Comprehensive Genomic Characterization and Expression Analysis of the Lipoxygenase Gene Family in Watermelon under Hormonal Treatments

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Abstract: Lipoxygenases (LOXs) are non-haem iron-containing dioxygenases and play vital roles in a variety of plant biological processes. Here, we first carried out the genome-wide identification of LOX genes in watermelon. A total of 16 LOX genes were identified, which could be classified into two categories according to phylogenetic analysis: the 9-LOXs (ClLOX1–4, 12, and 15) and 13-LOXs (ClLOX5–11, 13, 14, and 16). Furthermore, the protein structures, intrachromosomal distributions, and gene structures were thoroughly analyzed. Cis-element analysis of the promoter regions indicated that the expression of ClLOX genes may be influenced by stress and plant hormones. Bioinformatic and expression analyses revealed that the expression of ClLOX genes is tissue-specific and hormone-responsive. The detected LOX genes exhibited distinctive expression patterns in various tissues. Different ClLOX genes showed different responses to methyl jasmonate (MeJA), salicylic acid (SA), and ethylene (ET) treatments, particularly ClLOX7, which exhibited the most active response to the above treatments. This study provides valuable information for a better understanding of the functions of LOX genes and further exploration of the LOX gene family in watermelon.

Keywords: watermelon; lipoxygenase (LOX); phylogenetic analysis; hormone; gene expression

1. Introduction

Lipoxygenases (LOXs; EC 1.13.11.12) belong to a family of non-haem iron-containing dioxygenases that are widely present in plants, animals, fungi, and even in bacteria [1–4]. They can catalyze the oxidation of polyunsaturated fatty acids (PUFAs), such as linoleic acid (LA) and α-linolenic acid (α-LeA), into unsaturated fatty acid hydroperoxides [2,5]. In plants, LOX-mediated peroxidation of PUFAs undergoes a series of secondary reactions involving several multigene enzyme families, such as Allene oxide synthase (AOS), hydroperoxide lyase (HPL), divinyl ether synthase (DES), and allene oxide cyclase (AOC), and finally produces a large number of biologically active compounds such as jasmonic acid (JA) and its related chemical compounds [1,6,7].

Plant LOXs have a highly conserved lipoxygenase domain at the C-terminus and a PLAT/LH2 (polycystin-1, lipoxygenase, α-toxin domain, or lipoxygenase homology) domain at the N-terminus [8]. According to whether the substrate is oxygenated at carbon atom 9 or 13 of the fatty acid hydrocarbon backbone, plant LOXs are generally divided into two categories of 9-LOXs and 13-LOXs [2]. In addition, based on their primary structure and overall sequence similarity, plant LOXs can also be classified...
into two subfamilies: type I and type II. Type I LOXs have relatively higher sequence similarities with each other than type II LOXs and are lack of plastid transit peptide \[2,9\]. Type II exclusively comprises 13-LOXs, and all the proteins harbor an extra chloroplast transit peptide at the N-terminus \[10,11\].

By genome-wide analysis, previous studies have identified six LOX family genes in \textit{Arabidopsis} \[12\], eight in Tartary buckwheat \[10\], eight in pepper \[13\], 11 in either tea plant \[14\] or radish \[15\], 13 in maize \[16\], 14 in either rice \[17\] or tomato \[18\], 18 in either grape \[19\] or melon \[20\], 20 in poplar \[11\], and 23 in either cucumber \[21\] or pear \[22\]. In addition, many LOX genes have been cloned and functionally characterized to be involved in various growth and developmental processes in plants. For example, \textit{lox3 lox4} double mutants were male sterile and developed more inflorescence shoots and flowers, suggesting that they are essential for male fertility and flower development in \textit{Arabidopsis} \[23\]. Down-regulation of a rice LOX gene reduced the co-oxidation of \(\beta\)-carotene in carotenoid-enriched transgenic rice seeds during storage \[24\]. Moreover, some LOX members were found to be associated with resistance against various abiotic and biotic stresses. For example, silencing of pepper \textit{CaLOX2} gene reduced jasmonate accumulation and caused higher susceptibility to thrips feeding \[25\]. Overexpression of persimmon (\textit{Diospyros kaki}) \textit{DkLOX3} gene in \textit{Arabidopsis} contributed to higher resistance against various abiotic stresses, including osmotic stress, high salinity, and drought, as well as biotic stresses including \textit{Pseudomonas syringae} pv. tomato DC3000 and \textit{Botrytis cinerea} \[26,27\].

