Effects of abiotic stress and hormones on the expressions of five 13-CmLOXs and enzyme activity in oriental melon (Cucumis melo var. makuwa Makino)

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Abstract
Lipoxygenases (LOXs) are a group of non-heme, iron-containing enzymes and extensively involved in plant growth and development, ripening and senescence, stress responses, biosynthesis of regulatory molecules and defense reaction. In our previous study, 18 LOXs in melon genome were screened and identified, and five 13-LOX genes (CmLOX08, CmLOX10, CmLOX12, CmLOX13 and CmLOX18) were predicted to locate in chloroplast. Phylogenetic analysis result showed that the five genes have high homology with jasmonic acid (JA) biosynthesis-related LOXs from other plants. In addition, promoter analysis revealed that motifs of the five genes participate in gene expression regulated by hormones and stresses. Therefore, we analyzed the expressions of the five genes and LOX activity in leaves of four-leaf stage seedlings of oriental melon cultivar Yumeiren under abiotic stress: wounding, cold, high temperature and hydrogen peroxide (H2O2), and signal molecule treatments: methyl jasmonate (MeJA), abscisic acid (ABA) and salicylic acid (SA). Real time qPCR revealed that wounding and H2O2 induced the expressions of all the five genes. Only CmLOX08 was induced by cold while only CmLOX13 was suppressed by high temperature. ABA induced the expressions of CmLOX10 and CmLOX12 while inhibited CmLOX13 and CmLOX18. MeJA increased the 3 genes expressions except CmLOX08 and CmLOX13, whereas SA decreased the effect, apart from CmLOX12. All the abiotic stresses and signal molecules treatments increased the LOX activity in leaves of oriental melon. In summary, the results suggest that the five genes have diverse functions in abiotic stress and hormone responses, and might participate in defense response. The data generated in this study will be helpful in subcellular localization and transgenic experiment to understand their precise roles in plant defense response.

Keywords: oriental melons, lipoxygenase, abiotic stress, signal molecules, gene expression

1. Introduction
Lipoxygenases (LOXs, EC 1.13.11.12) are a group of non-heme, iron-containing enzymes which are ubiquitous in plants and animals. LOXs catalyze the polyunsaturated fatty acids (PUFAs) that contain a (1Z, 4Z)-pentadiene system to yield the corresponding (1S, 2E, 4Z)-hydroperoxides which are
collectively known as oxylipins (Liu et al. 2006). Oxylipins produced by LOXs have been reported to play an important role in plant defense responses (Christensen et al. 2013; García-Marcos et al. 2013). The allene oxide synthase catalyzes the fatty acid hydroperoxides produced by the 13-LOX to form unstable allene oxides which can be further metabolized to chiral (9S, 13S)-12-oxo phytodienoic acid (OPDA) by an allene oxide cyclase (AOC), and then products jasmonates (JAs) such as jasmonic acid (JA) and its derivative, methyl jasmonate (MeJA) (Feussner and Wasternack 2002; Demmig-Adams et al. 2013). JAs are now considered to be key regulators for stress-induced gene expression in plants (Browse 2005; Wasternack 2007; Wasternack and Hause 2013). Hydroperoxide lyases catalyze now considered to be key regulators for stress-induced synthesis of 13-hydroperoxy octadecatrienoic acid to form green leaf volatile (GLV) which act as signals to regulate the expression of defensive genes (Bate and Rothstein 1998) and plant-plant communication after insect elicitation (Engelberth et al. 2004).

Plant LOXs occur in a gene family and can be classified by two methods: For one method, LOXs are classified into 9-LOXs and 13-LOXs based on the specific site of the oxygen addition to linoleic acid (LA) at either the C-9 or C-13 residue in the plant (Feussner and Wasternack 2002). By the other method, LOXs are grouped into type I-LOXs and type II-LOXs according to comparison of the primary structure and overall sequence similarity. Enzymes designated type I have a high sequence similarity (>$75\%$) to one another and lack plastid transit peptide. Type II enzymes show only a moderate overall sequence similarity (<$35\%$) to one another and carry a chloroplast transit peptide sequence (Andreou and Feussner 2009). LOXs perform diverse physiological functions in plant growth, development, ripening, senescence, and stress responses (Hwang and Hwang 2010; Cho 2012; Vicente et al. 2012).

