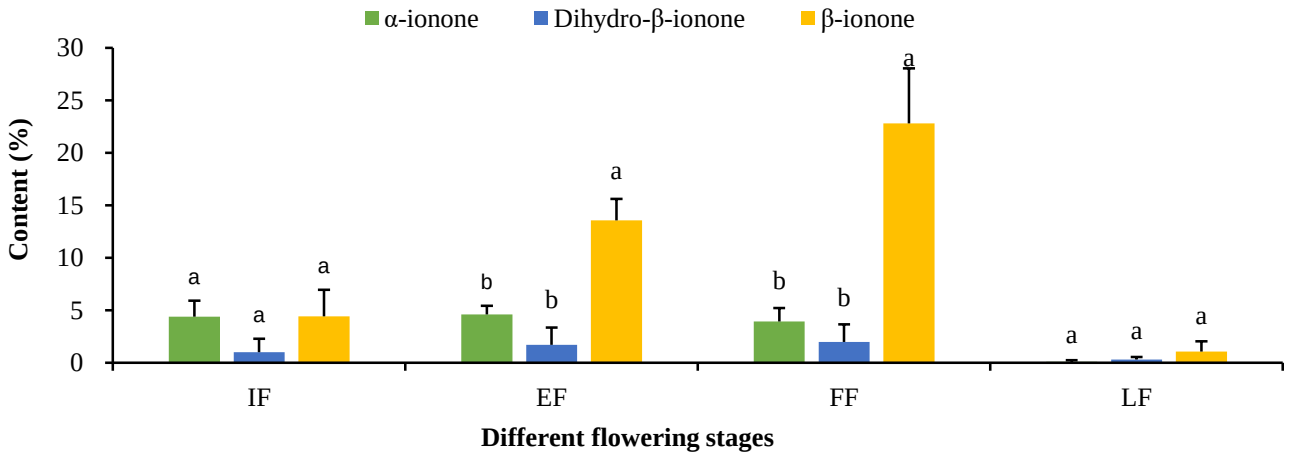

Fig. 1. Changes of ionone content in different flowering stages in sweet osmanthus.

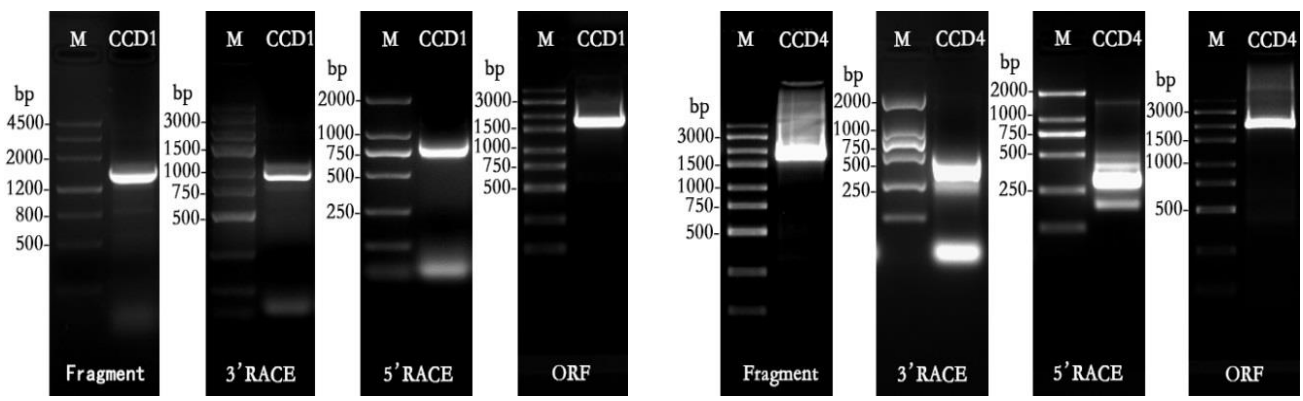

Fig. 2. Gene cloning of *OfCCD1* and *OfCCD4*.