Considering that LOX family members have important functions in different developmental processes and various stress responses as mentioned above, we conducted a genome-wide analysis of LOX genes in watermelon genome and systematically analyzed their phylogenetic relationships, protein structures, intrachromosomal localizations, and exon-intron arrangements. In addition, the cis-element analysis of the promoter regions was performed, and the expression patterns of watermelon LOX genes in different tissues and in response to various hormonal treatments were also determined. The results may lay a foundation for further elucidating the functions of the LOX genes and facilitate the molecular breeding of watermelon.

2. Materials and Methods

2.1. Identification of LOX Gene Family Members in Watermelon

To identify all the possible LOX genes in watermelon, the HMM (Hidden Markov Model) profile of the LOX domain (PF00305) was downloaded from the Pfam database (http://pfam.xfam.org/) and searched against the watermelon (97103) v1 proteome (http://cucurbitgenomics.org/organism/1) using the HMMER program with default parameters. Subsequently, the full-length LOX protein sequences in \textit{Arabidopsis} and rice were downloaded according to a previous study \[17\], and used as queries to search against the watermelon (\textit{Citrullus lanatus} subsp. \textit{vulgaris} cv. 97103) v1 proteome with the BLASTP program. The resulting sequences were further verified by SMART (http://smart.embl-heidelberg.de/) and Pfam to confirm the presence of both the LOX and PLAT/LH2 domains. Several redundant sequences were removed for not having the complete domain or shortness.

2.2. Analysis of Protein Properties, Phylogenetic Tree and Conserved Motifs

The isoelectric point (pI), molecular weight (MW) and grand average of hydropathicity (GRAVY) of watermelon LOX proteins were calculated by the ProtParam tool in ExPASy (https://web.expasy.org/protparam/). The subcellular localization of each member of watermelon LOX proteins was predicted using the ProtComp server (Version 9.0, New York, NY, USA) http://linux1.softberry.com/berry.phtml). Multiple sequence alignment was carried out by MAFFT (https://www.ebi.ac.uk/Tools/msa/mafft/) using the full-length sequences of LOX proteins from watermelon and other plant species, including tomato \[18\], pepper \[13\], \textit{Arabidopsis}, and rice \[17\]. A phylogenetic tree was constructed with the MEGA 7.0 using the neighbor-joining (NJ) method with a bootstrap value of 1000. The conserved motifs of watermelon LOX proteins were identified using the MEME tool (http://meme-suite.org/tools/meme),
and the parameter settings were set as follows: the number of motifs was 10, and the width range was 6–50. The MEME results were illustrated with the TBtools program [28].

2.3. Analysis of the Gene Structure and Putative Cis-Acting Regulatory Elements

The GSDS tool (Gene Structure Display Server, http://gsds.cbi.pku.edu.cn/) was employed to examine the gene structures of watermelon LOX genes by comparing their sequences of coding sequence (CDS) and corresponding genomic DNA (gDNA). To identify the potential stress- and hormone-related cis-elements, the 2000-bp DNA sequences of ATG site of watermelon LOX genes were obtained from the watermelon (97103) v1 genome database (http://cucurbitgenomics.org/organism/1) and analyzed by the PlantCARE server (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.4. Chromosome Mapping, Duplication and Synteny Analysis

The chromosomal information of watermelon LOX genes was downloaded from the watermelon genome database and the LOX genes were mapped to chromosomes with the MapChart software. Duplication analysis between the watermelon LOX genes and synteny analysis of LOX genes between watermelon and Arabidopsis were conducted with the MCScanX software (Athens, GA, USA) (http://chibba.pgml.uga.edu/mcscan2/) by referring to a previous report [29].

