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An oriental melon 9-lipoxygenase gene *CmLOX09* response to stresses, hormones, and signal substances*#

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Abstract: In plants, lipoxygenases (LOXs) play a crucial role in biotic and abiotic stresses. In our previous study, five 13-LOX genes of oriental melon were regulated by abiotic stress but it is unclear whether the 9-LOX is involved in biotic and abiotic stresses. The promoter analysis revealed that *CmLOX09* (type of 9-LOX) has hormone elements, signal substances, and stress elements. We analyzed the expression of *CmLOX09* and its downstream genes—*CmHPL* and *CmAOS*—in the leaves of four-leaf stage seedlings of the oriental melon cultivar "Yumeiren" under wound, hormone, and signal substances. *CmLOX09*, *CmHPL*, and *CmAOS* were all induced by wounding. *CmLOX09* was induced by auxin (indole acetic acid, IAA) and gibberellins (GA_3); however, *CmHPL* and *CmAOS* showed differential responses to IAA and GA_3. *CmLOX09*, *CmHPL*, and *CmAOS* were all induced by hydrogen peroxide (H_2O_2) and methyl jasmonate (MeJA), while being inhibited by abscisic acid (ABA) and salicylic acid (SA). *CmLOX09*, *CmHPL*, and *CmAOS* were all induced by the powdery mildew pathogen *Podosphaera xanthii*. The content of 2-hexynol and 2-hexenal in leaves after MeJA treatment was significantly higher than that in the control. After infection with *P. xanthii*, the diseased leaves of the oriental melon were divided into four levels—levels 1, 2, 3, and 4. The content of jasmonic acid (JA) in the leaves of levels 1 and 3 was significantly higher than that in the level 0 leaves. In summary, the results suggested that *CmLOX09* might play a positive role in the response to MeJA through the hydroperoxide lyase (HPL) pathway to produce C6 alcohols and aldehydes, and in the response to *P. xanthii* through the allene oxide synthase (AOS) pathway to form JA.

Key words: 9-Lipoxygenase (9-LOX); Hydroperoxide lyase (HPL); Allene oxide synthase (AOS); Green leaf volatile; Jasmonic acid

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1 Introduction

Lipoxygenases (LOXs, EC 1.13.11.12) are a group of non-heme iron-containing enzymes, which are widely distributed throughout animals, plants, and fungi (Liavonchanka and Feussner, 2006; Ivanov et al., 2010; Christensen and Kolomiets, 2011). In plants, LOXs are related to many biological processes, including growth and development (Keereetaweep et al., 2015), synthesis of aroma compounds (Shen et al., 2014), synthesis of ethylene (Griffiths et al., 1999), ripening and senescence (Hou et al., 2015), and they especially play a crucial role in defense against biotic and abiotic stresses (Wang et al., 2008; Maschietto et al., 2015). LOXs can catalyze the oxygenation of polyunsaturated fatty acids (PUFAs) with a *Z*,*Z*-1,4-pentadiene structure to form fatty acid hydroperoxides (Brash, 1999). These hydroperoxides act as

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substrates for seven downstream branches of the LOX pathway to form a wide variety of active compounds, collectively called oxylipins (Feussner and Wasternack, 2002). The hydroperoxide lyase (HPL) and allene oxide synthase (AOS) pathways responsible for green leaf volatiles (GLVs) and jasmonic acid (JA) production, respectively, are the predominant and comprehensible pathways activated in the stress response (Birkett et al., 2000). Overexpression of *CsiHPL1* greatly enhances the resistance of tomatoes to fungal *Aleuria aurantia* lectin (AAL), which indicates that *CsiHPL1* is involved in plant defense against fungal attack (Xin et al., 2014). The expression of AOS is greatly induced by wounding treatment and the level of JA in *Arabidopsis* wounding plants is limited by AOS expression (Park et al., 2002).

There are many studies suggesting that LOX gene expression and LOX activity are regulated by different stresses such as wounding, low and high temperatures, hormones, signaling substances, and biotic stress (Maccarrone et al., 2000; Porta et al., 2008; Bhardwaj et al., 2011; Christensen et al., 2013; Hu et al., 2013, 2015). In tomato plants, the expression of *TomloxD* has been rapidly induced by wounding (Heitz et al., 1997). *ZmLOX10* in maize was up-regulated by wounding and cold stresses (Nemchenko et al., 2006). Drought stress could increase LOX activity dramatically in brassica seedlings (Alam et al., 2014). Salt stress could also significantly increase LOX activity in rice (Mostofa et al., 2015). In *Arabidopsis*, LOX3 was dramatically induced under salt treatment (Ding et al., 2016). The LOX activity of soybean zygotic embryos was increased significantly after indole acetic acid (IAA) treatment (Liu et al., 1991). *CjLOX* from *Caragana jubata* could be regulated by signal substances such as abscisic acid (ABA), salicylic acid (SA), and methyl jasmonate (MeJA) (Bhardwaj et al., 2011). The LOX activity of oriental melon seedlings was increased after treatment with ABA, SA, hydrogen peroxide (H_2O_2), and MeJA (Liu et al., 2016). Cucumber mosaic virus (CMV) infection led to the up-regulated expression of LOX genes in *Arabidopsis thaliana* leaves (la Camera et al., 2009). *DkLOX3*-overexpression (*DkLOX3-OX*) in *Arabidopsis* showed more tolerance to *Botrytis cinerea* (Hou et al., 2018). In particular, the 9-LOX gene *CaLOX1* was distinctively induced in pepper leaves (*Capsicum annuum* L.) that had been inoculated with *Xanthomonas campestris* pv. *vesicatoria* (Hwang and Hwang, 2010). Also, after *X. campestris* pv. *malvacearum* infection, high LOX activity supported cell death in cotton (Sayegh-Alhamdia et al., 2008).