Results and Analysis

Changes of ionone content in different flowering stages in sweet osmanthus: In sweet osmanthus, we detected α-ionone, dihydro-β-ionone, and β-ionone in all four flowering stages (Fig. 1). The results showed that the aroma components were significantly different in the middle two flowering stages (EF and FF), but not in the outlying stages (IF and LF). The content of these three aroma substances was relatively high in the first three flowering stages and the lowest in the last (LF) stage. For α-ionone and dihydro-β-ionone, there was no significant difference in the four flowering stages. In the first three flowering periods, the α-ionone content was slightly more than that of dihydro-β-ionone. In addition, β-ionone was significantly high in the middle flowering stages (EF and FF), especially in the final FF stage. With its high proportion, reaching an average of 22.80%, β-ionone is obviously the major contributing component of the aroma substances.

The full-length cDNA sequence Cloning of the *OfCCD* genes in sweet osmanthus: By mining the previous transcriptome data, nine EST sequences for sweet osmanthus *CCD* genes were found. Through PCR amplification with specific primers, two intermediate fragments with lengths of 1588 bp and 1754 bp were successfully obtained. Using the middle fragment as a template, 3'RACE and 5'RACE were amplified to obtain 3'RACE fragments of 848 bp and 461 bp, and 5'RACE fragments of 813 bp and 476 bp. The ORF primers were designed for sequencing verification, and the obtained sequences were consistent with the spliced sequence, indicating that the spliced sequences were correct (Fig. 2). The two cloned *CCD* genes' encoded proteins were aligned in the NCBI Protein BLAST, and the obtained genes were named *OfCCD1* (GenBank No. OM256439) and *OfCCD4* (GenBank No. OM145981), according to the alignment results.

Analysis of *OfCCD* genes encoding proteins in sweet osmanthus: The physicochemical properties of the amino acid sequences of the cloned proteins encoded by the two CCD genes were analyzed. The results showed that the ORF of *OfCCD1* gene was 1632 bp long; the number of encoded amino acids was 543 aa; the molecular weight was 61.28 kDa; the theoretical isoelectric point was 6.27, and it was acidic protein. Its instability index was 25.65, and the protein was stable. Its aliphatic amino acid index was 80.77, and its average coefficient of hydrophilicity was -0.262, a hydrophilic protein. The ORF of *OfCCD4* gene was 1833 bp long; the number of encoded amino acids was 610 aa; the molecular weight was 66.68 kDa; the theoretical isoelectric point was 7.19, and it was a basic protein. Its instability index was 39.26, and the protein was stable. Its aliphatic amino acid index was 77.64, and the average hydrophilic coefficient was -0.260, a hydrophilic protein.

The protein secondary structure was analyzed, and the results showed that *OfCCD1* and *OfCCD4* were mainly composed of random coils. In *OfCCD1*, the alpha helix accounted for 22.47%, the extended strand for 29.28%, beta turn for 9.76%, and random coil for 38.49%. In *OfCCD4*, alpha helix accounted for 14.75%, the extended strand for 27.05%, beta turn for 10.00%, and random coil for 48.20%. The *OfCCD1* and *OfCCD4* sequences were submitted to the SWISS-MODEL website to predict the tertiary structure of the proteins. The results showed that the GMQE reliability scores were 0.72 and 0.66, respectively, which matched well with the template proteins in the database and indicated that the quality of the constructed tertiary structure model was good (Fig. 3).

Homology and phylogenetic tree analysis of *OfCCD* genes encoding proteins in sweet osmanthus: Conserved domain analysis of the *OfCCD1* and *OfCCD4* genes' encoding proteins showed that they both have a characteristic (retinal pigment epithelial membrane protein) RPE65 domain, which belongs to the carotenoid oxygenase family. The conserved domain of *OfCCD1* is located between F55-T533, and the conserved domain of *OfCCD4* is located between F132-S603 (Fig. 4).

