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Genome-wide identification and *in silico* expression analysis of *CCO* gene family in *Citrus sinensis* (orange) in response to citrus greening

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Abstract: *Citrus sinensis* (L.) Osbeck (sweet orange) is the most important cultivated citrus fruit in the world. However, Hanglongbing (HLB) disease, caused by *Candidatus Liberibacter asiaticus* (CLAs), poses a major threat to sweet orange production, by hindering colour, quality and export. Carotenoid cleavage oxygenases (CCOs), which include carotenoid cleavage dioxygenases (CCDs) and 9-cis-epoxycarotenoid dioxygenases (NCEDs), are essential for plant growth, development, and adaptation to phytohormonal, biotic, and abiotic stresses. This study identified 14 *CsCCO* genes in *C. sinensis.* Structural and conservation studies were conducted using gene structure and conserved domain analysis. Genomic localisation, gene duplication, and similarity among these genes were also examined. Gene ontology analysis predicted that CsCCOs could be involved in the carotene catabolic process. Analysis of cis--regulatory elements revealed that most *CsCCO* genes are involved in responses to stress, light signalling, and plant growth regulation. Genes in the 9-cis-epoxycarotenoid dioxygenase (NCED) subgroup are predominantly localised in chloroplasts, whereas genes in other subgroups are primarily found in the cytoplasm. All 13 of the *CsCCOs* genes identified were regulated by 25 microRNAs, indicating the crucial role of microRNAs in gene regulation in *Citrus sinensis*. The expression patterns of *CsCCO* genes in response to biotic and abiotic stress were studied. Transcriptome analysis demonstrated that *CsNCED3* and *CsNCED10* were up-regulated in response to HLB. This provides insight into the function of *CCO* genes in *C. sinensis* and identifies potential candidate genes for combating citrus greening.

Keywords: 9-*cis*-epoxycarotenoid cleavage dioxygenases; abscisic acid (ABA); *CCDs*; *CCOs*; carotenoid cleavage dioxygenases; carotenoid cleavage oxygenases; *NCEDs*

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Carotenoids, these intriguing isoprenoid compounds, have captured scientific curiosity ever since their unexpected discovery in the 19th century (Stafsnes et al. 2010). These chemicals serve varied biological activities in different organisms, including bacteria and plants (Misawa 2011). Carotenoids are crucial secondary pigments in photosynthetic organisms, shielding them from photo oxidation and allowing them to capture light more efficiently (Hashimoto et al. 2016). CCOs are categorised into two enzyme subfamilies based on their substrate epoxidation: the 9-cis-epoxycarotenoid (NCED) subfamily and the carotenoid cleavage dioxygenase (CCD) subfamily. These enzymes are crucial for transforming carotenoids into apocarotenoids (Yue et al. 2022). Carotenoid cleavage oxygenases (CCOs), as non-heme iron oxygenases, catalyse carotenoid degradation into apocarotenoids, including NCEDs, which play key roles in abscisic acid (ABA) biosynthesis and regulate plant responses to environmental signals (Pu et al. 2020). The *NCED* gene was initially cloned in the maize mutant Vp14, which leads to a better understanding of the production of ABA through *NCED* (Fei et al. 2021). Further research in *Arabidopsis thaliana* experiences nine members, 4 of which belong to *CCD* (*AtCCD1*, *AtCCD4*, *AtCCD7*, *AtCCD8*) and 5 belong to *NCED* (*AtNCED2*, *AtNCED3*, *AtNCED5*, *AtNCED6* and *AtNCED9*) (Hu et al. 2021). NCED subfamily is involved in ABA synthesis (Wang et al. 2021). The *NCED* gene family has been characterised in various plant species, such as *Arabidopsis* (Tan et al. 2003), cucumber (Zhang et al. 2022), cotton (Li et al. 2021), wheat, and tobacco (Zhang et al. 2014), citrus (Xian et al. 2014). CCDs are responsible for various biological functions, producing apocarotenoids with multiple biological activities (Wei et al. 2022). A new member of the CCO family, CCD-like (CCDL), was found in tomatoes for the first time (Cheng et al. 2022). RPE65 domain is found in all *CCD* genes, enzymes which is involved in carotenoid cleavage (Kim et al. 2016). The *CCD* research has been conducted in plant species like *Arabidopsis* (Chernys & Zeevaart 2000) and in grass species maize (*Zea mays*), rice (*O. sativa*), and sorghum (*Sorghum bicolor*) (Zhou et al. 2020), wheat (Takezawa 2000), watermelon (Cheng et al. 2022), pepper (Yao et al. 2022), tobacco (Zhou et al. 2019), rapeseed (Zhou et al. 2020), and maize (Sun et al. 2022). However, the research on *CCO* genes in citrus has not been precisely defined. *CCDL* proteins are not an ideal candidate for studying the function of CCD enzymes, because of their absence lately. Fortunately, *CCDL* genes have been discovered in *Citrullus lanatus* and *Cucumis melo*, allowing for future investigation (Xue et al. 2023).

Citrus Huanglongbing (HLB), also known as citrus greening, is one of the most destructive diseases affecting citrus crops worldwide. It is linked to phloem-limited, Gram-negative bacteria of the genus *Candidatus Liberibacter* (Bové 2006). Currently, there is no known cure for HLB. Infected fruits often exhibit irregular colour development, where ripening may only occur near the stem end, leaving much of the fruit green (Alvarez et al. 2016). Despite extensive research, the mechanisms by which HLB accelerates preharvest fruit drop remain unclear. However, recent studies suggest that carbohydrate shortages caused by HLB-related phloem blockages contribute to fruitlet drop, leading to substantial economic losses in the citrus industry (Zhao et al. 2015). Plant hormones like ethylene, jasmonic acid, and abscisic acid regulate abscission by activating genes in the abscission zone (AZ), with ethylene playing a key role in promoting this process in citrus.

Sweet orange (*Citrus sinensis*), a major fruit crop worldwide, faces increasing challenges from both biotic and abiotic stresses, including drought, salinity, extreme temperatures, and severe diseases like HLB – threats expected to intensify under global climate change (Etebu & Nwauzoma 2014). The availability of the sweet orange genome enabled genome-wide analysis of the *CsCCO* gene family, revealing their roles in ABA-mediated stress responses and seedling development. RNA-seq identified *CsNCED3* and *CsNCED10* as key candidates, with *CsNCED10* potentially enhancing ABA production and promoting preharvest fruit drop (Baldwin et al. 2018). While these *in silico* findings offer valuable insights into the functional diversity and evolutionary traits of the *CsCCO* gene family, further studies – such as gene cloning and functional validation – are essential. These discoveries provide a foundational resource for future research aimed at improving citrus resilience and productivity.

MATERIAL AND METHODS

Sequence retrieval. The genome annotation files of *Citrus sinensis* were retrieved from the Phytozome database (https://phytozome-next.jgi.doe.gov/). Peptide sequences of *CCO* genes and *Arabidopsis thaliana* genome data were obtained from NCBI (https://www.

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ncbi.nlm.nih.gov/). To identify *CCO* genes in *C. sinensis*, the RPE65 domain (PF03055.16) was used as a query from the Pfam database (http://pfam.xfam.org/) (Su et al. 2021), as the query to search against the protein sequences from *C. sinensis* using TBtool two sequence set BLAST tool. Fourteen sequences from the *C. sinensis* database were identified and NCBI CCD (Conserved Domain Database, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) with pre-set parameters was used to verify the precision of these sequences (Xue et al. 2023). Proteins that lacked the conserved domain were eliminated and evaluated further.