2.5. Expression Analysis of Watermelon LOX Genes Based on RNA-Seq Data

The RNA-seq data of the flesh and rind at 10, 18, 26, and 34 days after pollination (DAP) were obtained, and the expression annotation was performed as previously described [6,30]. FPKM (fragments per kilobase of exon model per million mapped reads) values of watermelon LOX genes were log2-transformed, and then the TBtools program was employed to visualize the expression values.

2.6. Plant Materials and Growth Conditions

Watermelon (Citrullus lanatus L. cv. Xinong 8) seeds were sown in pots containing nutritional soil within a greenhouse under the conditions of 25 ◦C/19 ◦C (12 h/12 h). For analysis of tissue-specific expression patterns, various tissues including leaves, roots, stems, flowers, and fruit were harvested from 2-month-old watermelon plants. For various hormone treatments, four-leaf stage watermelon seedlings were treated with 100 µM methyl jasmonate (MeJA), 1 mM salicylic acid (SA), and 500 µM ethylene (ET) by spraying according to our previous study [30]. Then, the leaves and roots were harvested from treated seedlings at 0, 1, 3, 9, and 24 h post-treatment with three biological triplicates. All of the samples were rapidly frozen in liquid nitrogen and stored at −80 ◦C until use.

2.7. RNA Extraction and Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was extracted with the total RNA Miniprep Kit (Axygen Biosciences, Union City, CA, USA) following the manufacturers’ protocol. The RNA (1 µg) was reverse-transcribed using the ReverTra Ace qPCR-RT Kit (TOYOBO, Osaka, Japan) for the synthesis of cDNA. qRT-PCR was performed on the iCycler iQTM Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) in three independent biological replicates. The primers used are described in Supplementary Table S1. Watermelon β-actin gene (Cla007792) was used as an internal control, and the relative expression was analyzed using by the $2^{-\Delta\Delta Ct}$ method [31].

3. Results

3.1. Identification of LOX Genes in Watermelon

By HMMER search and BLASTP, a total of 19 LOX genes were identified in watermelon (C. lanatus) genome, three of which were removed for shortness and not having the complete domain of LOX as tested by SMART and Pfam. Finally, the 16 LOX genes were denoted as CILOX1–16 in an ascending
order of the corresponding chromosomes (Table 1). The gDNA and CDS lengths of the ClLOX family genes varied from 2913 to 9824 bp and from 1686 to 2787 bp, respectively, encoding proteins ranging from 561 to 928 amino acids (aa) in length with an average length of 810.06 aa. In addition, the calculated MW, pI, and GRAVY values varied from 85.95 to 105.04 kDa, from 5.13 to 8.83, and from $-0.581$ to $-0.187$, respectively (Table 1). The subcellular localization analysis showed that the watermelon LOX proteins were located in cytoplasm and chloroplast (Table 1).

3.2. Evolutionary Relationship among LOX Family Members in Various Plant Species

To reveal the phylogenetic relationships of LOX family members in watermelon and other plant species, a phylogenetic tree was constructed based on the protein sequences of watermelon, pepper [13], tomato [18], Arabidopsis, and rice [17]. As a result, these LOX proteins could be clearly divided into two categories of 9-LOXs and 13-LOXs. Among the watermelon LOX proteins, six members (ClLOX1–4, 12, and 15) were grouped into the 9-LOX category, while other 10 members fell into the 13-LOX category (Figure 1). It is worth noting that ClLOX1–4 were clustered in the 9-LOX category, while ClLOX5–11 and ClLOX13 were clustered together in the 13-LOX category (Figure 1), suggesting that the ClLOX proteins may be evolutionarily conserved.