The amino acid sequences were aligned with the BLASTP in the NCBI database. The results showed that both had the most homology with *Olea europaea* (96.32% and 92.97%, respectively). *OfCCD1* has sound homology with *Sesamum indicum*, *Camellia sinensis*, *Vitis vinifera*, and *Coffea arabica* at 87.16%, 83.99%, 83.21%, and 82.90%, respectively, while *OfCCD4* has relatively little homology at 74.35%, 81.26%, 73.17%, and 75.73%, respectively. The amino acid sequences with the larger homology scores were downloaded, and the phylogenetic tree of *OfCCD1* and *OfCCD4* proteins was constructed by MEGA7.0 Software. The results showed that *OfCCD1* and *OfCCD4* proteins were clearly divided into two branches, both of which were closely related to *Olea europaea* (Fig. 5). The results of phylogenetic tree analysis were consistent with the classification of CCD1 and CCD4 subfamilies in the CCD family.

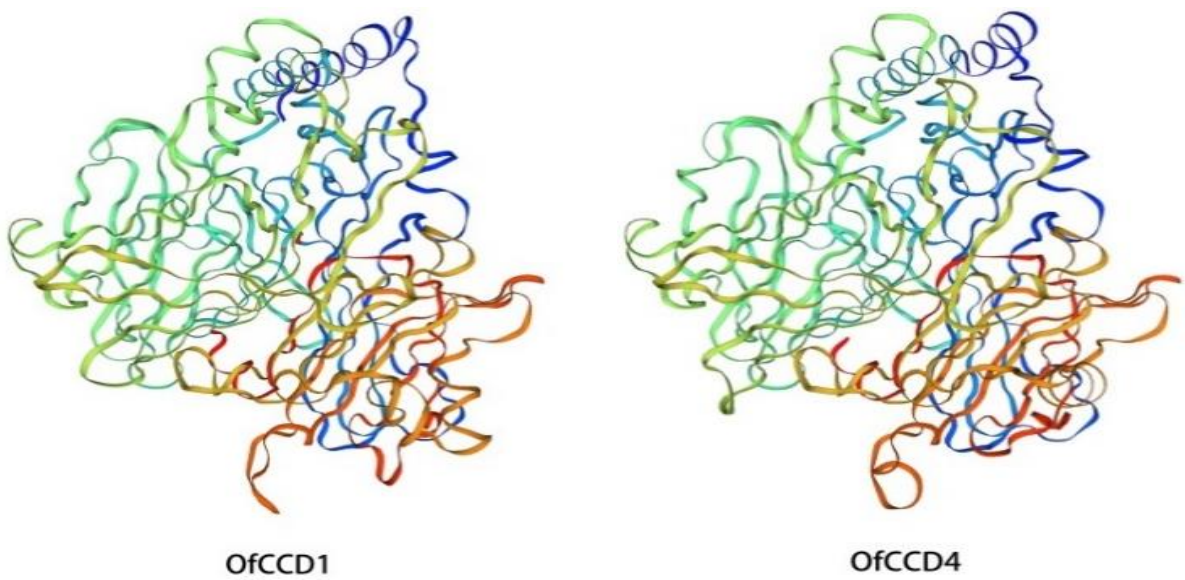

Fig. 3. Tertiary structure prediction of OfCCD1 and OfCCD4 protein.