Recent studies reported that the 13-LOX, especially chloroplastic LOXs, played an important role in plant defense response to stress (Halitschke and Baldwin 2003; Nemchenko et al. 2006; Christensen et al. 2013). TomLOXD, a chloroplast-localized LOX in tomato was up-regulated by wounding and MeJA (Heitz et al. 1997), a further study reported that overexpression of TomLOXD increased generation of endogenous JA and resistance to high temperature as well as expression of wound-responsive genes, elevated wound-induced JA biosynthesis (Hu et al. 2013; Yan et al. 2013). However a mutant phenotype results from a point mutation in the catalytic domain of TomLOXD exhibited a series of JA-dependent immune deficiencies, including the inability to express wound responsive genes, abnormal development of glandular trichomes, and severely compromised resistance to cotton bollworm (Helicoverpa armigera) and Botrytis cinerea which indicated that TomLOXD was involved in wound-induced and endogenous JA biosynthesis and played an important role in tomato defense response to stress (Yan et al. 2013). Similarly, in tobacco antisense expression of NaLOX3 specifically reduced JA accumulation, expression of JA-induced genes, and resistance to Manduca sexta attack (Halitschke and Baldwin 2003). In many other plants such as Arabidopsis thaliana (Bhardwaj et al. 2011), cucumber (Yang et al. 2012), potato (Royo et al. 1996), Arabidopsis (Melan et al. 1993; Bell et al. 1995) and common bean (Porta et al. 2008), the expressions of 13-LOX genes have been described in response to stress and signal compounds implicated defense responses.

Our previous study identified 18 LOX genes in melon genome (named CmLOX01–18) and analyzed the phylogeny and expression profiles of them, the study reported that these genes were differently expressed in vegetative and reproductive tissues and were divided as 9-LOX (CmLOX07 and CmLOX09) and 13-LOX (CmLOX01–06, CmLOX08, CmLOX10–16 and CmLOX18), among the 13-LOX genes there were five LOXs (CmLOX08, CmLOX10, CmLOX12, CmLOX13 and CmLOX18) predicted to locate in the chloroplast and possessed a chloroplast transit peptide (Zhang et al. 2014). As the chloroplast-localized LOX were reported to be a key factor during plant defense response described above, we analyzed expressions of the five 13-LOX genes (CmLOX08, CmLOX10, CmLOX12, CmLOX13 and CmLOX18) and LOX activity in leaves under abiotic stress and hormone treatments, namely wounding, cold, high temperature, hydrogen peroxide ($H_2O_2$), MeJA, abscisic acid (ABA), salicylic acid (SA).

2. Results

2.1. Phylogenetic analysis

To determine the relationship of the five genes with other known LOX sequences, we performed a phylogenetic tree using the full-length amino acid sequences of the five genes and other plant LOX genes (Fig. 1). The full-length of the five genes was listed in Table 1.

The phylogenetic tree showed that these LOXs were separated into two types, 9-LOXs and 13-LOXs. The five genes were all 13-LOXs. CmLOX08 and CmLOX18 had a high homology with Arabidopsis AtLOX3 and AtLOX4, NaLOX3 from tobacco, TomLOXD from tomato, CaLOX2 from potato, SiLOX3 from potato and VvLOX0 from grape. CmLOX10, CmLOX12 and CmLOX13 had more relationship with Arabidopsis AtLOX2, ZmLOX10 from maize, PVLOX6 from common bean, PdLOX1 and PdLOX2 from poplar.

2.2. Promoter sequence analysis

There were mainly two kinds of motifs in the five LOX genes,
which were known as cis-acting element and cis-acting regulatory element. Cis-acting element involved in defense and stress responsiveness and cis-acting regulatory element involved in the response to various hormones. CmLOX08, CmLOX10 and CmLOX13 possess motif involved in response to ABA and CmLOX08, CmLOX10 have MeJA element. CmLOX08, CmLOX10, CmLOX12 and CmLOX18 possess HSE binding site involved in response to heat (Table 2). CmLOX08 also has other motifs in response to SA and cold.