Determination of physio-chemical properties and subcellular localization of *CCO* gene. The physio-chemical properties, number of amino acids (AA), theoretical isoelectric point (pI)-value and molecular weight of CsCCO proteins were predicted using the ProtParam tool (http://web.expasy.org/protparam/). The information for gene IDs, chromosomal position, sequence of genes and proteins were retrieved from the Phytozome (Gasteiger et al. 2005). *CsCCO* genes were renamed based on their *Arabidopsis thaliana* orthologs, and their chromosomal positions, names, and protein sequences were identified using the *C. sinensis* genome database. Subcellular localisation of the 14 *CsCCO* genes was also predicted (Horton et al. 2006), a web-based tool WoLFPSORT (https://wolfpsort.hgc.jp/) was used.

Gene structure, *cis*-regulatory analysis and motif analysis. The gene structure of the *CCO* genes was displayed using the Gene Structure Display Server (GSDS) v2.0 (http://gsds.cbi.pku.edu.cn/) (Hu et al. 2015). For cis-regulatory analysis, phytozome was used to retrieve the promoter sequences of all 14 *CsCCO* genes with 1 000 bp upstream from the ATG as start codon (Shafqat et al. 2020). Cis-regulatory elements were obtained through signal scan search in the PlantCare database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Motifs are analysed using MEME suit programme (http://meme.nbcr.net/meme/) with a maximum value of 20 motifs. The TBtools was used to visualize the found motifs (Bailey et al. 2015).

Phylogenetic analysis. The phylogenetic tree was constructed using *CCO* amino acid (AA) sequences of *A. thaliana*, *C. sinensis* and *C. clementina*. *A. thaliana* and *C. clementina* were selected as reference species for comparative genomic analysis due to their well-annotated, fully sequenced genomes, facilitating accurate gene identification and evolutionary relationship assessment. The neighbour joining (NJ) option in MEGA X was used to create a tree from the aligned protein sequence with a bootstrapping value of 1 000 replications (Sun et al. 2015) (https://itol.embl.de/upload.cgi). Bootstrap support values in MEGA X are typically calculated using resampling (e.g. 1 000 replicates), and values above 70% are considered strong support for phylogenetic tree branches. To visualise the derived phylogenetic tree, iTOL is used (Letunic & Bork 2021).

Gene duplication and synteny analysis. Duplication of *CsCCO* genes was calculated using the nonsynonymous (*Ka*)/synonymous (*Ks*) substitution ratio with the help of TBtool (Chen et al. 2020). The paralogous gene pair was calculated by measuring the *Ka*/*Ks* ratio. Then, using the formula:

$$T = Ks/2r$$

where:
T – time since divergence;
r – the neutral substitution rate.

The time of divergence (DT) was determined (Wang et al. 2019). The MCScanX v1.0 (Multiple Collinearity Scan toolbox) was used with default settings to evaluate gene duplication occurrences (Wang et al. 2013). To visualise the duplication pattern of the *CCO* gene family, Advance Circos package of TBtools is used (Wang et al. 2013). Dual synteny analysis is done using *A. thaliana*, *C. sinensis* and *C. clementine*. Synteny graph was built with the help of TBtools circos module.

Gene ontology analysis and protein-protein interaction. The functions of *CCO* genes in *C. sinensis* were determined using gene ontology (GO) term enrichment analysis utilising GO annotations from the uniport website (https://www.uniprot.org/) (Langenbacher et al. 2020). To identify the molecular actions and biological procedures of *CCO* genes with the use of the website ShinyGo v0.741 (Mazhar et al. 2023). The activities of the *CCO* genes were further examined using GO annotations by GO term enrichment study. Moreover, expanded studies on protein-protein interactions have been carried out by the String Database (https://string-db.org/) (Szklarczyk et al. 2021).

Chromosomal mapping. All the predicted *CsCCO* genes were mapped according to chromosome length, gene position on the chromosome derived from the Phytozome database (https://phytozome-next.jgi.

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doe.gov/n) (Shafqat et al. 2020). The chromosomal mapping of the gene was done using the TBtools (Chen et al. 2020).

Expression analysis. Differential expression analyses were performed to identify important *CsCCO* genes involved in tissue-specific function. High-Throughput-rna-seq data, were obtained from the citrus-pan-genom database (http://citrus.hzau.edu.cn/). The expression levels were normalised using the FPKM method (Fragment per kilobase of transcripts per million reads) to account for transcript length and sequence depth. This normalisation allowed for accurate comparison of gene expression across tissues and conditions. Second, differential expression analyses were performed to identify important *CsCCO* genes involved in tissue-specific function (Liu et al. 2022). The Citrus Pan-genome to Breeding Database (CPBD) (http://citrus.hzau.edu.cn/) was a helpful tool for understanding the phases of organ development and their response to citrus greening.

RESULTS

Identification of *CCO* gene in *Citrus sinensis*. To identify the *CCO* gene family in *C. sinensis*, conserved CCO domains were used to search its genome via Phytozome, complemented by BLAST analysis using *A. thaliana CCO* gene sequences against the *C. sinensis* v1.1 genome. This approach led to the identification of 14 *CCO* genes, which were renamed according to their *A. thaliana* orthologs. Detailed information on these genes, including accession numbers, chromosomal locations, amino acid lengths, molecular weights, and isoelectric points (pI), is presented in Table 1. The identified proteins ranged in length from 1 005 AA (*CsCCD7b*) to 1 845 AA (*CsCCD1a*), all containing conserved domains. Their pI values varied between 5.79 (*CsCCD7b*) and 8.75 (*CsNCED14*), reflecting acidic, basic, and neutral properties essential for post-translational modifications and biochemical functions in plants.

Analysis of *CCO* conserved domain and motif in *Citrus sinensis*. The MEME analysis of *Citrus sinensis CsCCO* genes identified 15 distinct conserved motifs, with all proteins containing the RPE65 domain characteristic of the RPE65 superfamily. Specifically, proteins such as CsCCD4a, CsCCDL, CsCCD4b, CsCCD1, CsCCD8, CsCCD7a, CsCCD7b, and the CsNCED group (2, 3, 10, 11, 12, 13, 14) were classified under this superfamily, indicating strong

Table 1. Information of 7 *CCD*, 7 *NCED* genes discovered from the genome of *Citrus sinensis*

Source		Scaffold	Chromosome location		Direction	pI	Aliphatic index	GRAVY	No. of amino acids	Molecular weight	Size (AA)
Accession No.	rename	No.*	start	end							
orange1.1g047920 m	*CsCCD4a*	5	878 867	882 500	forward	6.05	79.73	–0.322	589	66 677.4	1 671
orange1.1g008275 m	*CsCCDL*	5	779 540	784 020	reverse	6.33	81.62	–0.293	597	67 203.0	1 716
orange1.1g010461 m	*CsCCD4b*	5	859 124	862 280	forward	6.87	81.33	–0.236	603	66 452.9	1 533
orange1.1g043705 m	*CsCCD1*	17	530 796	533 764	forward	8.53	79.54	–0.316	597	66 347.1	1 845
orange1.1g007605 m	*CsCCD7a*	3	4E+06	4 252 242	reverse	8.34	79.96	–0.245	563	63 060.2	1 791
orange1.1g019900 m	*CsCCD7b*	3	4E+06	4 246 057	forward	5.79	82.13	–0.314	611	69 176.6	1 005
orange1.1g046348 m	*CsNCED2*	254	9 994	11 071	forward	5.93	80.47	–0.282	510	56 800.6	1 077
orange1.1g007291 m	*CsNCED3*	7	2E+06	1 683 076	forward	8.27	82.46	–0.187	410	46 734.1	1 830
orange1.1g044684 m	*CsCCD8*	496	22 269	23 907	reverse	5.98	82.41	–0.276	556	61 964.5	1 638
orange1.1g007379 m	*CsNCED10*	206	221 163	223 346	reverse	6.07	82.90	–0.243	590	66 508.9	1 821
orange1.1g044599 m	*CsNCED11*	9 487	199	2 328	forward	6.29	80.96	–0.275	542	59 888.4	1 629
orange1.1g040986 m	*CsNCED12*	898	26 036	27 581	reverse	7.25	77.67	–0.279	412	45 860.3	1 545
orange1.1g044992 m	*CsNCED13*	15	130 153	131 947	forward	7.96	79.21	–0.305	597	66 184.8	1 794
orange1.1g039955 m	*CsNCED14*	15	146 896	148 543	forward	8.75	79.40	–0.341	420	46 659.2	1 263