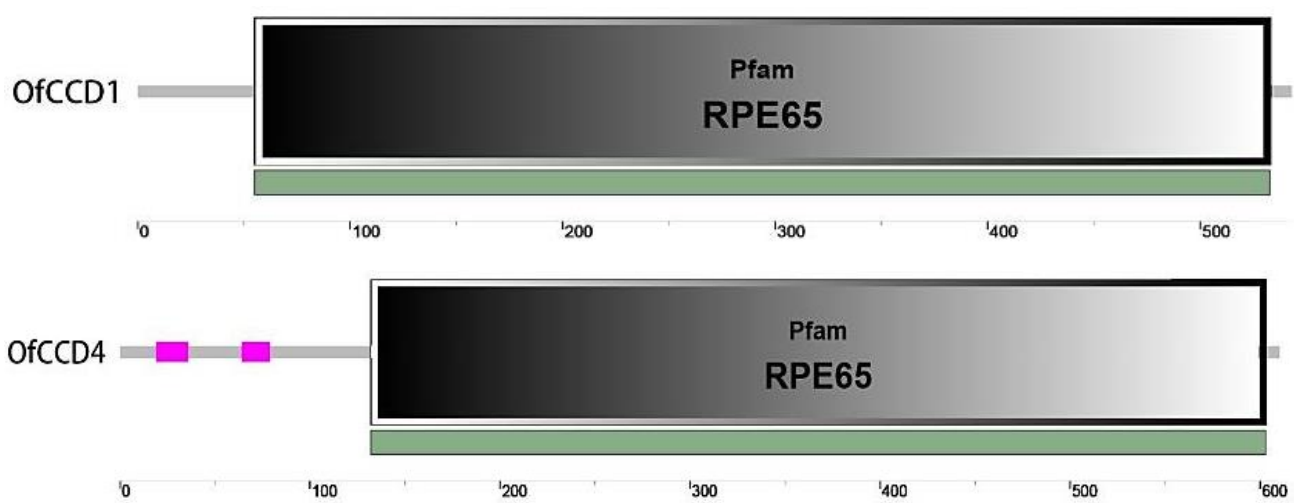

Fig. 4. Conserved domain of OfCCD1 and OfCCD4 protein.