2.3. LOX activity assay

All the treatments caused the total LOX activity of seedling leaves increase in varying degrees (Fig. 2). The LOX activity of leaves treated with cold increased significantly at 12 h, and increase from 3 to 24 h with high temperature, similarly, wounding increased LOX activity from 1.5 to 12 h. MeJA and SA both increased LOX activity from 3 to 24 h, while ABA treatment only induced at 6 h. H$_2$O$_2$ induced LOX activity...
at 6 h which peaked at 72 h then declined to the control level at 168 h.

2.4. The five 13-LOX genes expressions

Since there were cis-acting elements and cis-acting regulatory elements in the promoter of the five LOX genes, we checked whether the five genes were regulated by the abiotic stress and signal molecules.

The expression of CmLOX08 was induced by wounding, cold, high temperature and H₂O₂ while inhibited by MeJA and SA (Fig. 3), and no effect under ABA treatment. A slight increase of CmLOX08 mRNA was observed at 1.5 h after wounding and reached a maximum at 3 h. The transcripts level reached a maximum at 6 h at both 4 and 45°C, while decreased to the lowest point at 6 h after SA treatment. H₂O₂ treatment induced steady-levels of CmLOX08 mRNA accumulation starting at 6 h and reached a maximum value at 72 h, and then decreased to the level of control. CmLOX10 was also induced by wounding, high temperature and H₂O₂, but unlike CmLOX08, the expression was up-regulated by MeJA and SA and decreased by cold (Fig. 4). The mRNA transcripts increased after both wounding and MeJA treatments and peaked at 6 h, and

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<th>Table 2</th>
<th>List and description of nucleotide motifs discovered in promoter region of five melon LOX genes</th>
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<tr>
<td>Motif name</td>
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<tr>
<td>TC-rich repeats</td>
<td>CmLOX08</td>
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<td>ARE</td>
<td>CmLOX10</td>
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<td>CGTCA-motif</td>
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<td>TGACG-motif</td>
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<td>CCAAT-box</td>
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<td>WUN-motif</td>
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then decreased to normal level at 24 h. The expression decreased by SA and cold treatments. 

*CmLOX12* was induced by all the treatments except cold (Fig. 5). The expression level increased immediately after wounding and ABA treatment, whereas SA and MeJA induced its expression, reached a maximum value at 12 and 3 h, respectively.

*CmLOX13* was induced by wounding and H$_2$O$_2$ and suppressed by ABA, cold and high temperature treatments, and suppressed after SA treatment, but increased at 24 h and then recovered to the control level (Fig. 6).

Similar to *CmLOX10*, the expression of *CmLOX18* was increased by wounding, high temperature, MeJA and H$_2$O$_2$ and suppressed by cold and SA, but its expression was declined by ABA (Fig. 7).

3. Discussion

There are 18 LOXs identified in melon genome and among them five LOXs (*CmLOX08*, *CmLOX10*, *CmLOX12*, *CmLOX13* and *CmLOX18*) are predicted to locate in the chloroplast and possessed a chloroplast transit peptide (Zhang et al. 2014). To gain knowledge of the possible role of the five LOXs under stress, we analyzed the five gene expression and LOX activity under abiotic stress and hormone treatments.

The phylogenetic tree showed that the five genes exhibited a close genetic relationship with Arabidopsis AtLOX2, NaLOX3 from tobacco and TomLOX0 from tomato (Fig. 1). Antisense-mediated depletion of AtLOX2 reduced wound-induced accumulation of JA and a wound- and JA-inducible
gene (Bell et al. 1995). *NaLOX3* and *TomLOXD* are known to provide linolenic acid hydroperoxide substrates for the synthesis of JA (Halitschke and Baldwin 2003; Hu et al. 2013; Yan et al. 2013). The results suggest that the five melon LOX genes might participate in the biosynthesis of JA and defense response to abiotic stress.

The expressions of the five genes under abiotic stress and hormone treatments were in accordance with their motifs (Table 2, Figs. 3–7). Interestingly, the promoter sequences analysis showed that *CmLOX08* contained CGTCA-motif (MeJA) and TCA-element (SA) while the expression of *CmLOX08* was declined by MeJA and SA treatments, which suggest the two motifs negative regulate the expression of *CmLOX08*. On the contrary, the expression of *CmLOX10* was positive regulated by CGTCA-motif (MeJA) and HSE (heat) as the accumulation of *CmLOX10* mRNA increased upon MeJA and high temperature treatments. *CmLOX13* was down regulated by ABRE (ABA) and *CmLOX18* was up regulated by HSE (heat).