AA – amino acid sequence length; pI – isoelectric point; *values refer to scaffold numbers as the *Citrus sinensis* genome is not fully assembled into chromosomes

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conservation. The presence of shared motifs across these genes suggests their involvement in similar biological functions and highlights the potential role of gene expansion in the evolutionary history of the CsCCO family. Notably, *CsCCD1* contained fewer motifs (specifically motifs 4, 6, 9, and 11), while the remaining 14 genes maintained a highly conserved motif pattern, underscoring their possible functional significance as shown in Figure 1.

Phylogenetic analysis of *Citrus sinensis CCO*. We investigated the evolutionary relationship between the identified *CCO* genes of *Citrus sinensis*, using MEGA X software. Phylogenetic trees were constructed using protein sequences of respective genes of *C. sinensis*, *A. thaliana*, and *C. clementina* by the neighbour-joining approach (Figure 2). All 14 *CCOs* were grouped into 7 clades (Figure 2). We named the clades (*CCD1*, *CCD4*, *CCDL*, *NCED2*, *CCD7*, *CCD8* and *NCED3*) according to the *Arabidopsis NCED*. Among the two *CsCCD4a* and *CsCCD4b* was clustered in clade 1 (*CCD4*) with *AtCCD4*, *CsCCD1* was present in clade 2 (*CCD1*) and closely clustered with *AtCCD1*. On the other hand, *CsCCD8* is clustered with *AtCCD8*. Through phylogenetic analysis, we demonstrate that there is 1 *CCDL* gene present in clade 3. *CsNCED12*, *CsNCED2*, *CsNCED14*, *CsNCED11* and *CsNCED13* were grouped in clade 4 (*NCED2*). *CsNCED3* and *CsNCED10* were present in clade 7 (*NCED3*), while *CsCCD7a* and *CsCCD7b* were identified in clade 5 (*CCD7*).

Gene structure and chromosomal localisation analysis. Exon and intron patterns are important indicators of evolutionary relationships within gene families. In *Citrus sinensis*, gene structure analysis of *CsCCO* genes revealed significant variation in intron numbers. For example, *CsCCD4a* and *CsCCD4b*

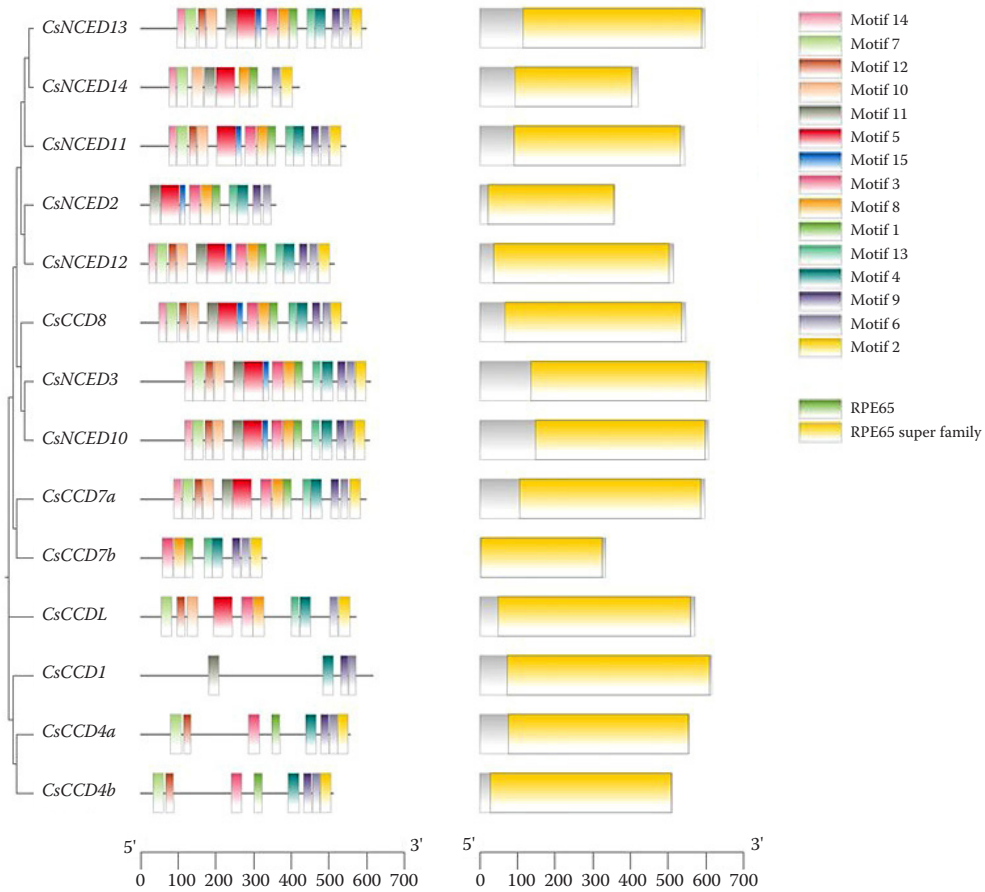

Figure 1. Meme version 5.5.2 was used to construct a colour-coded bar graph depicting the motif distribution analysis of *Citrus sinensis CsCCO* genes; the research revealed a total of 15 unique motifs; to further comprehend the relationship between the CsCCO proteins and motif distribution, the graph was linked to a phylogenetic tree, which provided more information about the CsCCO protein's evolutionary pattern and functional links

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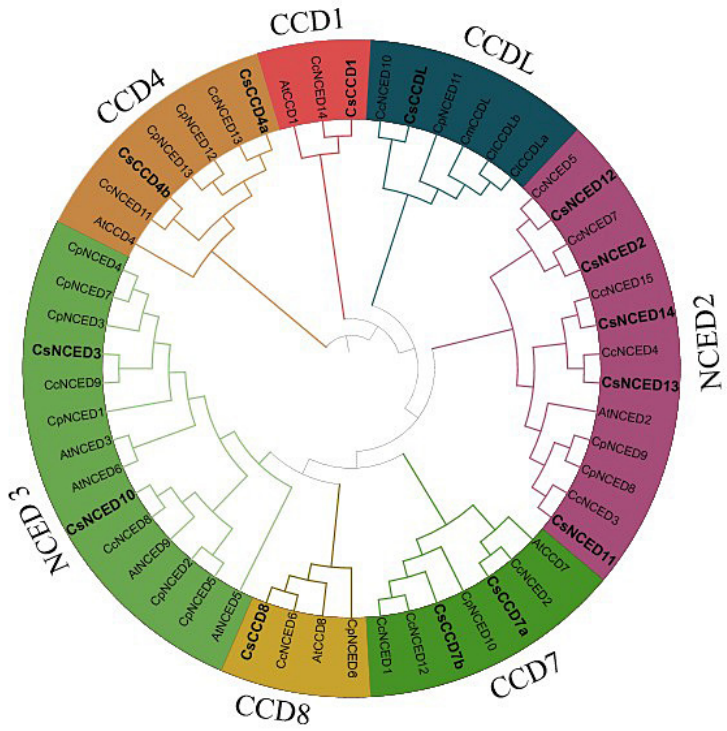

