Isolation and functional validation of the CmLOX08 promoter associated with signalling molecule and abiotic stress responses in oriental melon, Cucumis melo var. makuwa Makino

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Abstract

**Background:** Lipoxygenases (LOXs) play significant roles in abiotic stress responses, and identification of LOX gene promoter function can make an important contribution to elucidating resistance mechanisms. Here, we cloned the CmLOX08 promoter of melon (Cucumis melo) and identified the main promoter regions regulating transcription in response to signalling molecules and abiotic stresses.

**Results:** The 2054-bp promoter region of CmLOX08 from melon leaves was cloned, and bioinformatic analysis revealed that it harbours numerous cis-regulatory elements associated with signalling molecules and abiotic stress. Five 5′-deletion fragments obtained from the CmLOX08 promoter—2054 (LP1), 1639 (LP2), 1284 (LP3), 1047 (LP4), and 418 bp (LP5)—were fused with a GUS reporter gene and used for tobacco transient assays. Deletion analysis revealed that in response to abscisic acid, salicylic acid, and hydrogen peroxide, the GUS activity of LP1 was significantly higher than that of the mock-treated control and LP2, indicating that the −2054- to −1639-bp region positively regulates expression induced by these signalling molecules. However, no deletion fragment GUS activity was induced by methyl jasmonate. In response to salt, drought, and wounding treatments, LP1, LP2, and LP4 promoted significantly higher GUS expression compared with the control. Among all deletion fragments, LP4 showed the highest GUS expression, indicating that −1047 to −1 bp is the major region regulating promoter activity and that the −1047 to −418-bp region positively regulates expression induced by salt, drought, and wounding, whereas the −1284 to −1047-bp region is a negative regulatory segment. Interestingly, although the GUS activity of LP1 and LP2 was not affected by temperature changes, that of LP3 was significantly induced by heat, indicating that the −1284- to −1-bp region is a core sequence responding to heat and the −2054- to −1284-bp region negatively regulates expression induced by heat. Similarly, the −1047- to −1-bp region is the main sequence responding to cold, whereas the −2054- to −1047-bp region negatively regulates expression induced by cold.

**Conclusions:** We cloned the CmLOX08 promoter and demonstrated that it is a signalling molecule/stress-inducible promoter. Furthermore, we identified core and positive/negative regulatory regions responding to three signalling molecules and five abiotic stresses.

**Keywords:** Oriental melon, Lipoxygenase, Promoter, Signalling molecule, Abiotic stress
Background

Lipoxygenases (LOXs: EC 1.13.11.12) are a class of non-haem iron-containing dioxygenases. Plant LOXs have been classified as 9-LOX and 13-LOX, according to their different oxygen positions, and are involved in enzymatic reactions associated with the oxidation of polyunsaturated fatty acids (PUFAs) during transmutation to unsaturated fatty acid hydroperoxides [1]. Plant LOXs, which are encoded by multigene families, have been shown to play roles in the response to abiotic stresses [2–4]. Overexpression of TomLOxD in transgenic tomatoes clearly indicated that this gene is involved in endogenous jasmonic acid (JA) synthesis, and in turn regulates the expression of plant defence genes and resistance to high temperature [5, 6]. Similarly, pepper CalLOX1 has been shown to increase resistance to osmotic, drought, and high salinity stress [7]. Furthermore, the persimmon DkLOX3 gene has been found to play positive roles in enhancing tolerance to salt and drought, and the stress-responsive expression of this gene in the DkLOX3-OX line of Arabidopsis was shown to be higher than that in the wild type [8]. Overexpression and silencing of the japonica rice OsLOX1 gene has indicated that this gene is involved in resistance to wounding associated with JA biosynthesis [9]. LOX gene expression has been shown to be regulated in response to different abiotic stresses such as heat, cold, and wounding, or different signalling molecules such as methyl jasmonate (MeJA), abscisic acid (ABA), hydrogen peroxide (H₂O₂), and salicylic acid (SA) [10–13].

The promoter is a specific sequence of DNA upstream of the protein-coding region of a gene that contains numerous cis-regulatory elements and initiates transcription [14]. To date, a number of signalling molecule/stress-inducible elements have been identified in promoter regions, examples of which include a salicylic acid-responsive element (TCA) identified in tobacco [15], ABRE, a cis-acting element involved in ABA responsiveness, in wheat and rice [16, 17], and ethylene-responsive elements (EREs), containing an 11-bp sequence (TAAGAGCCGCC), that act as transcriptional activators or repressors of gene expression under ethylene treatment in tobacco and Arabidopsis [18, 19]. Heat-shock promoters have been appraised during high-temperature stress experiments in transgenic soybean and Arabidopsis [20, 21]. Furthermore, many core functional promoter regions have also been identified. The −148-bp region of the grape C4C4-type RING-finger gene promoter has been demonstrated to be the core functional promoter region and plays a key role in response to heat stress [22], whereas the CsslSip promoter from Citrus sinensis was found to be induced in response to wounding of the phloem tissue of transgenic tobacco plants [23]. Deletion analysis of the maize type-II H⁺-pyrophosphatase gene promoter in transgenic tobacco plants has revealed that a 71-bp segment (−219 to −148 bp) is the key region regulating the ZmGAPP response to NaCl or PEG stress [24]. Furthermore, the expression levels of GUS in transgenic tobacco indicated that a 348-bp fragment of the ShGSTU promoter could be used for both constitutive and stress-inducible expression of genes [25]. Similarly, GUS transient assays in tobacco leaves have indicated that a 113-bp segment (−467 to −355 bp) from the maize phosphatidylinositol synthase gene (ZmPIS) is sufficient for the NaCl or PEG stress response and is considered to be the key sequence for the ZmPIS response to NaCl or PEG treatment [26]. In addition, stress-inducible gene expression requires the interaction between transcription factors and cis-acting elements in the promoter, thereby highlighting the need for functional validation of gene promoter activity in response to signalling molecules and abiotic stresses.