Figure 1. Phylogenetic analysis of LOX family members in watermelon and other plant species. The NJ phylogenetic tree was created based on the full-length amino acid sequences by using MEGA 7.0 with 1000 bootstrap replicates. The information of LOX proteins in various plant species is presented in Table S2.
Table 1. Identification and characterization of LOX family genes in watermelon.

| Nomenclature | Locus   | Predicted LOX Class | Chromosomal Position | gDNA (bp) | CDS (bp) | Protein Properties | Protein Properties | Protein Properties | Protein Properties | Protein Properties | Protein Properties | Protein Properties |
|--------------|---------|---------------------|----------------------|-----------|----------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|             |         |                     |                      |           |          |                     |                     |                     |                     |                     |                     |                     |
| CILLOX1     | Cla019908 | 9-LOX               | Chr2: 25963474 .. 25968681 (+) | 5208      | 2628     | 875 99.03 40–181 192–858 6.14 −0.365 Cytoplasm |
| CILLOX2     | Cla019907 | 9-LOX               | Chr2: 25991122 .. 25996850 (+) | 5729      | 2640     | 879 99.70 40–181 192–862 5.41 −0.364 Cytoplasm |
| CILLOX3     | Cla019897 | 9-LOX               | Chr2: 26087261 .. 26097084 (-) | 9824      | 2589     | 862 97.49 18–162 173–845 5.67 −0.432 Cytoplasm |
| CILLOX4     | Cla019896 | 9-LOX               | Chr2: 26108726 .. 26117164 (-) | 8439      | 2259     | 752 85.95 7–97 82–735 5.13 −0.361 Cytoplasm |
| CILLOX5     | Cla008520 | 13-LOX              | Chr2: 33497808 .. 33502790 (-) | 4983      | 2709     | 902 102.74 74–207 218–885 5.76 −0.413 Chloroplast |
| CILLOX6     | Cla008519 | 13-LOX              | Chr2: 33513361 .. 33519238 (-) | 5878      | 1686     | 561 63.98 66–187 342–561 5.16 −0.187 Cytoplasm |
| CILLOX7     | Cla008517 | 13-LOX              | Chr2: 33543209 .. 33546121 (-) | 2913      | 2355     | 784 89.41 1–89 100–767 5.44 −0.447 Chloroplast |
| CILLOX8     | Cla008516 | 13-LOX              | Chr2: 33565766 .. 33569015 (-) | 3250      | 2511     | 836 95.13 3–129 142–819 5.72 −0.579 Chloroplast |
| CILLOX9     | Cla003211 | 13-LOX              |Chr2: 33577135 .. 33581164 (-) | 4010      | 2493     | 830 94.30 3–129 140–813 7.02 −0.483 Chloroplast |
| CILLOX10    | Cla003210 | 13-LOX              |Chr2: 33590798 .. 33596027 (-) | 5200      | 1989     | 662 75.43 1–68 79–645 6.57 −0.448 Chloroplast |
| CILLOX11    | Cla003209 | 13-LOX              |Chr2: 33605180 .. 33608324 (-) | 3345      | 2574     | 857 97.86 7–140 151–840 6.02 −0.558 Chloroplast |
| CILLOX12    | Cla009402 | 9-LOX               |Chr6: 6667738 .. 6672962 (+) | 5225      | 2652     | 883 100.89 36–180 191–861 6.00 −0.352 Cytoplasm |
| CILLOX13    | Cla005400 | 13-LOX              |Chr7: 27624656 .. 27628157 (+) | 3782      | 1755     | 584 67.73 4–128 158–584 6.55 −0.581 Chloroplast |
| CILLOX14    | Cla015542 | 13-LOX              |Chr9: 581154 .. 585338 (-) | 4185      | 2787     | 928 103.02 80–234 245–911 6.65 −0.376 Cytoplasm |
| CILLOX15    | Cla014845 | 9-LOX               |Chr9: 6640629 .. 6649232 (+) | 8604      | 2517     | 838 96.18 16–143 154–816 6.61 −0.459 Cytoplasm |
| CILLOX16    | Cla022987 | 13-LOX              |Chr11: 16214820 .. 16219598 (+) | 4779      | 2787     | 928 105.04 105–229 240–911 8.83 −0.475 Cytoplasm |
3.3. Characterization and Conserved Domain Analysis of Watermelon LOX Proteins

To determine the evolutionarily conserved domains, the full-length amino acid sequences of CILOX proteins were submitted to pfam and SMART tools. The results showed that all watermelon 9-LOXs and 13-LOXs harbored both a conserved LH2/PLAT and a LOX domain, while CILOX9 seemed to have a truncated LOX domain (Figure 2A,B).