Plant LOXs are encoded by multigene families (Andreou and Feussner, 2009) and classified as 9-LOXs and 13-LOXs, according to the position, at which oxygen is inserted into linoleic acid or linolenic acid (Feussner and Wasternack, 2002). In our previous study, we have identified 18 LOX genes in the melon genome (named *CmLOX01–18*) (Zhang et al., 2014). In addition, five 13-LOX genes were found to respond to abiotic stress and signal substances (Liu et al., 2016). However, the defense-related functions of 9-LOXs in oriental melon are still poorly understood. An oriental melon (*Cucumis melon* var. *makuwa* Makino) 9-LOX gene, *CmLOX09*, which encodes a 9-specific LOX, was isolated from melon fruits (Zhang et al., 2015). The phylogenetic analysis of *CmLOX09* showed that it is closely related to cucumber *CsLOX09*, and responds to low temperatures and 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang et al., 2012; Zhang et al., 2015). Moreover, the pepper 9-LOX gene *CaLOX1* also plays a significant role in osmosis, drought, high salinity, and microbial pathogens' stress responses (Lim et al., 2015). The promoter analysis revealed that the *CmLOX09* promoter has various elements of abiotic stress, hormones, signal substances, and biotic stress. Therefore, we analyzed the effects of wounding, hormones treatments, signal substances treatments and inoculation with a powdery mildew pathogen *Podosphaera xanthii* on oriental melon seedlings in order to elucidate the role of *CmLOX09* in various stress responses. Furthermore, the expression of two downstream genes *CmHPL* and *CmAOS* was measured. The GLVs of HPL-derived oxylipins served as signals to defend against wounding, fungal and insect attack (Christensen et al., 2013; Ameye et al., 2015). Based on the high-level expression patterns of *CmLOX09* and *CmHPL* under MeJA treatment compared with other treatments, the levels of GLVs in leaves under MeJA treatment were determined in order to investigate whether *CmLOX09* responded to MeJA through the HPL pathway to produce GLVs. Moreover, 13-LOXs have been shown to be associated with the production of JA in resistance to microbial pathogens (Hu et al.,

2013). To test whether the 9-LOX gene *CmLOX09* was involved in pathogen-induced production of JA, the levels of JA were measured after inoculation with *P. xanthii*.

2 Materials and methods

2.1 Plant materials and treatments

All the treatments were carried out with oriental melon (*Cucumis melon* var. *makuwa* Makino), cultivar "Yumeiren" (Changchun, China). Seedlings were grown in pots (compost:peat:soil=1:1:1) in a greenhouse (temperature 25 °C (day)/22 °C (night), natural light, relative humidity 60%–70%). The melon seedlings with a four-leaf stage were treated as follows.

To induce wounding, the four expanded true leaves were wounded with a pair of surgical scissors and the wounded leaves were collected, and the healthy leaves from uninjured seedlings were collected as controls. The third and fourth true leaves were sampled at 0, 0.5, 1.5, 3, 6, 12, and 24 h after treatment.

For hormone treatments, 100 μmol/L IAA and 100 μmol/L gibberellins (GA_3) were applied to oriental melon seedlings (10 ml for each plant) and the control seedlings were sprayed with 0.1% (v/v) ethanol solution (10 ml for each plant). For all the seedlings, the third and fourth true leaves without veins were harvested at 0, 1, 3, 6, 12, 24, 72, 120, and 168 h.

For signal substance treatments, seedlings were sprayed (10 ml for each seedling) with 100 μmol/L ABA, 5 mmol/L SA, 10 mmol/L H_2O_2, and 100 μmol/L MeJA, and the control seedlings of ABA, SA, and MeJA treatments were sprayed with 0.1% (v/v) ethanol solution (10 ml for each seedling), while the controls of H_2O_2 were sprayed with sterile water (10 ml for each seedling). Plants treated with MeJA were tightly sealed in a plastic bag. For all the seedlings, after 0, 1, 3, 6, 12, 24, 72, 120, and 168 h the third and fourth true leaves without veins were harvested.

P. xanthii was collected from a naturally infected plant, and then true leaves were sprinkled in order to inoculate the melon seedlings sampled at different disease levels. The disease grade of inoculation with *P. xanthii* in the leaves of oriental melon was as follow: level 0, no lesions; level 1, a mild disease, lesion area 0%–25%; level 2, the onset of mild, lesion area 25%–50%; level 3, moderate incidence, lesion area 50%–75%; level 4, severe disease, lesion area >75%.

All plant materials were frozen in liquid nitrogen immediately after collecting and stored at −80 °C until used.

2.2 Promoter analysis

Firstly, the 9-LOX gene *CmLOX09* (GenBank: MELO3C0014482) and the NCBI program were used to search for the DNA skeleton of the *CmLOX09*. Secondly, the translation start site is defined as +1; the promoter sequence was 2000 bp upstream of the translation start site. Finally, the PLANT CARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) was used to analyze the response element of the promoter sequence (Fig. S1).

2.3 RNA extraction and gene expression analysis by qRT-PCR

Total RNA was extracted from 0.5 g treated leaves under liquid nitrogen using the RNAprep Pure Plant Total RNA Extraction Kit (Kangwei, Beijing, China) according to the manufacturer's instructions. The complementary DNA (cDNA) was synthesized from 2 μg of RNA using the M-MLV RTase cDNA Synthesis Kit (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. Primer sequences are listed in Table 1. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in a 20-μl reaction volume using PrimeScript™ RT Master Mix (Perfect Real Time) (TaKaRa, Tokyo, Japan) on an ABI PRISM 7500 Sequence-Detection System according to the manufacturer's protocol. The PCR program was followed by 45 cycles of 95 °C for 30 s, 95 °C for 5 s and 60 °C for 34 s, 95 °C for 5 s, 60 °C for 1 min, and 95 °C for 30 s. A constitutively expressed 18S RNA gene was used as an internal reference. Three independent biological experiments were used as replicates for the qRT-PCR. The relative gene expression of *CmLOX09*, *CmHPL*, and *CmAOS* was calculated using the $2^{-\Delta\Delta C_T}$ method.

2.4 Green leaf volatile analysis

The GLVs of oriental melon leaves were analyzed using gas chromatography-mass spectrometry (GC-MS; Trace GC Ultra-ITQ 900, Thermo Scientific, Waltham, MA, USA). About 3 g of frozen leaves were

Table 1 Sequence of primers used for gene expression analysis by qRT-PCR

Name	Oligonucleotide	Sequence
CmLOX09	CmLOX09-F	5'-CAGATCCATCTTGTGAAC-3'
	CmLOX09-R	5'-AGTTGGTAGAGTCATTCC-3'
CmHPL	CmHPL-F	5'-AGCGTTTTACTCCTCTTCTGGC-3'
	CmHPL-R	5'-CTTCTTCCTTCACGGTTGTCCT-3'
CmAOS	CmAOS-F	5'-TTCTTCCTCGTCTTCTTCCTCTC-3'
	CmAOS-R	5'-CAAATACTCTTCCCTCCCCTGAT-3'
18S RNA	18sRNA-F	5'-AAACGGCTACCACATCCA-3'
	18sRNA-R	5'-CACCAGACTTGCCCTCCA-3'

GenBank: *CmLOX09* (MELO3C0014482); *CmHPL* (AF081955); *CmAOS* (AF081954)

ground and then transferred to a 20-ml glass vial (Thermo, USA), and 10 ml 20% (0.2 g/ml) NaCl solution was then added to the samples, along with 1-octanol (50 μl, 59.5 mg/L) as an internal standard. The mixture was completely homogenized and the glass vial was sealed with a crimp-top cap with a silicone/aluminium septa seal (20 mm, Thermo, USA). Then, the volatiles were extracted as described by Tang et al. (2015).