Figure 2. Phylogenetic tree analysis of *CCO* gene families from *Citrus sinensis* (Cs), *Arabidopsis thaliana* (At), and *Citrus clementina* (Cc); groups are named according to the *A. thaliana* genes; the *C. sinensis* genes were specifically identified by bold letters; neighbour-joining methods are used to construct trees, and the evolutionary history was inferred using the unweighted pair group method with arithmetic mean (UPGMA) with 1 000 bootstrap; evolutionary analyses were conducted in MEGA X software

both have 5 introns, while *CsCCD7a* and *CsCCDL* contain 13 and 11 introns, respectively. Notably, six genes (*CsNCED10*, *CsNCED12*, *CsNCED13*, *CsNCED2*, *CsCCD8*, and *CsNCED3*) lack introns entirely, whereas *CsNCED11* and *CsNCED14* have 2 and 4 introns in Figure 3. The similarities in exon-intron organisation between some *CsCCO* genes and their orthologs suggest functional conservation.

All 14 *CCO* encoding genes were located on 10 out of 18 *Citrus sinensis* chromosomes. There is 1 gene precisely present on scaffold 17, scaffold 254, scaffold 7, scaffold 496, scaffold 206, scaffold 9 487, and scaffold 898. Similarly, two genes are on scaffold 3 and 15, respectively. *CsCCDs* were located on 5 scaffold, among all *CsCCD4a* genes *CsCCD4b* and *CsCCDL* suggested tandem duplication. The details of each gene location on the scaffold are present in Figure 4.

Assessment of nonsynonymous (*Ka*) and synonymous (*Ks*) substitution rates and gene duplication. Gene duplication has a crucial role in plants for the evolution and expansion of their gene families. *Citrus sinen-*

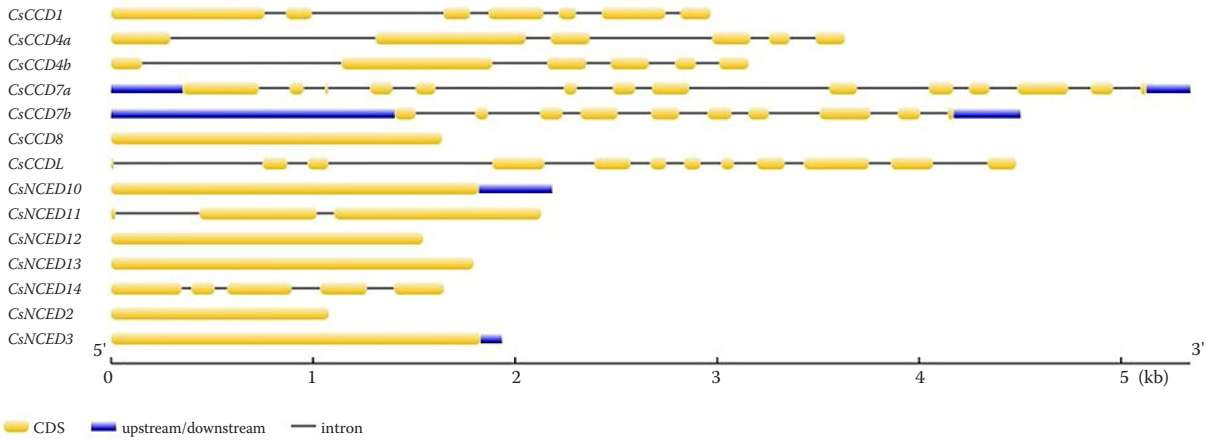

Figure 3. Gene structure analysis of *Citrus sinensis* (sweet orange) and *Arabidopsis thaliana*
Yellow boxes indicate exons, black lines indicate introns, and blue lines indicate upstream and downstream regions

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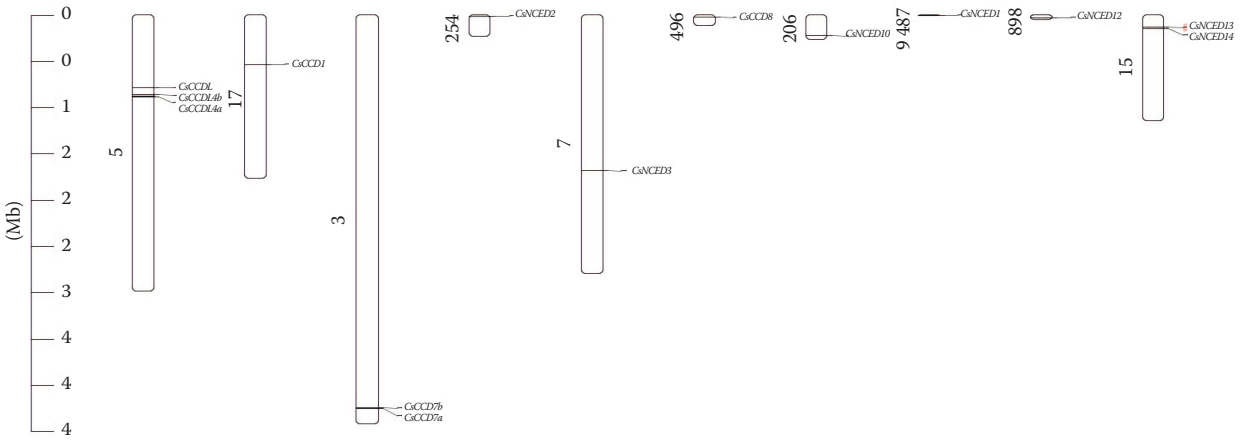

Figure 4. Distribution of *CCO* genes on *Citrus sinensis* (sweet orange) scaffolds
The scale represents a bp (base pair) scaffold distance; a map was generated using TBtool

sis's segmental duplications of the *CCO* genes originated from *CsCCD7a_CsCCD7b*; *Ka*/*Ks* = 1.86 (1 496.228669 million years ago; MYA) to *CsNCED3_CsNCED10*; *Ka*/*Ks* = 0.08 (1 721.253720 MYA), the *Ka*/*Ks* of tandem duplications ratio was less than 1 indicates purifying selection, which means that natural selection is acting to preserve the amino acid sequence because it is important for the protein's function in Figure 5. This type of selection is also known as negative selection, and it operates to eliminate deleterious mutations