![Figure 2. Phylogenetic relationships (A), conserved domains (B) and motif compositions (C) of CILOX proteins.](image)

The structures of the CILOX proteins were further examined by using the MEME server. A total of 10 motifs (designated as motif 1–10) were identified for the 16 CILOX proteins (Figure 2C). Amongst them, motifs 1–9 corresponded to the LOX domain, while motif 10 was part of the LH2/PLAT domain (Supplementary Figure S1). Motif 1 included a representative motif of 38 amino acids [His-(X)4-His-(X)4-His-(X)17-His-(X)8-His] with five conserved His (H) residues, which was demonstrated to play a vital role in iron binding and is necessary for enzyme stability and activity [5,32,33]. The 10 motifs were widely present in all CILOX proteins, and their distributions exhibited certain degrees of specificity (Figure 2). The majority of CILOX proteins harbored motif 1, except for CILOX6 (Figure 2C). Besides, some other CILOX proteins were also lack of certain conserved motifs. For example, motif 4, motif 5, motif 6, and motif 9 were absent in CILOX13, while motif 10 was not found in CILOX4. In addition, CILOX1 and CILOX2 had an additional motif 8 in their N-terminus. These differences in motif arrangement may account for the functional differentiation among LOX proteins in watermelon.

To further characterize the structures of watermelon LOX proteins, all full-length CILOX protein sequences were aligned by MAFFT, and the representative motif of 38 amino acids was shown in Figure S2. The watermelon 13-LOXs had a conserved F residue that is indicative of the 13-LOX activity of LOX enzymes, while the 9-LOXs had V/H/L in the corresponding position (Supplementary Figure S2).

3.4. Intrachromosomal Localization and Structural Analysis of Watermelon LOX Genes

The 16 CILOX genes were mapped on five of the eleven chromosomes in watermelon genome, and the number of genes on each chromosome was highly uneven (Figure 3). Chromosome 2 comprised the largest number of CILOX genes (11 genes). Chromosome 9 included two CILOX genes, while each of chromosomes 6, 7, and 11 only had one CILOX gene (Figure 3).
Figure 3. Locations of the LOX genes in watermelon chromosomes.

Further, we carried out a synteny analysis of LOX genes between watermelon and Arabidopsis. All Arabidopsis LOX genes had syntetic copies in watermelon (Supplementary Figure S3), and these syntetic relationships may originate from whole genome triplication or segmental duplication events [15]. To further trace the evolutionary history of CILOX genes, we investigated the duplication information of them using MCScanX. The results showed that a total of 10 CILOX genes (CILOX1, 2, 3–10) could be determined as tandemly duplicated genes.

The structural features of CILOX genes were examined by the GSDS tool. Generally, all CILOX genes harbored introns in their genomic sequences, and the intron number varied from two to eight (Figure 4). All 9-LOX genes (6 out of 9) contained eight introns. Most of the 13-LOX genes comprised 7–8 introns, except for CILOX7, CILOX10, and CILOX14, which had 6, 5, and 6 introns, respectively (Figure 4).

Figure 4. Exon-intron arrangements of watermelon LOX genes according to phylogenetic analysis. The exons and introns are shown as blue boxes and black lines, respectively.
3.5. Cis-Element Analysis in the Promoter Regions of CILOX Genes