2.5 Extraction and determination of JA

Approximately 0.5–1.0 g fresh leaves were ground in liquid nitrogen to a fine powder, and an extraction buffer composed of isopropanol/hydrochloric acid was then added to the powder. The extract was shaken at 4 °C for 30 min. Dichloromethane was then added and shaken at 4 °C for 30 min and centrifuged at 13000 r/min for 5 min at 4 °C. The lower organic phase was extracted, then dried with nitrogen and dissolved in 200 μl methanol (0.1% methanoic acid), followed by filtering with a 0.22-μm filter membrane. Finally, the product was analyzed using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Methanol (0.1% methanoic acid) was used to prepare different concentrations of standard solution. JA was analyzed using a ZORBAX SB-C18 (Agilent Technologies, USA) column (2.1 μm×150 μm; 3.5 μm). A volume of 2 μl of sample solution was injected for HPLC analysis. Methanol/0.1% methanoic acid and ultrapure water/0.1% methanoic acid served as mobile phase A and mobile phase B, respectively. The spray voltage was 4500 V, the pressure of the aux gas, nebulizer, and air curtain were 70, 65, and 15 psi (pound per square inch; 1 psi=6895 N/m^2), respectively, and the atomizing temperature was 400 °C.

2.6 Statistical analysis

Three replicates of each treatment were carried out. The data were analyzed using the SPSS 13.0 statistics program, and significant differences were compared using the Duncan's multiple range test for each experiment at $P<0.05$ level. The charts were made using Origin 8.0.

3 Results

3.1 *Cis*-regulatory element analysis of *CmLOX09* promoter

The promoter analysis revealed that the *CmLOX09* promoter has various *cis*-regulatory elements as shown in Fig. S1, such as light responsive elements (3-AF1 binding site, Box 4, ACE, AT1-motif), fungal elicitor responsive elements (Box-W1 element), hormone elements (P-box element), signal substance elements (ABRE element, TCA element), and *cis*-acting elements involved in defense and stress responsiveness (TC-rich repeats).

3.2 Effects of wounding on expression of *CmLOX09*, *CmHPL*, and *CmAOS* in leaves of oriental melon

The expression patterns of *CmLOX09*, *CmHPL*, and *CmAOS* after wounding treatment were examined to verify whether *CmLOX09* is related to wounding treatment. After wounding treatment, the expression of *CmLOX09*, *CmHPL*, and *CmAOS* began to increase at 0.5 h, and all reached the highest level at 1.5 h. The expression of *CmLOX09* and *CmHPL* was both significantly higher than the controls at 0.5, 1.5, 3, and 6 h (Figs. 1a and 1b). The expression of *CmAOS* was significantly higher than the controls at 0.5, 1.5, and 3 h, and then returned to normal levels at

6 h (Fig. 1c). This finding suggested that *CmLOX09* might play a positive role in plants against wounding treatment through the HPL and AOS pathways.

3.3 Effects of hormone treatments on expression of *CmLOX09*, *CmHPL*, and *CmAOS* in leaves of oriental melon

Since there were *cis*-acting elements, such as hormone elements (P-box element) in the promoter of *CmLOX09*, we wanted to check whether the *CmLOX09* was regulated by hormones. The expression of *CmLOX09*, *CmHPL*, and *CmAOS* in oriental melon leaves at the four-leaf stage was analyzed in response to IAA and GA_3.

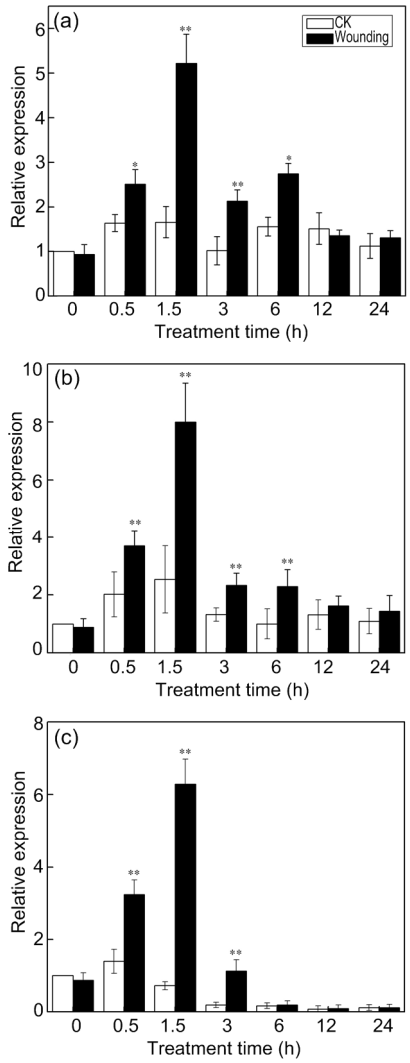

Fig. 1 Expression patterns of *CmLOX09*, *CmHPL*, and *CmAOS* after wounding treatment

(a) Expression pattern of *CmLOX09* after wounding treatment. (b) Expression pattern of *CmHPL* after wounding treatment. (c) Expression pattern of *CmAOS* after wounding treatment. Data are presented as mean±standard error from three replicates with three biological repeats. * $P<0.05$, ** $P<0.01$, compared to the respective control (CK) at each point

After IAA treatment, *CmLOX09* expression was significantly higher than the controls at 6, 72, 120, and 168 h, and reached its highest level at 168 h (Fig. 2a). Basically, the expression trends of *CmHPL* and *CmAOS* were similar; the expression was significantly higher than the controls at 6, 72, 120, and 168 h, while it was significantly lower than the controls at 1, 3, and 12 h (Figs. 2b and 2c).