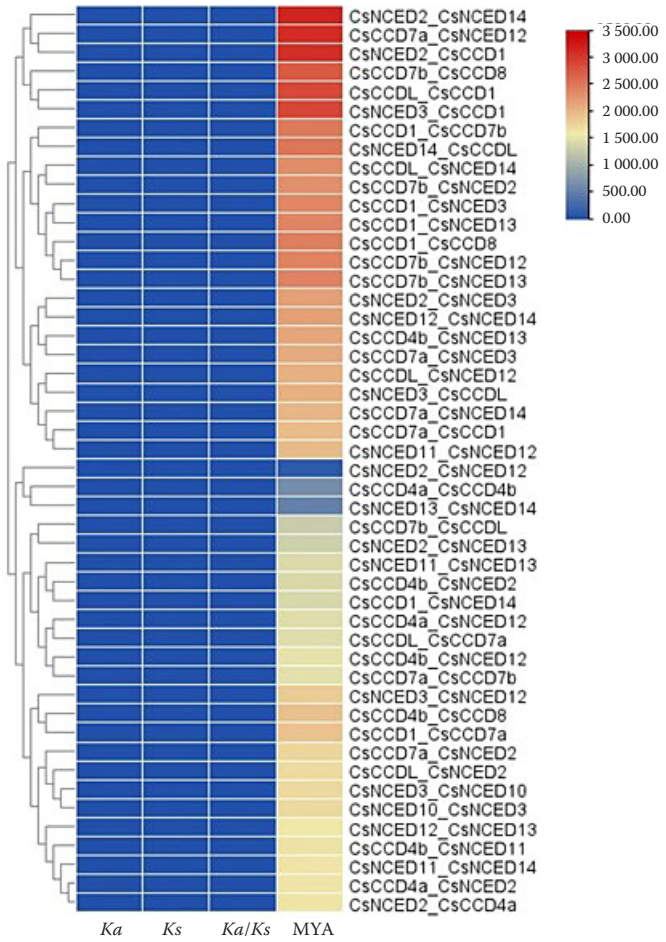

Figure 5. The *Ks* (synonymous substitution rate) and *Ka* (nonsynonymous substitution rate) were estimated with TBtool; *Citrus sinensis* has a rectangular-like rate of 6.1 × 10–9; the date of the duplication event was determined using the formula $T = Ks/2r$; this approach sheds light on the timing and evolutionary dynamics of gene duplications in *C. sinensis*
MYA – million years ago

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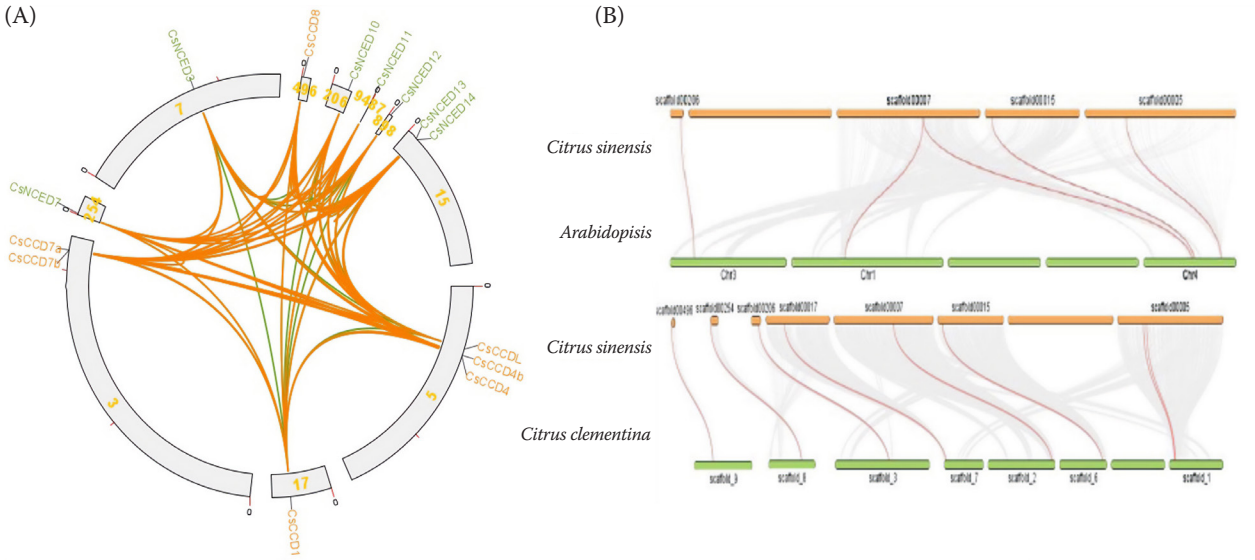

Figure 6. The distribution of *CsCCO* genes on *Citrus sinensis* chromosomes, with lines indicating potential gene duplications (A), synteny and dual synteny of *CsCCO* genes between *C. sinensis*, *Arabidopsis thaliana*, and *C. clementina* are illustrated, with red lines indicating duplicate gene pairs; the analysis highlights that segmental duplications are more prevalent than tandem duplications, providing valuable insights into the evolutionary history of the *CsCCO* gene family (B)

that may affect protein function (Liu et al. 2019). In *CsCCO* gene pairs, the highest time of divergence value was shown by *CsNCED14_CsCCDL* (338.64 MYA). Under purifying selection, a protein is anticipated to exhibit a *Ka*/*Ks* ratio of less than one due to a higher rate of synonymous substitutions than non-synonymous substitutions.

Further to find out the orthologous genes of *Citrus sinensis* in other species, we constructed the comparative dual synteny of *C. sinensis* with *A. thaliana* and *C. clementina* (Figure 6B). Through dual synteny analysis of *C. sinensis* and *Arabidopsis thaliana*, we have found 5 orthologue gene pairs (Figure 6B). There was a total of 8 orthologue gene pairs between *C. sinensis* and *C. clementina* gene pair (Figure 6B). Additionally, advanced circos of predicted *CCO* genes of *C. sinensis* showed the paralogous genes present in the genome (Figure 6A).

Analysis of cis-regulatory elements. Various cis-regulatory elements were identified in the 1 000 bp promoter regions of predicted *CsCCO* genes using the PLANT CARE database. Core elements like CAAT-box and TATA-box were present in all *CsCCO* genes, while others were categorised into plant growth and development, stress-responsive, and light-responsive groups. Common stress-related cis-elements (MYC, MYB, MBS, ABRE) were detected in several genes, including *CsCCD7a*, *CsNCED3*, and *CsNCED10*. Over ten light-responsive cis-elements were found in multiple genes such as *CsCCD4*, *CsNCED12*, and *CsNCED14*. Defence-related elements like TC-rich repeats and WUN-motifs were observed, linked to pathogen and mechanical stress responses. Additionally, cis-elements responsive to low temperature, heat, ethylene, auxin, and gibberellin were identified, highlighting their regulatory roles in stress adaptation (Figure 7).

Subcellular localisation analysis of the predicted gene. The subcellular location of predicted *CCO* genes revealed that they were mostly localised in the nucleus, cytoplasm, and chloroplast (Figure 8). CsCCO proteins CsCCDL, CsCCD1, CsCCD8, CsNCED10 and CsNCED11 were reported in the nucleus except CsNCED14 and CsCCD1 which was reported in the cytoplasm. All genes, except *CsCCD4b*, *CsCCD7b* and *CsNCED7* were located in the chloroplast.

Gene ontology analysis of *CCO* genes. GO analysis was used to predict the cellular, molecular, and biological functions of the predicted *CCO* genes, as detailed in Figure 9 and Table S1 in Electronic Supplementary Material (ESM). Based on GO categories, the functions of all CCO proteins, including biological process, molecular function, and cellular component, were determined. In the biological pro-