To reveal the possible transcriptional regulation patterns of the CILOX genes, the 2.0-kb sequence upstream of the ATG site of CILOX genes was retrieved and analyzed by using the PlantCARE program. A total of 15 kinds of cis-elements involved in responses to stresses and plant hormones were identified, and all CILOX genes contained 2–8 kinds of cis-elements in their promoter regions, with the exception of CILOX7 (Figure 5). Among the stress- and hormone-related cis-elements, anaerobic induction element (ARE) and ET responsive element (ERE) were the most frequently detected in CILOX genes. In addition, 1, 3, 3, 4, 5, and 5 CILOX genes had the dehydration-responsive element (DRE), low temperature responsive element (LTR), defense and stress-responsive elements (TC-rich repeats), MYB binding site involved in drought and stress (MBS), WRKY binding site (W-box), and wound-responsive element (WUN-motif), respectively (Figure 5), implying the possible roles of CILOX genes in responses to various stresses. Besides the ERE element, seven other important hormone-related cis-elements were also widely identified, including ABRE, CGTCA-motif, TCA-element, AuxRR-core, TGA-element, P-box, and GARE-motif, suggesting that the CILOX genes may be regulated by various plant hormones. Notably, over half of CILOX genes (nine out of 16) contained the TCA-element in their promoters, and ABA responsive element (ABRE), MeJA responsive element (CGTCA-motif), auxin-responsive element (AuxRR-core and TGA-element), and gibberellin responsive element (P-box and GARE-motif) were found in the promoter regions of 7, 5, 4, and 6 CILOX genes, respectively (Figure 5).

![Figure 5. Analysis of stress- and hormone-related cis-elements in the promoter regions (−2000 bp) of CILOX genes. The numbers of cis-elements are boxed.](https://example.com/figure5.png)
3.6. Expression Analysis of ClLOX Genes in Different Tissues and during Fruit Development

Four ClLOX genes (one from 9-LOXs and three from 13-LOXs) were selected to examine their expression levels in various tissues, including leaves, roots, stems, flowers, and fruit. The qRT-PCR results indicated that some ClLOX genes may have tissue-specific expression patterns. For example, ClLOX14 was highly expressed in fruit; ClLOX4 and ClLOX7 exhibited the most abundant transcripts in flowers; while ClLOX8 showed evident expression specificity in roots (Figure 6). We further determined the expression patterns of ClLOX genes during fruit development in watermelon based on RNA-seq data by referring to our previous study [6]. During the development of flesh, ClLOX5 and ClLOX15 showed decreases in transcript levels. During rind development, ClLOX4, ClLOX5, and ClLOX16 displayed increases in transcripts at some time points, particularly at 26 DAP (Supplementary Figure S4).

![Figure 6](image_url)

Figure 6. qRT-PCR analysis of the expression of selected ClLOX genes (A–D) in different watermelon tissues. L, leaves; R, roots; S, stems; F, flowers; Fr, fruit. Error bars indicate standard deviation (SD) based on three biological replicates.

3.7. Expression Analysis of Several Watermelon ClLOX Genes in Response to JA, SA and ET Treatment

Considering that the ClLOX genes harbor a large number of hormone-related cis-elements, qRT-PCR was carried out to investigate the expression patterns of selected ClLOX genes under JA, SA, and ET treatments. Upon JA treatment, the expression levels of ClLOX7, ClLOX12, and ClLOX14 showed notable increases in both the leaf and root, with the most significant increases being observed for ClLOX7, suggesting that ClLOX7 plays a primary role in JA signaling in leaves and roots (Figure 7A,B). However, ClLOX4 and ClLOX16 showed different expression patterns in leaves and roots under JA treatment. The expression of ClLOX4 observably decreased in leaves but gradually increased and reached the highest level at 9 h in roots (Figure 7A,B). For ClLOX16, the expression increased and peaked at 24 h in leaves, while exhibited a significant decrease in roots at 3 h (Figure 7A,B). Upon SA treatment, ClLOX7 and ClLOX16 showed similar expression patterns, and their transcripts dramatically increased and peaked at 3 h, while the expression of ClLOX14 showed remarkable declines at certain time points (Figure 7C). Upon ET treatment, the expression of ClLOX7 and ClLOX16 showed significant
increases, while that of CILOX4 and CILOX12 displayed significant decreases at certain time points (Figure 7D). In addition, the expression of CILOX14 was observably induced at earlier time points (1 h), but showed sharp decreases thereafter (Figure 7D). These results indicated that CILOX genes may be involved in diverse hormonal responses.

Figure 7. qRT-PCR analysis of the expression of selected CILOX genes under various hormone treatments, including JA (A,B), SA (C), and ET (D). Error bars indicate standard deviation (SD) based on three biological replicates.