The analysis of expressions of the five genes showed that they were differently regulated by abiotic stress and hormones. MeJA, ABA, SA and H₂O₂ are important signal compounds regulating defense response to stress in plants (Jia 2012; Yang et al. 2012). Our results showed that *CmLOX10*, *CmLOX12* and *CmLOX18* were inducible by wounding and simultaneously up-regulated with MeJA (Figs. 4, 5 and 7), which are also reported in many plants such as cucumber, *Arabidopsis* and tomato (Melen et al. 1993; Heitz et al. 1997; Yang et al. 2012), suggesting they might participate in the biosynthesis of wound-induced JAs, which would be verified by transgenic experiments in the near future. All the five genes were induced by both wounding and H₂O₂, which is a key molecule in response to wounding and pathogens, triggering plant cell death to

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**Fig. 3** Expressions of *CmLOX08* at various time points under wounding, cold (4°C), high temperature (45°C), MeJA (100 μmol L⁻¹), ABA (100 μmol L⁻¹), SA (5 mmol L⁻¹) and H₂O₂ (10 mmol L⁻¹) were analyzed by real time qPCR using the primers specific to the five *CmLOX* genes.
defense to stress (Montillet et al. 2005). LOXs in common bean (PvLOX6) and pepper (CaLOX2) are also induced by H$_2$O$_2$ (Porta et al. 2008; Bhardwaj et al. 2011).

SA is a key signaling molecule required in the development of systemic acquired resistance (SAR) (Ryals et al. 1995) and it also mediates defence reactions against biotrophic pathogens (Glazebrook 2005). JA is a key component of resistance mechanisms against necrotrophic pathogens and insects (Dong 1998). Numerous researches report that JA and SA act antagonistically in defence reactions (Pena-Cortes et al. 1993; Feys and Parker 2000; Cipollini et al. 2004) because JA inhibits SA-induced expression of SAR (Traw et al. 2003). It is suppressed that the present study reported CmLOX12 was induced by both MeJA and SA (Fig. 5). However, more recent studies suggest that SA and JA had a cooperative effect (Salzman et al. 2005; Mur et al. 2006). In several plants LOX genes induced by both SA and JA are reported, such as barley, maize and common (Weichert et al. 1999; Nemchenko et al. 2006; Porta et al. 2008). Therefore, CmLOX12 might participate in both JA and SA signal pathways. It would be interesting to confirm the function of CmLOX12 in these two signal pathways in the future.

ABA plays an important role in integrating various stresses like cold and high temperature and controlling downstream stress responses (Tuteja 2007). The present work showed that CmLOX13 and CmLOX18 were down-regulated by ABA and cold while CmLOX10 and CmLOX12 were up-regulated by ABA and high temperature. However we observed that the time points at which these genes reached the lowest expression level in response to cold were earlier than in response to ABA (Figs. 6 and 7), while reached the maximum in response to high temperature latter than in response to ABA (Figs. 4 and 5). That may be the result of
the more direct effect of cold on membrane deterioration and the tolerance to high temperature of melon. Similarly, the LOX genes reached the maximum in response to cold earlier than in response to ABA in cucumber (Yang et al. 2012).

The total LOX activity of leaves was all increased by the treatments. The expression pattern of the five genes treated with wounding, high temperature, ABA and MeJA were mainly consistent with the change of activity. Most of the five genes were suppressed by cold and SA but the LOX activity was increased by these two treatments. That might be the other genes of CmLOX induced by these two treatments.