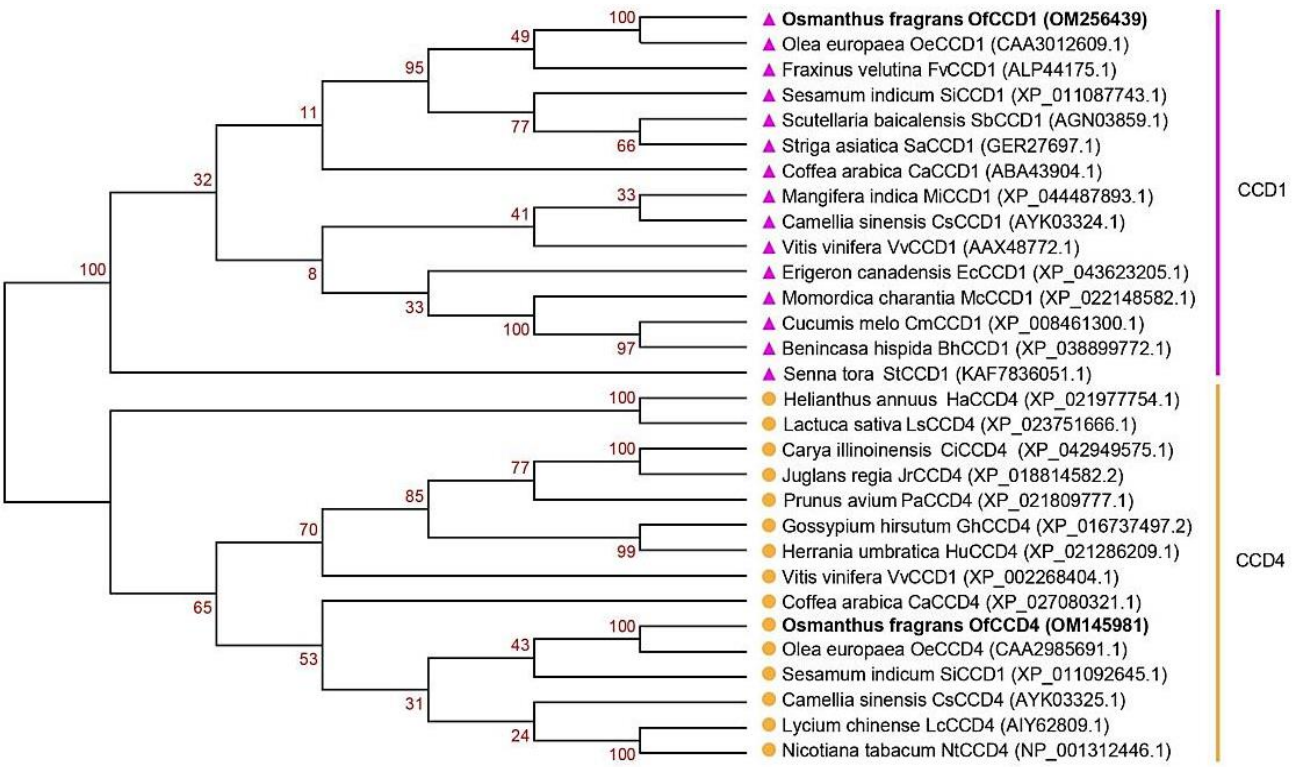

Fig. 5. Phylogenetic tree of OfCCD1 and OfCCD4 protein.

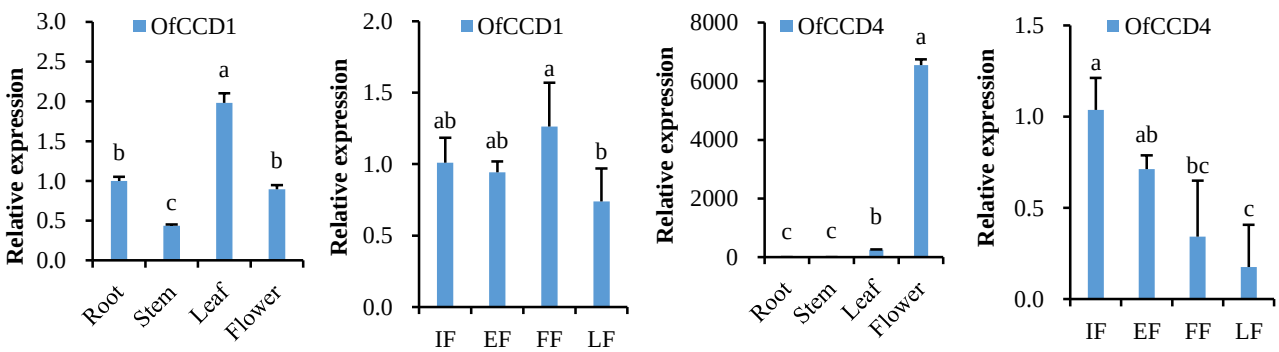

Fig. 6. Expression level of *OfCCD4* and *OfCCD4* in different organs and flowering stages.

Expression analysis of *OfCCD1* and *OfCCD4* genes: The expression levels of the *OfCCD1* and *OfCCD4* genes in different tissues and different flowering stages were detected by qRT-PCR (Fig. 6). The expression of the *OfCCD1* gene was largest in the leaves, followed by roots and inflorescences, and least in the stems. The *OfCCD1* gene was expressed in all four flowering periods. It decreased slightly from the IF stage to the EF stage, then increased to reach the peak in the FF stage, and then fell to the lowest level in the LF stage. Among the four tissues, the *OfCCD4* gene had the largest expression in the inflorescence, 6549 times more than in the root. The *OfCCD4* gene was expressed throughout the four flowering periods but gradually decreased to its lowest value at the last, the LF stage. Based on these results, it can be seen that the *OfCCD1* gene is expressed in different tissues and in different flowering stages, though the differences between the samples are small. The tissue specificity of *OfCCD4* gene was obvious, and its expression level decreased as the flowering progressed.

Discussion

There are many kinds of floral aroma compounds in flowering plants, including terpenoids, phenylpropane/ benzene compounds, and aliphatic compounds (Dudareva *et al*., 2006). In sweet osmanthus cultivars, terpenoids, alcohols, and ketones constitute most volatile components and account for 56.6-95.06% of the tested components (Fu *et al*., 2019). Among them, ionone and its derivatives are significant floral aroma substances (Nisar *et al*., 2015). Among various sweet osmanthus cultivars, β-ionones dominate the active substances during flowering (Zhou *et al*., 2017). Sweet osmanthus releases ionones and their derivatives with a high content of aroma substances during the initial and early flowering stages (Shi *et al*., 2018).