On the contrary, after GA_3 treatment, the expression of *CmLOX09* was down-regulated at 1 and 3 h. However, the expression of *CmLOX09* was significantly higher than the controls from 6 to 168 h, and reached the highest level at 168 h (Fig. 2d). The expression of *CmHPL* was significantly lower than the controls at all time except at 72 h (Fig. 2e). The expression of *CmAOS* was significantly lower than the controls except at 72 and 168 h (Fig. 2f). Our results implied that *CmLOX09*, *CmHPL*, and *CmAOS* were differentially induced by hormones, which may be caused by the GA elements (P-box element) in the promoter of *CmLOX09* showing a differential response to IAA and GA_3.

3.4 Effects of signal substances on expression of *CmLOX09*, *CmHPL*, and *CmAOS* in leaves of oriental melon

ABA, SA, H_2O_2, and MeJA are important signaling molecules that regulate defense against stresses in plants (Yang et al., 2012; Liu et al., 2016). According to *cis*-acting elements, ABRE and TCA elements in the promoter of *CmLOX09*, to test whether the *CmLOX09* was regulated by the signal molecules, the expression of *CmLOX09*, *CmHPL*, and *CmAOS* in oriental melon leaves at the four-leaf stage in response to ABA, SA, H_2O_2, and MeJA was analyzed.

After ABA treatment, the expression of *CmLOX09* was down-regulated significantly at 1 and 3 h (Fig. 3a). The expression of *CmHPL* reached its maximum level at 12 h and was significantly higher than the controls at 12, 24, and 72 h while being significantly lower than the controls at 1 and 3 h (Fig. 3b). The expression of *CmAOS* was significantly higher than the control at 72 h and reached the highest level while being significantly lower than the controls at 1 and 3 h (Fig. 3c).

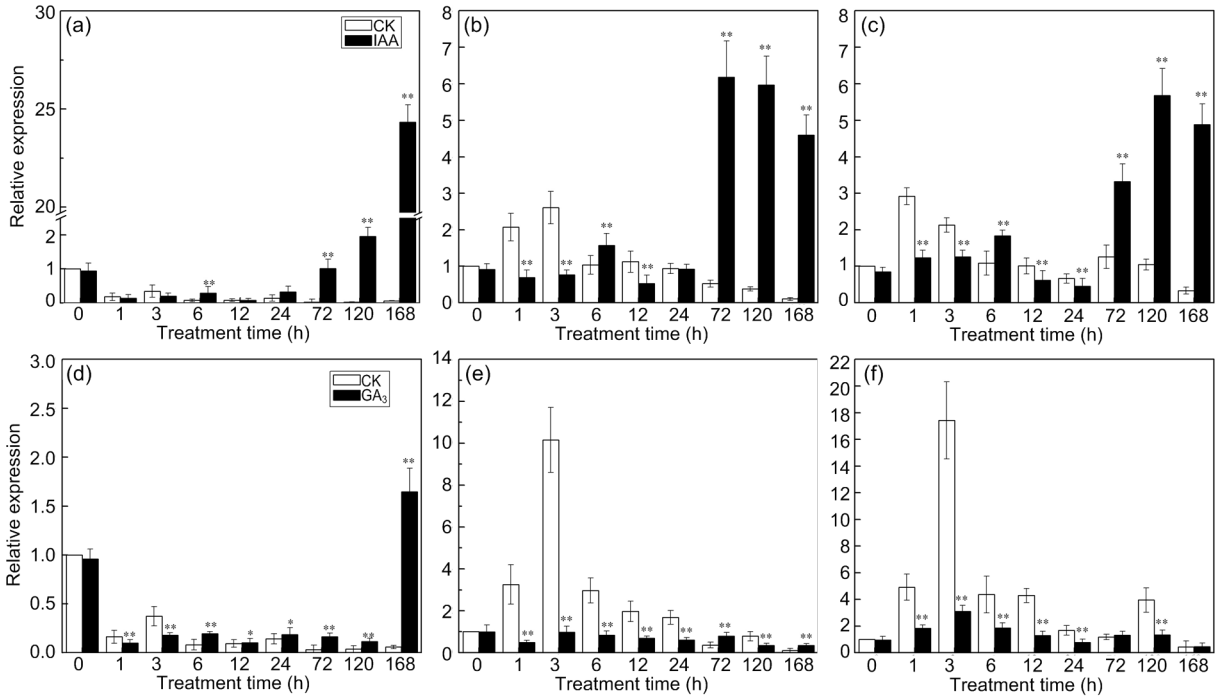

Fig. 2 Expression patterns of *CmLOX09*, *CmHPL*, and *CmAOS* after IAA (100 μmol/L) and GA_3 (100 μmol/L) treatments
(a) Expression patterns of *CmLOX09* after IAA treatment. (b) Expression patterns of *CmHPL* after IAA treatment. (c) Expression patterns of *CmAOS* after IAA treatment. (d) Expression patterns of *CmLOX09* after GA_3 treatment. (e) Expression patterns of *CmHPL* after GA_3 treatment. (f) Expression patterns of *CmAOS* after GA_3 treatment. Data are presented as mean±standard error from three replicates with three biological repeats. $^{*}P<0.05$, $^{**}P<0.01$, compared to the respective control at each point

The expression of *CmLOX09*, *CmHPL*, and *CmAOS* was down-regulated by SA at different degrees. The expression of *CmLOX09* was significantly lower than the controls from 1 to 72 h (Fig. 3d). The expression of *CmHPL* was significantly lower than the controls at the treatment time except at 6 and 168 h (Fig. 3e). The expression of *CmAOS* was significantly lower than the controls at the treatment time except at 6 and 24 h (Fig. 3f).

The expression of *CmLOX09*, *CmHPL*, and *CmAOS* was largely up-regulated by H_2O_2. Basically, the expression trends of *CmLOX09*, *CmHPL*, and *CmAOS* were similar and significantly higher than the controls at 1 and 3 h, showed its maximal level at 3 h and then declined at 6 h after treatment (Figs. 3g–3i). The expression of *CmLOX09*, *CmHPL*, and *CmAOS* was up-regulated by MeJA in different degrees. The expression of *CmLOX09*, *CmHPL*, and *CmAOS* was significantly higher than the controls at the treatment time, and reached its highest levels at 168, 24, and 3 h, respectively (Figs. 3j–3l).

In summary, the expression of *CmLOX09*, *CmHPL*, and *CmAOS* was induced by H_2O_2 and MeJA, while inhibited by SA. The expression of *CmLOX09* was down-regulated by ABA. The expression of *CmHPL* and *CmAOS* was down-regulated by ABA in the early stage while up-regulated in the late stage. Our results showed that *CmLOX09* might respond to stresses through the production of signal substances such as H_2O_2 and MeJA rather than through ABA and SA.