The AOS- and HPL-derived signaling compounds execute co-operative defence signaling (Halitchke et al. 2004). C6-volatiles have been suggested to be essential to act wound-related pathways, some of which are JA independent (Bate and Rothstein 1998). In maize GLVs have been shown to play a strong signaling role for the induction of JA (Engelberth et al. 2004, 2007). ZmLOX10 is specialized in generating GLVs, furthermore, it has been shown to be required for wound-induced JA biosynthesis. The impaired ability of ZmLOX10 mutants to produce GLVs and JA led to compromised resistance to insect attack, suggesting that ZmLOX10 is important in defense response of maize (Christensen et al. 2013). CmLOX10, CmLOX12 and CmLOX13 was close to ZmLOX10 and other LOXs participating GLVs generation. Moreover, CmLOX12 was induced by MeJA, ABA and wounding, which was the same as ZmLOX10 (Nemchenko et al. 2006). CmLOX10 and CmLOX13 were also regulated by these treatments. Based on the data above, CmLOX10, CmLOX12 and CmLOX13 might participate in plant defense response to stress and GLVs biosynthesis. Overexpression of tomato TomLOXD increases the resistance to high temperature and generation of endogenous JA. CmLOX08, CmLOX10, CmLOX12 and

![Fig. 5 Expressions of CmLOX12 at various time points under wounding, cold (4°C), high temperature (45°C), MeJA (100 μmol L⁻¹), ABA (100 μmol L⁻¹), SA (5 mmol L⁻¹) and H₂O₂ (10 mmol L⁻¹) were analyzed by real time qPCR using the primers specific to the five CmLOX genes.](image-url)
CmLOX18 possess HSE binding site involved in response to heat (Table 2) and were induced by high temperature. So we suggest that the expression of these genes increased the biosynthesis of endogenous JA, and then enhanced the resistance to stresses.

4. Conclusion

Here we report the effect of abiotic stresses (wounding, cold, high temperature and H$_2$O$_2$) and signal molecules (ABA, MeJA and SA) on the expressions of five LOXs (CmLOX08, CmLOX10, CmLOX12, CmLOX13 and CmLOX18) and LOX activity. The expressions of five genes and LOX activity in leaves were differently regulated by these treatments, indicating their diverse functions in stress response. CmLOX10 and CmLOX18 might participate in the biosynthesis of JA. CmLOX12 is required in several responses because it was induced by all the treatments except cold. CmLOX10, CmLOX12 and CmLOX13 might participate in GLVs biosynthesis and plant defense response to stress. Further studies through genetic engineering are needed to learn more about the function of the five genes in plant defense and signaling compounds biosynthesis.

5. Materials and methods

5.1. Plant materials and treatments

All the experiments were carried out with Yumeiren, oriental melons (Cucumis melo var. makuwa Makino), from the Yijianpu Mishijie Melon Research Institution (Changchun, China). All the seedlings were grown in pots (soil:peat:compost=1:1:1) in a greenhouse after seed germination. They were then moved into a growth chamber kept at 25°C/22°C
(day/night) with 14 h light/10 h dark photoperiod at a photosynthetic photon flux of 250 μmol m⁻² s⁻¹, and the relative humidity was 60–70%. For all of the expression studies, melon seedlings at the four-leaf stage were used.

To induce wounding, the expanded true leaves were clipped with a pair of surgical scissors. Both sides of the leaves were clipped from leaf edge to leaf central without hurting veins and the wounded leaves were collected at indicated time intervals, whereas healthy and intact leaves from other uninjured seedlings were collected as controls.

For cold and high temperature treatments, seedlings were moved into a climate chamber kept at 4°C or 45°C for 24 h with 14 h light/10 h dark photoperiod at a photosynthetic photon flux of 250 μmol m⁻² s⁻¹, and the relative humidity was 60–70%, and control plants were kept at 25°C with the same conditions described above.

MeJA (Sigma, W341002, USA), ABA (Sigma, A1049, USA) or SA were dissolved in ethanol (100 mmol L⁻¹ stock solution) and added to the hydroponics solution to 100 μmol L⁻¹ final concentration while SA added to 5 mmol L⁻¹ final concentration. For MeJA, ABA and SA treatments leaves of seedlings were sprayed (10 mL per seedling) with the configured MeJA, ABA or SA solutions. For H₂O₂ treatment leaves of seedlings were sprayed (10 mL per seedling) with 10 mmol L⁻¹ H₂O₂ water solution. Control seedlings of MeJA, ABA and SA treatments were sprayed (10 mL per seedling) with 0.1% (v/v) ethanol solution while controls of H₂O₂ were sprayed with water. Seedlings treated with MeJA and its controls were tightly sealed in plastic bags. For all the treatments and controls, the second and third true leaves (functional leaves) without veins were sampled at various time points after treatment. All the samples were frozen immediately after being collected in liquid N₂ and stored at −80°C.