The CCDs genes have been identified and characterized in many plants, including *Arabidopsis* (Tan *et al*., 2003), *Nicotiana* (Zhou *et al*., 2019), *Brassica napus* (Zhou *et al*., 2020), *Solanum Lycopersicum* (Wei *et al*., 2016), *Populus trichocarpa* (Wei *et al*., 2022), *Vitis*

vinifera (Lashbrooke *et al*., 2013), etc. In tomato, two *CCD1* genes were cloned and identified, namely *LeCCD1A* and *LeCCD1B*. It was found that the *LeCCD1* gene contributes to the synthesis of volatile substances, β-ionone, pseudoionone, and geranyl acetone (Simkin *et al*., 2004). Rice overexpressing the *AtCCD4* transgene showed lowered levels of β-carotene and lutein along with the changed levels of the two-fold increase of β-ionone (Song *et al*., 2016). In chrysanthemum, a flower color difference gene *CmCCD4a* found to be specific to petals was remarkably expressed in chrysanthemum varieties with white flowers and minimally in chrysanthemum varieties with yellow flowers. The white flowers became yellow after RNAi interference (Ohmiya *et al*., 2006).

Currently, there are many homologous *CCD* genes in plants, and sequence alignment analysis shows that the protein sequence of this gene is conservative. Compared to *OfCCD4*, the *OfCCD1* homologous sequence alignments are more conservative in sweet osmanthus. Both *OfCCD1* and *OfCCD4* contain the conserved carotenoid oxygenase domain RPE65, a typical enzyme involved in carotenoid cleavage (Kim *et al*., 2016). Carotenoids are directly related to plant traits, widely distributed in nature, and are also precursors of much biosynthesis. CCDs participate in the catalytic cracking process of plant carotenoids, producing aroma components, pigments, and signal regulators (Maoka, 2009). Although *OfCCD1* and *OfCCD4* belong to the same family of CCDs, they only have a protein sequence alignment similarity of 40%. The phylogenetic tree analysis also shows that *OfCCD1* and *OfCCD4* belong to two branches. *OfCCD1* and *OfCCD4* were closely related to the evolution of *O. europaea*, which was consistent with the analysis of the sweet osmanthus genome (Yang *et al*., 2018). Previous studies have shown that CCD1 has a distant evolutionary relationship with CCD4, CCD7, and CCD8, while CCD4 has a relatively close evolutionary relationship with CCD7 and CCD8 (Priya *et al*., 2014).

The results of gene expression analysis showed that the *OfCCD1* tissue specificity and difference over the flowering stages were not pronounced; the divergence was less than two-fold. In contrast, *OfCCD4* has distinct tissue-specific and flowering changes. *OfCCD4* showed the greatest expression level in the inflorescence, where it greatly exceeded that of other tissues and gradually trended downward over the flowering period. For the *OfCCD1* gene of sweet osmanthus, prokaryotic expressed recombinase can cleave carotene to generate α-ionone and β-ionone *In vitro*, and it was found that the release of aromatic substances and the expression of the *OfCCD1* gene followed changes in light rhythm and increased with light time (Baldermann *et al*., 2010). *In vitro* experiments showed that β-ionone was produced by overexpression of the sweet osmanthus gene, *OfCCD4* (Zhang *et al*., 2016).

In this study, the full-length cDNA sequences of *OfCCD1* and *OfCCD4* genes of sweet osmanthus were successfully cloned by using the transcriptome EST sequence and the RACE technique, and their bioinformatics was analyzed. The acquisition of the full-length cDNA gene sequence and the expression pattern analysis provides a basis for further research on the function of *OfCCD* genes and floral aroma metabolism.

Acknowledgments

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