3.5 Effect of MeJA treatment on GLV content in leaves of oriental melon

Based on the expression patterns of *CmLOX09* and *CmHPL*, we found that *CmLOX09* and *CmHPL* exhibited a high-level expression under MeJA treatment compared with other treatments. We therefore hypothesized that *CmLOX09* might be involved in MeJA-induced GLV synthesis. To test this hypothesis, we analyzed the emission of GLVs in oriental melon leaves after MeJA treatment. The treatment time of 6, 72, and 168 h was selected to verify whether the

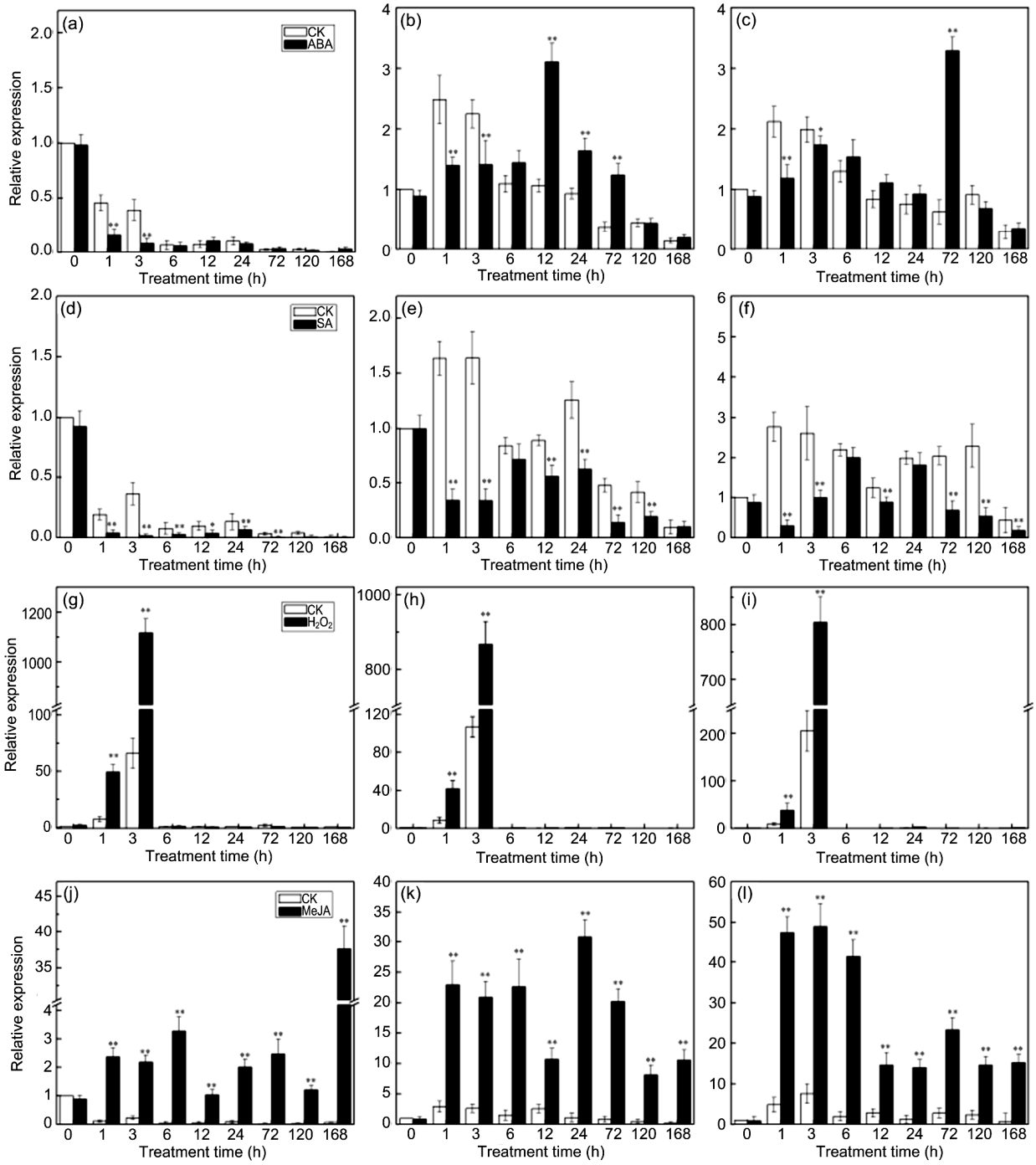

Fig. 3 Expression patterns of *CmLOX09*, *CmHPL*, and *CmAOS* after ABA (100 μmol/L), SA (5 mmol/L), H_2O_2 (10 mmol/L), and MeJA (100 μmol/L) treatments

(a) Expression patterns of *CmLOX09* after ABA treatment. (b) Expression patterns of *CmHPL* after ABA treatment. (c) Expression patterns of *CmAOS* after ABA treatment. (d) Expression patterns of *CmLOX09* after SA treatment. (e) Expression patterns of *CmHPL* after SA treatment. (f) Expression patterns of *CmAOS* after SA treatment. (g) Expression patterns of *CmLOX09* after H_2O_2 treatment. (h) Expression patterns of *CmHPL* after H_2O_2 treatment. (i) Expression patterns of *CmAOS* after H_2O_2 treatment. (j) Expression patterns of *CmLOX09* after MeJA treatment. (k) Expression patterns of *CmHPL* after MeJA treatment. (l) Expression patterns of *CmAOS* after MeJA treatment. Data are presented as mean±standard error from three replicates with three biological repeats. * $P<0.05$, ** $P<0.01$, compared to the respective control at each point

accumulation of GLVs is induced by high-level *CmHPL* expression. The results of GC-MS showed that 10 alcohols, 3 aldehydes, 3 esters, 2 ketones, 1 other and 10 alcohols, 1 aldehyde, 5 esters, 1 ketone, 4 others were measured in the control and leaves with MeJA treatment, respectively (Table S1). However, after MeJA treatment, the content of 2-hexynol and 2-hexenal was significantly higher than that of the controls at 6, 72, and 168 h, with the highest level found at 72 h (Figs. 4a and 4b). Taken together, these findings suggested that *CmLOX09* response to MeJA might occur through the HPL pathway to produce C6 alcohols and aldehydes. Hence, we speculated that high-level *CmHPL* expression induced by wounding and H_2O_2 might promote the accumulation of GLVs to respond to stress.