4. Discussion

LOX proteins are widely present in plants, and are generally encoded by a multigene family. In this study, a total of 16 LOX proteins were identified in watermelon through HMMER combined with BLASTP search (Table 1), and several proteins possessing either one LOX domain or the PLAT/LH2 domain were excluded based on the criteria in previous reports [11,13]. Watermelon has a comparatively larger number of LOX family members relative to other plant species, such as Arabidopsis (six members) [12], pepper (eight members) [13], tea plant (11 members) [14], tomato (14 members) [18], and melon (18 members) [20], and the number of LOX genes in these plants is not proportional to their genome sizes. Notably, 11 out of the 16 CILOX genes were located on chromosome 2 and formed two distinct tandem duplicate gene clusters (Figure 3). Moreover, over half of the CILOX genes (10 in 16) were identified as tandemly duplicated genes, indicating that tandem duplication events are the main driving force for the evolution of CILOX genes. This feature has also been found in some other plants, such as poplar [11], maize [16], tomato [18], cucumber [34], and radish [15]. In addition, according to the collinear module of watermelon and Arabidopsis, all of the six AtLOX genes have syntenic copies in CILOX genes (Supplementary Figure S3). The larger number of AtLOX-CILOX orthologous events indicates that CILOX genes may have similar structure and function to AtLOX genes.

Through a phylogenetic analysis of the LOX gene family in watermelon and other plant species, LOX family members can be divided into the categories of 9-LOXs and 13-LOXs (Figure 1), which
13-LOX members contain the F residue at the active site (Figure S2), which is indicative of the 13-LOX activity of LOX enzymes [35,36]. However, the 9-LOXs had V/H/L in the corresponding position instead (Figure S2). The conserved V residue associated with 9-LOX activity is a characteristic of LOX enzymes, while some 9-LOXs possess no V residue at the position [37–39]. In addition, the analysis of conserved motif distribution and exon-intron arrangement further supported the phylogenetic results. The LOXs clustered together tended to have similar conserved motif distributions and structural features (Figures 2 and 4). However, some phylogenetically related CILOX genes had similar exon/intron structures but variable numbers of introns, such as CILOX12 and CILOX13, CILOX17 and CILOX19 (Figure 4), suggesting that gain or loss of introns may occur during the evolution of CILOX genes, which may be responsible for their indispensable roles in watermelon.

Previous studies have shown that some LOX genes have distinctive expression patterns, which may provide important clues for understanding their physiological functions. For example, over half of the PILOX genes were found to be preferentially expressed in mature leaves and male catkins [11]. Most of the GhLOX genes were expressed in vegetative tissues, while several GhLOX genes were only expressed in specific tissues, such as GhLOX4, GhLOX5, and GhLOX15 in root, GhLOX7 in stem, and GhLOX17 and GhLOX19 in stigma [32]. In the present study, the identified CILOX genes also displayed significantly higher expression only in specific tissues, such as CILOX14 in fruit, CILOX4 and CILOX7 in flowers, and CILOX8 in roots (Figure 6), suggesting their specific roles in these tissues. In addition, two tandemly duplicated genes, CILOX7 and CILOX8, exhibited diverse tissue expression patterns (Figure 6), implying that they are functionally distinctive and have undergone non-functionalization, sub-functionalization, or neofunctionalization [40,41]. In addition, several CILOX genes showed decreases in transcript abundance during fruit development (Supplementary Figure S4), and similar results were also found in kiwifruit [42], grape [19], melon [20], and tomato [18,43]. The higher expression levels of these LOX genes indicate that plants may require the LOX activity for cell division and fruit enlargement during early developmental periods. Instead, CILOX4, CILOX5, and CILOX16 showed increases in transcripts during the late stage of rind development, implying their regulatory roles in fruit ripening. Previous reports have revealed that the LOX activity is associated with membrane degradation during fruit ripening, and ethyl and butyl acetates would increase with fruit ripening [20,44]. Therefore, the continuous increase in the expression of LOX genes during fruit development may be associated with cell degradation and senescence during fruit ripening.