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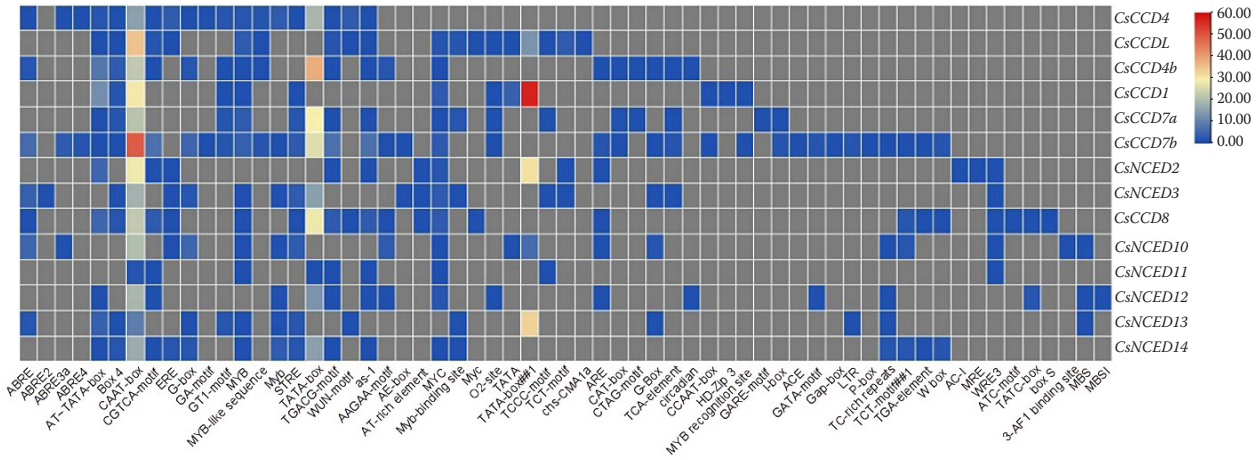

Figure 7. Cis-acting regulatory elements analysis (CAREs) of growth and development, light, hormones, temperature, defence and drought responsive associated elements of 1 000 bp promoter sequences of *CCO* genes

cess category, the maximum number of genes of CCO proteins was involved in the carotene metabolic process and the terpene catabolic process, respectively. In the molecular process category, CCO proteins were involved in carotenoid dioxygenase (GO: AT4G19170) and 9-cis-epoxycarotenoid dioxygenase activity (GO: AT3G63520), respectively. In terms of cellular components, the chloroplast stroma was mostly enriched. Therefore, these CCO proteins have a wide range of roles in cell metabolism.

MiRNA target site prediction and validation. In genome-wide analysis, miRNAs are recognised as key regulators of gene expression, influencing plant growth, development, and stress responses. This study identified 25 miRNAs targeting *CsCCO* genes, except *CsNCED12*. These miRNAs, ranging from 20 to 24 nucleotides, showed high specificity, with some genes targeted by multiple miRNAs. *CsNCED13* was regulated by 4 miRNAs, while *CsCCD7*, *CsNCED3*, and *CsCCD8* each had 3 miRNA targets. *CsNCED10*, *CsCCD4b*, and *CsCCD1* were targeted by 2 miRNAs, whereas *CsNCED14*, *CsNCED11*, *CsCCDL*, *CsNCED2*, *CsCCD7a*, and *CsCCD4a* had single miRNA regulators. These interactions suggest miRNAs play a critical role in modulating hormone biosynthesis and maintain-

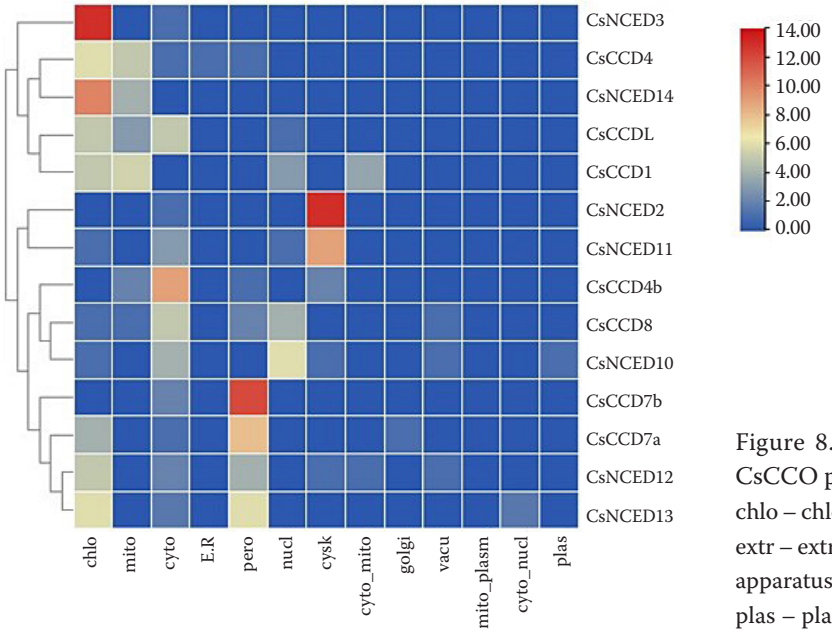

Figure 8. Sub-cellular localisation analysis for CsCCO proteins

chlo – chloroplast; nucl – nucleus; cyto – cytoplasm; extr – extracellular; cysk – cytoskeleton; golgi – golgi apparatus; mito – mitochondria; pero – peroxisome; plas – plastid; vacu – vacuole

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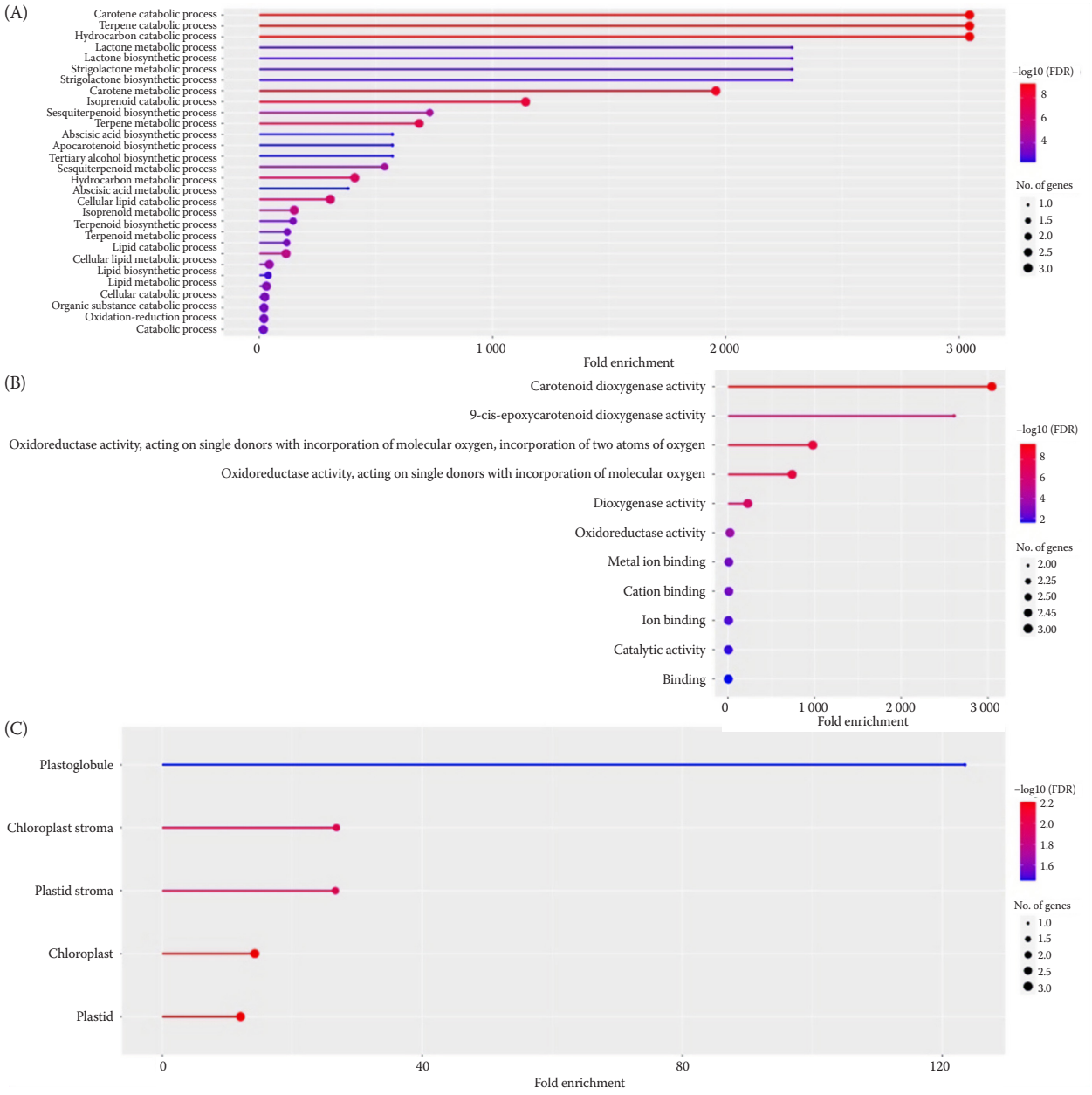