3.6 Effects of inoculation with *P. xanthii* on expression of *CmLOX09*, *CmHPL*, and *CmAOS* in leaves of oriental melon

Similarly, there was a fungal elicitor responsive element in the promoter of *CmLOX09*. To investigate whether *CmLOX09* was regulated by fungus, the expression of *CmLOX09*, *CmHPL*, and *CmAOS* in oriental melon leaves after inoculation with *P. xanthii* was analyzed. As shown in Fig. 5, the expression of *CmLOX09*, *CmHPL*, and *CmAOS* was significantly induced by *P. xanthii*. The expression of *CmLOX09* and *CmHPL* was strongly induced at the mild morbidity level and later tended to be stable but also significantly higher than that at level 0 (Figs. 5b and 5c). The expression of *CmAOS* was higher in the incidence levels of 1, 2, and 3 than in level 4; however, the expression of level 4 was also significantly higher than that of level 0 (Fig. 5d). These findings indicated that the 9-LOX gene *CmLOX09* was involved in biotic stress and might respond to this stress through the HPL or AOS pathway.

3.7 Effect of inoculation with *P. xanthii* on content of JA in leaves of oriental melon

Studies showed that 13-LOXs were involved in JA accumulation in defense against microbial pathogens (Hu et al., 2013). In this study, the endogenous JA, after inoculation with *P. xanthii*, was determined to verify whether the 9-LOX gene *CmLOX09* was involved in the fungus-induced production of JA. The level of JA in diseased leaves was analyzed using HPLC-MS/MS (Fig. S2). As expected, the content of

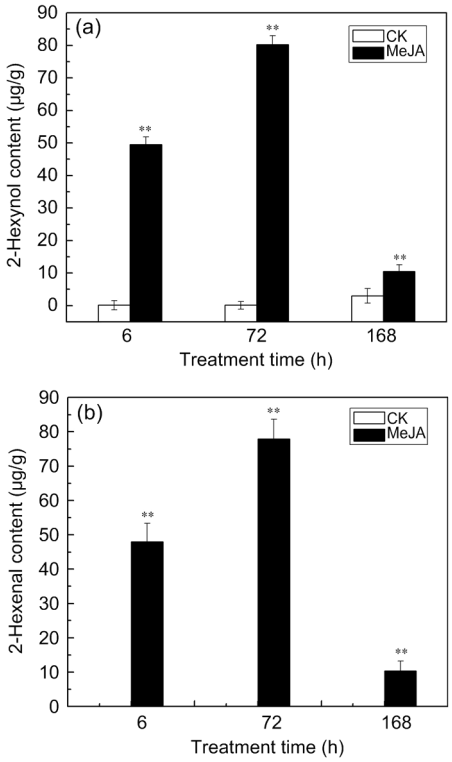

Fig. 4 Levels of 2-hexynol and 2-hexenal in oriental melon treatment and control leaves after MeJA treatment
(a) 2-Hexynol release from oriental melon leaves after treatment at 6, 72, and 168 h. (b) 2-Hexenal release from oriental melon leaves after treatment at 6, 72, and 168 h. Data are presented as mean±standard error from three replicates with three biological repeats. ** $P<0.01$, compared to the respective control at each point

JA in levels 1 and 3 was significantly higher than that in level 0, and the content of JA in level 4 was significantly lower than that in level 0. However, the content of JA in level 2 did not differ from that in level 0, and the content of JA in diseased leaves was basically consistent with the trend of *CmAOS* expression (Fig. 6). Collectively, these findings demonstrated that the *CmLOX09* response to *P. xanthii* might work through the AOS pathway to form JA. Whereas, based on the expression patterns of *CmLOX09* and *CmHPL*, the possibility that the *CmLOX09* response to *P. xanthii* occurs through the HPL pathway to produce GLVs could not be denied.

4 Discussion

The promoter analysis indicated that the *CmLOX09* promoter contained stress response element

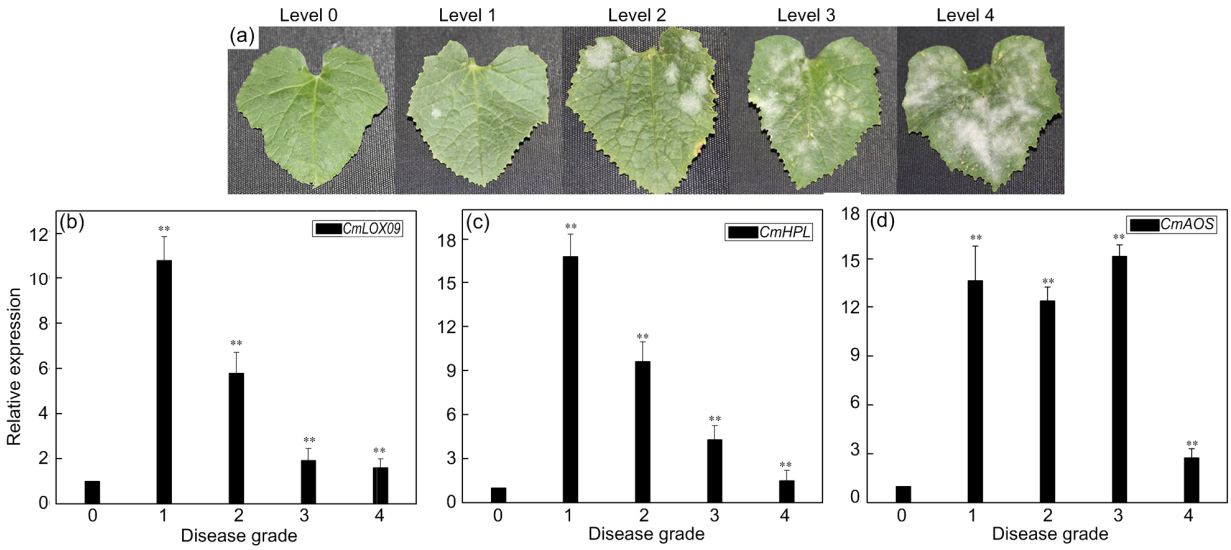

Fig. 5 Expression patterns of *CmLOX09*, *CmHPL*, and *CmAOS* after inoculation with *Podosphaera xanthii* treatments