The LOX pathway is involved in the early steps of plant responses to pathogen and insect attacks. The analysis of cis-elements uncovered many hormone-related cis-elements in the promoter regions of the CILOX genes (Figure 5). Therefore, we determined the expression profiles of some selected CILOX genes upon different plant hormone treatments. Under JA treatment, the expression of all the genes was altered, particularly CILOX7, which exhibited more dramatic increases of expression in both leaves and roots relative to CILOX genes (Figure 7A,B). CILOX7 is orthologous to AtLOX2 in the 13-LOX group (Figure 1), both of which were localized in chloroplasts (Table 1). Therefore, compared with other CILOX genes, CILOX7 might play a primary role similar to that of AtLOX2, which can provide linolenic acid hydroperoxide substrates for JA biosynthesis in vivo [45]. JA, SA, and ET are three main defense-associated phytohormones that mediate signal transduction to combat attackers such as pathogens and herbivorous insects, and the SA- and ET/JA-mediated defense response pathways were reported to act antagonistically, synergistically, or additively [46–48]. In this study, the selected CILOX genes were also found to be regulated in response to SA and ET treatments (Figure 7C,D). Our previous reports have shown that other oxylipin pathway genes, such as CIAOCs, CIAOS and CHIPLs, are also regulated in response to JA, SA, and ET, which may play important roles in watermelon defense against root-knot nematode (RKN) infection [6,30]. The roles of LOXs in the defense against pathogens and pests might be associated with the synthesis of a number of compounds from the oxylipin pathway [1,7]. For example, OsLOX1 could lead to the production of more JA, (Z)-3-hexenal, and colheneic acid after brown plant hopper (BPH) feeding, and transgenic rice plants with enhanced
OsLOX1 expression were more resistant to BPH attack [49]. Tea plant CsLOX1 was induced in response to feeding by the tea green leafhopper, and the expression profile showed a clear association with the emission pattern of LOX-derived volatiles [50]. ZmLOX10 provides substrate to several LOX branches and produces a series of 13-oxylipin products, and the zmlox10 plants were unable to produce green leaf volatiles (GLVs) and JA, resulting in a dramatic decline in herbivore-induced plant volatiles and attractiveness to parasitoid wasps [51,52]. A recent study has revealed that GLVs and JA contribute to maize susceptibility to Colletotrichum graminicola due to the suppression of SA-related defense [53]. Hence, the ClLOX genes may participate in the protection of plants from biotic stresses by catalyzing the synthesis of some oxylipins through the regulation of the SA- and ET/JA-mediated defense response pathways. In addition, some DRE and ABRE and ERE cis-elements were found in the promoters of LOXs, suggesting that these LOXs may participate in watermelon defense against abiotic stress. A recent report has revealed that tomato LOXs were up-regulated or down-regulated in response to heat, salt, or drought stress [54]. In future work, we will focus on the functions of watermelon LOX genes in defense against abiotic stress.

5. Conclusions

In summary, a total of 16 LOX genes were identified in watermelon, which could be divided into two categories: 9-LOXs (ClLOX1–4, 12, and 15) and 13-LOXs (ClLOX5–11, 13, 14, and 16). Their phylogenetic relationships, protein structures, intrachromosomal distributions, gene structures, and cis-element compositions in the promoters were thoroughly analyzed in this work. The results improve our understanding of the LOX gene family in watermelon. In addition, the expression analysis of some selected ClLOX genes showed that their expression is tissue-specific as well as hormone-responsive. These findings may expand the understanding of the functions of ClLOX genes and lay a foundation to select candidate genes for watermelon genetic improvement.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0472/10/10/429/s1. Figure S1. Logo of 10 motifs of watermelon LOX proteins by MEME. Figure S2. Multiple sequence alignments of the representative 38 amino acid motif of watermelon LOX proteins. The representative 38 amino acid motif is boxed with purple. The five essential His residues involved in the binding of the iron atom in the active site are denoted by asterisks. The residues indicative of LOX enzymes with 9- or 13-LOX activity are boxed with red. Figure S3. Collinear relationships of LOX genes in watermelon and Arabidopsis. Figure S4. Expression patterns of ClLOX genes during the development of flesh and rind at different stages. The expression levels are indicated as log2-based FPKM+1 values. DAP, days after pollination.

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