Figure 9. A fold enrichment graphic that illustrates the functionality of the overlapping *CsCCO* genes; the more genes participating in that process are represented by red lines, and the opposite is true for tiny blue sizes: gene ontology (GO) biological function (A), GO cellular function (B), GO molecular function (C)

ing plant adaptability under environmental stress, as detailed in Table S2 in ESM.

Protein-protein interaction. The *CsCCD4b*, *CsCCD4a* and *CsCCD1* genes have the most associations in the protein-protein interaction network. *CsCCD4b*, *CsCCD4a* and *CsCCD1* form a network within the broader protein-protein interaction network, indicating a unique biological process or coordinated functional link between these proteins (Figure 10). Meanwhile *CsNCED14*, *CsNCED10*, *CsNCED13*, *CsNCED12*, *CsNCED11*, *CsNCED7*, *CsCCD7b*, *CsNCED8*, *CsCCD8a*, *CsCCD7a* and *CsCCDL* form a sub-network, interacts specifically with one another.

Expression analysis of *CsCCO* genes against HLB disease. The expression analysis of 14 *CsCCO* genes in *Citrus sinensis* under Huanglongbing (HLB) disease revealed that *CsNCED2*, *CsNCED3*, *CsCCD4b*, *CsCCD7a*, *CsCCD7b*, *CsNCED10*, *CsNCED11*, and

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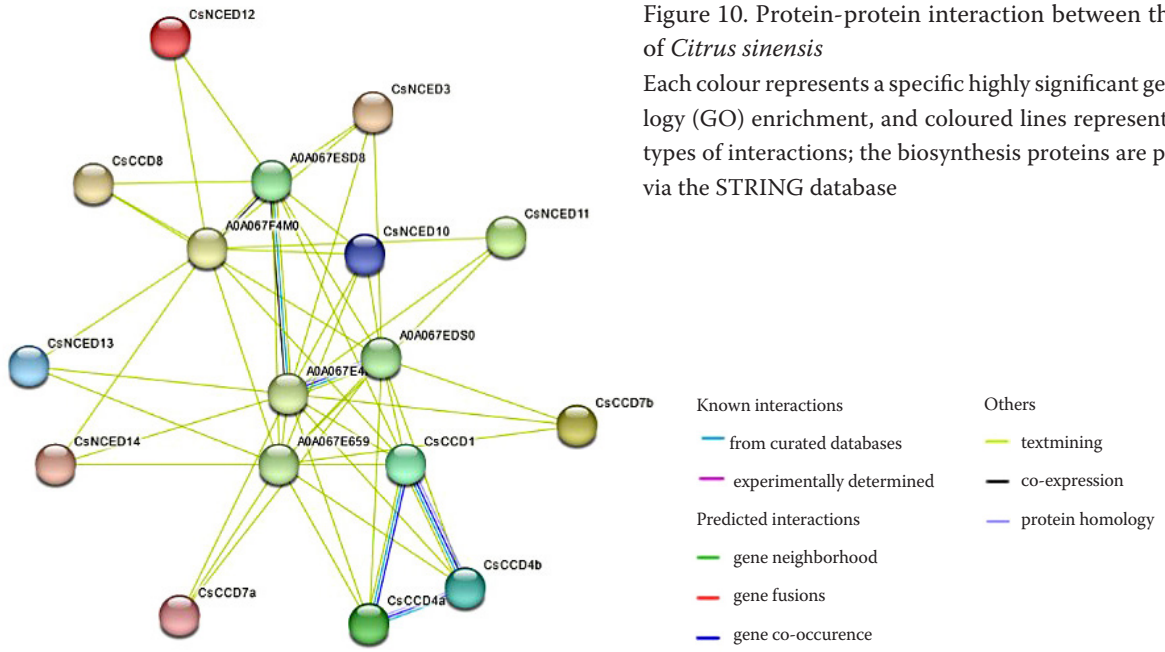

Figure 10. Protein-protein interaction between the genes of *Citrus sinensis*

Each colour represents a specific highly significant gene ontology (GO) enrichment, and coloured lines represent various types of interactions; the biosynthesis proteins are predicted via the STRING database

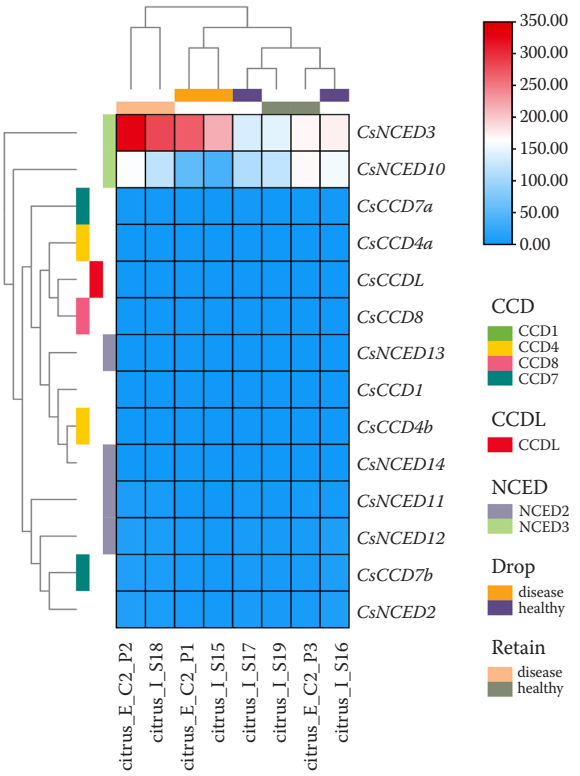

Figure 11. Samples from *Citrus sinensis* calyx abscission zones include: HLB-infected drop (citrus_E_C2_P1, citrus_I_S15), HLB-infected retain (citrus_E_C2_P2, citrus_I_S18), healthy drop (citrus_I_S16, citrus_I_S17), and healthy retain (citrus_E_C2_P3, citrus_I_S19), all from the Hamlin variety

CsNCED12 were highly expressed in various plant parts. Notably, *CsNCED10* promotes abscisic acid biosynthesis, contributing to fruit abscission and preharvest drop. These genes also influence pigmentation, causing browning at the calyx abscission zone and uneven fruit colouration, characteristic of citrus greening. Among them, *CsNCED3* was upregulated, while *CsNCED2*, *CsNCED12*, *CsNCED11*, *CsCCD7a*, and *CsCCD7b* were downregulated during HLB infection in Figure 11.