(a) Different disease grades in oriental melon leaves after inoculated with *P. xanthii*. (b) Expression patterns of *CmLOX09* after inoculated with *P. xanthii*. (c) Expression patterns of *CmHPL* after inoculated with *P. xanthii*. (d) Expression patterns of *CmAOS* after inoculated with *P. xanthii*. Numbers indicate the different disease grades (0, no lesions; 1, a mild disease, lesion area 0%–25%; 2, the onset of mild, lesion area 25%–50%; 3, moderate incidence, lesion area 50%–75%; 4, severe disease, lesion area >75%). Data are presented as mean±standard error from three replicates with three biological repeats. ** $P<0.01$ compared to the healthy leaves (0)

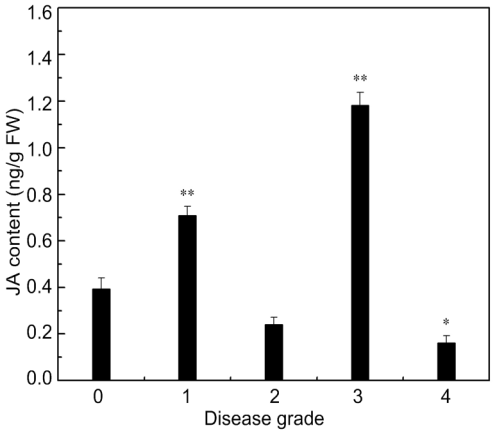

Fig. 6 Level of JA in oriental melon leaves after inoculation with *Podosphaera xanthii*

Data are presented as mean±standard error from three replicates with three biological repeats. * $P<0.05$, ** $P<0.01$, compared to the healthy leaves (0)

TC-rich repeats, and the expression pattern of *CmLOX09* on hormones, signal substances, and fungi stress was consistent with the prediction results of the promoter analysis. The *CmLOX09* promoter had a P-box element and the expression was induced by GA_3. *CmLOX09* had ABRE and TCA elements and that the expression was inhibited by ABA and SA may be due to the response elements playing a negative role in the expression of *CmLOX09*. *CmLOX09* had Box-W1 elements and its expression was induced by *P. xanthii*. Although there were no wounding or MeJA elements, the results showed that *CmLOX09* was induced by wounding and MeJA.

To date, the best characterized LOX pathways are the HPL and AOS pathways, which were involved in the stress response. The GLVs produced by the HPL pathway played a vital role in defense against herbivores, and showed a certain antibacterial activity (Kallenbach et al., 2011). *ZmLOX10* is involved in the wound-induced regulation of GLV biosynthesis (Christensen et al., 2013). LOX-H1 co-suppression decreased the release of GLVs (León et al., 2002). Similar to GLVs, the synthesis of JA, by the AOS pathway, as a signal substance was also involved in the defense response. Studies showed that the tomato *TomloxD* was involved in wound-induced JA biosynthesis (Yan et al., 2013). The LOX activity increased and the JA content accumulated dramatically in a short time in *Arabidopsis thaliana* leaves by wounding treatment (Bell et al., 1995). Antisense expression of *NaLOX3* significantly reduced wound-induced JA accumulation (Halitschke and Baldwin, 2003). Ginseng responded to wounding that may be involved in the production of GLVs and JA at the

wounded sites (Bae et al., 2016). In this study, the expression of *CmLOX09* and its downstream genes *CmHPL* and *CmAOS* was all induced by wounding, suggesting that *CmLOX09* may respond to wounding by both HPL and AOS pathways.

LOXs showed a differential response to hormones. Ethylene, IAA, GA_3, and 6-benzylaminopurine (6-BA) are well-known hormones that participate in plant biological processes. Previous studies reported that *CaLOX1* expression was significantly induced by ethylene treatment. In this study, *CmLOX09* was up-regulated in the late stage of IAA and GA_3 treatments; interestingly, *CmHPL* and *CmAOS* were up-regulated by IAA treatment and down-regulated by GA_3 treatment. The LOX activity of soybean was greatly increased by IAA treatment (Liu et al., 1991). After GA_3 treatment, LOX activity was inhibited, the expression of *DkLOX01* and *DkLOX03* was down-regulated, and then fruit ripening was delayed and fruit firmness was increased (Lv et al., 2014). Studies indicated that various LOX member responses to IAA and GA_3 were different, while the specific mechanism still remains unclear.

ABA, SA, H_2O_2, and MeJA are central signaling molecules in the defense responses to abiotic stresses (Neill et al., 2002; Durrant and Dong, 2004; Zhang et al., 2006; Guo and Stotz, 2007). Studies showed that after ABA, SA, H_2O_2, and MeJA treatments, *CmLOX09* responded rapidly, and the two downstream genes *CmHPL* and *CmAOS* actively participated in the stress. The expression of *CmLOX09* was inhibited by ABA, but the expression of *CmHPL* and *CmAOS* was induced by ABA. Previous studies showed that *CmLOX10* and *CmLOX12* were up-regulated by ABA; meanwhile, *CmLOX13* and *CmLOX18* were down-regulated by ABA (Liu et al., 2016). *ZmLOX6* declined dramatically starting at 1 h and reached its lowest level at 6 h after treatment with ABA (Gao et al., 2008). In this study, the expression of *CmLOX09*, *CmHPL*, and *CmAOS* was down-regulated by SA. The expression of *CmLOX09* was significantly inhibited from 1 to 72 h. Our previous study showed that only *CmLOX12* was induced by SA among the five 13-LOXs; others were inhibited by SA (Liu et al., 2016). However, the 9-LOX gene *CaLOX1* was induced by SA treatment (Hwang and Hwang, 2010). The specific reasons for the above differences are not clear; it may be caused by the difference between species and genes. The expression of *CmLOX09*, *CmHPL*, and *CmAOS* was strongly induced by H_2O_2 at 3 h and declined after this time. H_2O_2 was added to the medium and the LOX gene expression and activity of lentil in the roots were increased to some extent (Maccarrone et al., 2000). The expression of *CmLOX09*, *CmHPL*, and *CmAOS* was induced by MeJA. The expression of *CsLOX2* was rapidly up-regulated with MeJA and reached its maximal level up to 100-fold at 3 h after treatment (Yang et al., 2012). *CjLOX* was strongly induced after spraying with MeJA (Bhardwaj et al., 2011).