Fig. 7 Expressions of CmLOX18 at various time points under wounding, cold (4°C), high temperature (45°C), MeJA (100 μmol L⁻¹), ABA (100 μmol L⁻¹), SA (5 mmol L⁻¹) and H₂O₂ (10 mmol L⁻¹) were analyzed by real time qPCR using the primers specific to the five CmLOX genes.
5.2. RNA Isolation and real time qPCR

Total RNA was isolated from leaves using RNA Kit (Kangwei Biotech, Beijing, China) according to the manufacturer’s instructions. Four micrograms of RNA were pretreated with RQ 1DNase I (Promega, USA) to remove contaminating genomic DNA. The RNA concentration was measured by the NanoDrop spectrophotometer ND-1000 (NanoDrop, USA) and quality was checked by agarose gel electrophoresis (Biowest, Spain) (28S rRNA/18S rRNA ratios).

Reverse transcription was performed with 1 μg of total RNA using M-MLV RTase cDNA Synthesis Kit following the manufacturer’s instructions (Cat#D6130, TaKaRa, Tokyo, Japan). The gene-specific primers of real-time quantitative PCR (qPCR) were the same with our previous study (Zhang et al. 2014), and 18S rRNA DNA fragment (148 bp) of melons was used as the internal standard for each analyzed gene. Primer sequences are listed in Table 3.

Real time qRT-PCR was performed in a 20-μL reaction volume using SuperReal PreMix Plus (SYBR Green) (Cat. FP205, Tiangen Biotech, Beijing, China) on an ABI PRISM 7500 Sequence-Detection System according to manufacturer’s instructions. The PCR program was at 95°C for 1 min, followed by 45 cycles at 95°C for 15 s and 60°C for 34 s. No template controls for each primer pair were included in each run. At least three different RNA isolations and cDNA synthesizes were used as replicates for the qPCR. Samples were run in triplicate on each 96-well plate. The real time PCR data were calibrated relative to the corresponding gene expression level of control seedlings at zero time for each treatment, which were set to 1, following the 2^ΔΔCt method for relative quantification.

5.3. LOX activity

LOX activity was determined as previously described (Axelrod 1981) with some modifications. Approximately 1 g of fresh leaves was ground to a fine powder in liquid nitrogen. All the powdered tissue was homogenized in 10.0 mL of ice-cold 0.1 mol L⁻¹ phosphate buffer (pH 6.8). The mixture was centrifuged at 15 000×g for 20 min at 4°C and the supernatant was collected as the enzyme source. LOX activity was determined spectrophotometrically by measuring the formation of conjugated dienes during oxidation of polyunsaturated fatty acids at 234 nm at 30°C. Linoleic acid was used as the substrate of the reaction. The reaction was started by addition of 0.2 mL of crude leaves extract to 25 μL of substrate containing 2.775 mL of 0.1 mol L⁻¹ phosphate buffer (pH 6.8) as reaction buffer. One activity unit (U) was defined as the increase in one unit of absorbance per minute and results were expressed as specific activity (mU mg⁻¹ protein). Three biological replicates were included and three readings of absorbance for each replicate were recorded.

5.4. Protein content

Protein concentration of enzyme extracts was determined by using Coomassie brilliant blue G-250 with the method described by Bradford (1976) for protein assay with modifications (BioRad Protein Assay Kit) according to manufacturer’s instructions, using bovine serum albumin (BSA) as a standard.

5.5. Phylogenetic analysis of the five CmLOXs

MEGA 4 (http://www.megasoftware.net/mega.html) was used to construct and align the five CmLOX full-length protein sequences, using the neighbor joining (NJ) with the following parameters: poisson correction, complete deletion, and none-bootstrap (Tamura et al. 2007). TreeView 1.66 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) was used to visualize the genes and BioEdit 5.06 software was used for gene analysis (Page 1996; Hall 1999). PLANTCARE program was used to carry out promoter analysis.

5.6. Statistical analysis

Experiments were performed in a completely randomized design. The data were analyzed by the analysis of variance (ANOVA) using the SPSS 13.0 statistics program, and significant differences were compared by a one-way ANOVA following Duncan’s multiple range tests for each experiment at P<0.05 level. The charts were performed by using Origin (ver. 8.0).

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