DISCUSSION

Within the carotenoid metabolism pathway, members of the carotenoid cleavage oxygenase (CCO) family oxidise and cleave carotenoids into apocarotenoids and their derivatives. These apocarotenoids, as major components of plant hormones, pigments, flavours, and defence substances, possess significant biological importance in plant growth and development (Akram et al. 2023). CCO proteins catalyse carotenoid cleavage and aid in regulating plant stress responses. Generally, the *CCO* gene family exists in most eukaryotes, notably in various plants. For instance, 9, 21, 19, 9, 23, and 15 *CCO* genes have been identified in *Arabidopsis thaliana*, *Malus domestica*, *Vitis vinifera*, *Cucumis melo*, *Populus trichocarpa*, and *Litchi chinensis* (Cheng et al. 2022). In grapevine, *CCO* genes linked to aroma formation and ABA are

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involved in berry ripening, while in tomato, they make contributions to carotenoid degradation and modulate ABA levels at some stage in fruit maturation (Vannozzi et al. 2012). Despite its larger genome size compared to *A. thaliana*, *C. sinensis* exhibits unique genetic traits, making it valuable for studying *CCO* gene evolution and function. This study establishes a comprehensive foundation for understanding *CsCCO* gene expression, particularly under CLas infection, and supports future research on their functional divergence.

In this study, 14 *CsCCO* genes were identified in *C. sinensis*, categorised into seven subfamilies (*CCD1*, *CCD4*, *CCD7*, *CCD8*, *CCD-like*, *NCED2*, and *NCED3*) based on phylogenetic analysis with *A. thaliana* and *C. clementina*. A total of 54 CCO proteins were characterised, including 40 CsNCEDs, 10 CsCCDs, and 4 CsCCDLs. Functional similarities exist between CsCCO proteins and their *Arabidopsis* counterparts, while CsCCDL proteins may have roles similar to ClCCDLa, ClCCDLb, and CmCCDL (Shafqat et al. 2020).

Say et al. (2025), Tunç et al. (2024, 2025a, b) highlighted the function of ISSR markers in assessing genetic diversity for crop improvement. Khadivi et al. (2025) on pistachio cultivars, along with Yaman et al. (2024) on Berberis genotypes, spotlight the importance of genetic and biochemical characterisation for enhancing crop trends. Similarly, information on the genetic diversity and regulation of *CsCCO* genes in cucumber under HLB stress can provide insights into improving stress resistance and fruit aroma, aligning with the wider efforts in crop development via genetic evaluation. Moreover, such analyses enable the transfer of genomic data from a well-researched taxonomic group to one that is not extensively studied (Zhou et al. 2020). In the current research, 48 paralogous gene pairs were detected within *CsCCO*, indicating gene replication via gene duplication (Yue et al. 2022). The duplication events observed in the *CsCCO* gene family provide valuable insights into their evolutionary expansion and functional diversification. Comparative analysis within subgroups highlights potential similarities in their biological roles. Most CsCCO proteins range from 1 077 to 1 265 amino acids and consistently contain the conserved RPE65 domain, essential for carotenoid cleavage activity. Subcellular localisation analysis indicates that these proteins are predominantly located in the chloroplast and cytoplasm, suggesting their key involvement in metabolic processes within these organelles.

The positioning of exons and introns within gene families is important for evolution (Wei et al. 2022). This study's analysis of motifs found that the motifs among members of the same population and clade were compatible with the shape of the phylogenetic tree. Every *CCO* gene contained exons and introns with the maximum number of 13 introns in *CsCCD7a*. The motifs of the NCED subfamily were noted to be more maintained than the CCD subfamily in keeping the traits of plants. Cis-regulatory elements are present in the promoter region and are essential at the transcriptional level study of gene expression (Wang et al. 2019). Research on cis-regulatory elements revealed that a significant portion of the largest group contained motifs such as ABRE, MYB, STRE, TGACG-motif, and WRE3, which had a stress response. Meanwhile, the second-largest group, such as A-box, Box 4, and TCCC motif, focused on metabolism and development (Tan et al. 2003). Additionally, *CsCCO* has a variety of motifs that are responsible for distinct reactions, including the CGTCA-motif, TGACG-motif, and TCA-element for the salicylic acid response, GARE-motif, TATC-box, and P-box for the Gibberellic acid response, and the ABRE, TGA-element for the auxin response. The presence of ABRE, MYB, and MBS motifs in *CsCCO* promoters highlights their stress-responsive regulatory ability. ABRE allows ABA-dependent transcription, while MYB and MBS are related to broader stress signals like drought. These elements facilitate transcription factor binding under pressure, enhancing *CsCCO* expression. This regulation supports a rapid ABA-mediated defence and fruit abscission response at some stage in HLB pressure (Wang et al. 2019).

The GO enrichment analysis showed the involvement of *CsCCO* genes in carotene metabolic and terpene catabolic processes, having roles in pigment synthesis, vitamin A production, and plant defence mechanisms (Sun et al. 2015). The high enrichment of *CsCCO* genes in the chloroplast stroma suggests their critical participation in different metabolic processes within this organelle, including photosynthesis and pigment biosynthesis (Sun et al. 2022). *CsCCD4b*, *CsCCD4a*, and *CsCCD1* appear to be involved in shared cellular processes, indicating specific functional relationships within the broader interaction network. This study highlights the functional diversity and evolutionary significance of the *CCO* gene family in *C. sinensis*. The comprehensive genome-wide analysis provides a valuable foundation for future functional studies and gene cloning

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efforts. Plants can undergo citrus greening, affected by *Candidatus Liberibacter* bacteria, resulting in reduced crop yields. *Citrus sinesis* plants under citrus greening were examined for the functions of various genes, including *CsCCO* genes, using transcriptome data (Horton et al. 2006). Interestingly, while *CCD* genes did not show any impact during HLB, *CsNCED3* and *CsNCED10* genes were identified as possible candidates for developing citrus greening resistant varieties based on the RNA-seq data analysis. *CsCCO* genes in cucumber play an essential role in ABA biosynthesis by catalysing carotenoid cleavage, specifically under HLB stress. Different ABA levels trigger stress-responsive pathways, which include those leading to fruit abscission. The upregulation of CsCCO under HLB may boost this system as an adaptive response. For this reason, *CsCCO* genes are crucial regulators of the specific fruit detachment mechanisms. In citrus greening, brown discolouration may be present in the calyx abscission zone (AZ–C) located at the pedicel-fruit interface. The plugging of sieve pores and phloem collapse have been documented in leaves of HLB-affected sweet orange (Gasteiger et al. 2005). Sucrose, the major photoassimilate transported in the phloem, has been shown to accumulate in symptomatic leaves of HLB-affected trees compared with healthy trees, suggesting that sugar transport in the phloem is blocked (completely or partially) in the presence of *Candidatus Liberibacter asiaticus* (CLas) infection (Cheng et al. 2022). In sweet orange, the sucrose content in the peels of mature fruit from trees exhibiting HLB symptoms was lower than that of mature fruit collected from healthy trees (Bové 2006). Abscisic acid is an abscission accelerator. By producing ABA hormone, these genes facilitate abscission, resulting in fruit drop, thereby impacting rice physiology and development (Akram et al. 2023).

CONCLUSION

This research uncovered fourteen *CsCCO* genes within *Citrus sinensis*. Examination of their structure showed a range of intron counts, from a single intron to thirteen. Analysis of the promoter regions revealed cis-regulatory elements associated with responses to light, developmental cues, and hormones, implying a role for *CsCCO* genes in the plant's reaction to abiotic stress. RNA-seq data analysis identified *CsNCED3* and *CsNCED10*, which hold potential for genetic manipulation to create citrus greening-resistant cultivars and improve crop productivity. While transcriptome analysis indicated both increases and decreases in *CsCCO* gene expression, further investigation is needed to solidify their importance in diverse physiological and biological functions. Overall, this computational study provides a comprehensive, genome-wide understanding of *CsCCO* genes in *Citrus sinensis*.

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