Furthermore, based on the expression patterns of the three genes after signal substance treatment, we also investigated the emission of GLVs at 6, 72, and 168 h after MeJA treatment. The 2-hexynol and 2-hexenal content was significantly higher than that of the controls in oriental melon leaves in response to MeJA treatment; these data suggested that *CmLOX09* may contribute to the production of C6 volatiles. After wounding treatment, the levels of *Z*-3-hexenal and *Z*-3-hexan-1-ol in the leaves of two LOX lines and wild type (WT) plants were significantly higher than those in the untreated leaves; however, the emission of *Z*-3-hexenal and *Z*-3-hexan-1-ol from transgenic plants did not result from levels released by the WT plant—this finding suggested that *OsHI-LOX* does not supply substrates for the production of GLVs in rice (Zhou et al., 2009). The LOX10-2 and LOX10-3 mutants provide substrates for the HPL pathway for GLV production in maize leaves in response to wounding or herbivory by *Salix exigua* (Christensen et al., 2013).

The 9-LOX gene *NaLOX1* was induced in tobacco leaves infected with *Phytophtora parasitica* var. *nicianae* (Rancé et al., 1998). The 9-LOX gene *GhLOX1* is associated with the hypersensitive reaction of cotton cell death responses to *X. campestris* pv. *malvacearum* (Marmey et al., 2007). The *CaLOX1* of pepper was distinctively induced by *X. campestris* pv. *vesicatoria*, the number of cell death reactions decreased, and it was more susceptible to infection by pathogens in *CaLOX1* silence pepper plant, which indicated that *CaLOX1* was involved in the defense response of pepper to pathogenic microorganisms and cell death response (Hwang and Hwang, 2010). In the present study, after inoculation with *P. xanthii*, the expression of *CmLOX09*, *CmHPL*, and *CmAOS* at different disease levels was significantly higher than

that at level 0, which indicated that *CmLOX09* was involved in the response to fungus.

Overexpression of *TomloxD* in transgenic tomatoes increased the content of endogenous JA and enhanced resistance to *Cladosporium fulvum* compared with non-transformed tomato plants, which suggested that *TomloxD* was involved in the synthesis of JA (Hu et al., 2013). Tomato plants with suppressed expression of the *TomloxD* gene have diminished LOX activity and the content of endogenous JA, which finally lead to increase in their susceptibility to microbial pathogens (Hu et al., 2015). Antisense expression of *AtLOX2* decreased wound-induced JA accumulation (Bell et al., 1995). CMV inoculation increased the expression of two LOXs genes that participate in JA biosynthesis (la Camera et al., 2009). In *Arabidopsis*, LOX3 and LOX4 contribute to JA synthesis (Chauvin et al., 2016). In this study, the expression of *CmLOX09* was strongly induced by *P. xanthii*, JA analysis showed that levels 1 and 3 contain higher endogenous JA compared with the level 0, the trend of JA was similar to the expression pattern of *CmAOS*, and the results indicated that the disease leaves might response to fungal stress by the 9-LOX-AOS pathway; however, the role of *CmLOX09* in responses to biotic and abiotic stress needed to be verified using gene editing or overexpressed technique. Since lipid peroxidation closely related to LOX, we will measure malondialdehyde (MDA) or thiobarbituric acid reactive substances (TBARS) in next work.

5 Conclusions

Here we reported the expression of *CmLOX09*, *CmHPL*, and *CmAOS* in the leaves of melon seedlings that were regulated by wounding, hormones (IAA, GA_3), signal substances (ABA, SA, H_2O_2, MeJA), and biotic stresses (*P. xanthii*), which indicated that *CmLOX09*, *CmHPL*, and *CmAOS* were all induced by mechanical wounding and differentially induced by hormones and signal substances. In addition, we analyzed the content of GLVs after MeJA treatment and the content of JA after inoculation with *P. xanthii*. Taken together, these results implied that the *CmLOX09* response to MeJA might occur through the HPL pathway to produce C6 alcohols and aldehydes; and the *CmLOX09* response to *P. xanthii* might occur through the AOS pathway to form JA. Currently, the molecular mechanism of the *CmLOX09*-mediated pathway still remains unclear in plant responses. Moreover, the relationship between *CmLOX09* and signal molecules is needed to be clarified. In further studies we will use transgenic plant to better understand the role of *CmLOX09* in responses to biotic and abiotic stresses.

Compliance with ethics guidelines

Li-jun JU, Chong ZHANG, Jing-jing LIAO, Yue-peng LI, and Hong-yan QI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Fig. S1 *Cis*-regulatory elements analysis of *CmLOX09* promoter

Fig. S2 Level of JA in oriental melon leaves after inoculation with *Podosphaera xanthii* analyzed with HPLC-MS/MS

Table S1 Emission of GLVs in control and MeJA treatment leaves at 6, 72, and 168 h

中文概要

题　目：薄皮甜瓜 9-脂氧合酶（9-LOX）类型的 *CmLOX09* 对逆境、激素和信号类物质的响应

目　的：研究 *CmLOX09* 及其下游基因 *CmHPL* 和 *CmAOS* 对逆境、激素和信号类物质的响应，进一步测定茉莉酸甲酯（MeJA）处理后叶片中绿叶挥发物的含量以及接种白粉病菌后叶片中茉莉酸含量的变化，探讨脂氧合酶（LOX）响应这两种胁迫的可能途径。

创新点：通过对 *CmLOX09* 启动子中顺式作用原件的分析预测，首次研究薄皮甜瓜 9-LOX 类型的 *CmLOX09* 对机械损伤、激素、信号类物质以及生物胁迫的响应。

方　法：利用 Plant CARE 软件对 *CmLOX09* 启动子响应元件进行预测分析（图 S1）；利用荧光定量聚合酶链反应（qRT-PCR）技术分析甜瓜在机械损伤、激素、信号类物质以及生物胁迫处理后叶片中 *CmLOX09*、*CmHPL* 和 *CmAOS* 的表达模式；利用气相色谱-质谱连用仪（GC-MS）测定叶片中绿叶挥发物的含量（图 4）；利用高效液相色谱-串联质谱法（HPLC-MS/MS）分析和测定叶片中茉莉酸的含量（图 6）。

结　论：本研究结果显示：*CmLOX09* 参与机械损伤、激素、信号类物质及白粉病菌的防御反应（图 1~3，5）。9-LOX 类型的 *CmLOX09* 可能通过氢过氧化物裂解酶（HPL）途径产生的绿叶挥发物（GLV）来响应 MeJA（图 4），并通过丙二烯合酶（AOS）途径产生的茉莉酸来响应真菌胁迫（图 6）。综上所述，9-LOX 类型的 *CmLOX09* 可能在生物和非生物胁迫反应中起重要作用。

关键词：9-脂氧合酶；氢过氧化物裂解酶；丙二烯合酶；绿叶挥发物；茉莉酸