The oriental melon (Cucumis melo var. makuwa Makino), which has shallow roots and large thin leaves, is an important agricultural commodity and widely grown in China and other eastern Asian countries. Abiotic stresses, such as low temperature, drought, high salinity, and mechanical damage, are all unfavourable to the growth and development of melon. With the release of the entire genome sequence of melon, a new resource for the functional analysis of LOX genes in this plant has emerged [27]. To date, 18 candidate LOX genes have been identified in the melon genome, which have been grouped into three categories (type I 9-LOX, type I 13-LOX, and type II 13-LOX) based on phylogenetic analysis [28]. Previous studies have shown that the expression levels of melon CmLOX8, CmLOX10, CmLOX12, CmLOX13, and CmLOX18 genes differ in response to different signal molecule and abiotic stress treatments, indicating that these five genes may play diverse functional roles in melon [29].

Phylogenetic analysis has indicated that CmLOX08 (MELO3C011885) is a member of the type II 13-LOX genes, and is known to play an important role in biotic and abiotic stress responses [4, 28, 30, 31]. It has previously been shown that CmLOX08 is induced in response to various stresses, including wounding, heat, and cold, or signalling molecules such as H₂O₂ [29]. To date, however, there have been no reports describing the mechanisms whereby the expression of CmLOX08 is regulated in response to abiotic stress. In this study, we cloned the CmLOX08 promoter from young leaves of the ‘Yumeiren’ cultivar of oriental melon based on sequences in melon genome databases and sought to identify putative cis-regulatory elements that respond to signalling molecules and abiotic stresses. To identify regions of the CmLOX08 promoter that play a role in regulating transcription, we constructed five promoter 5′-deletion vectors using pBI121 and examined their responses in tobacco leaves subjected to a range of different signalling molecules and abiotic stresses using Agrobacterium-mediated transient assays.
The findings of these analyses will contribute to gaining a better understanding of the molecular mechanisms underlying the response of \textit{CmLOX08} to abiotic stress in oriental melon.

**Results**

**Isolation and sequence analysis of the \textit{CmLOX08} promoter**

On the basis of the publicly available sequence in the melon genome database (http://melonomics.net), we obtained the 2054-bp 5' flanking sequence of \textit{CmLOX08} upstream of the ATG start codon from melon genomic DNA. Two alignments of the promoter sequence of \textit{CmLOX08}-pro were performed based on the sequence obtained and that from the melon (\textit{Cucumis melo} L.) genome database (\textit{GeLOX08}-pro) using DNAMAN software. The results showed that the nucleotide sequences of \textit{CmLOX08}-pro and \textit{GeLOX08}-pro shared 99.61% identity. However, compared with \textit{GeLOX08}-pro, \textit{CmLOX08}-pro was found to have a larger number of nucleotides containing a single adenine and fewer nucleotides containing three thymines (Additional file 1).

**Analysis of cis-regulatory elements in the \textit{CmLOX08} promoter**

The \textit{CmLOX08} promoter sequence was characterized using the online software PlantCARE and PLACE. Thirty types of potential cis-acting elements were detected within the 2054-bp region of the \textit{CmLOX08} promoter (Fig. 1 and Table 1).

Multiple core cis-acting elements, including 11 TATA and 11 CAAT boxes, were identified at numerous positions. Furthermore, a series of putative cis-regulatory elements that facilitate the inductive expression of \textit{CmLOX08} were detected, including eight types of light-responsive elements (GAG-motif, MRE, TBOXATGAPB, Box I, IBOXCORE, REALPHALGHC21, TCCC-motif, and GT1-motif), nine types of hormone-responsive elements (WRKY71OS, WBOXATNPRI, TCA-element, TGA-element, PYRIMIDINE, TATCCAOASAM, GARE-motif, ABRE, and CGTC A-motif), two cis-acting elements involved in heat stress responsiveness (HSE and CCAATBOX1), a cis-acting element involved in low-temperature-induced expression (LTR), an element involved in pathogen- and salt-induced SCAm-4 gene expression (GT1GMSCAM4), a MYB binding site involved in drought inducibility (MBS), an ATMYB2 binding site and an element related to dehydration-responsive genes (MYB2AT and ACGTATD1, respectively), an enhancer-like element involved in anaerobic inducibility (ARE), a fungal-inducible element (Box-W1), a binding site of OsBIHD1 involved in disease resistance responses, and three TC-rich repeats involved in defence and stress responsiveness.

**Activities of the \textit{CmLOX08} promoter in response to signalling molecule treatments**

To determine the role of putative cis-regulatory elements in the response of the \textit{CmLOX08} promoter to signalling molecules, tobacco leaves infiltrated with \textit{Agrobacterium} harbouring \textit{CmLOX08} promoter fragments of five different deletion lengths were treated with SA, ABA, MeJA, and H$_2$O$_2$, followed by GUS histochemical staining and fluorometric assay analyses (Fig. 2).

In response to treatment with SA, each of the five deletion structure was intensely stained and the GUS activity of the LP1–LP5 promoter fragments was significantly increased by 9.18-, 9.48-, 18.65-, 10.99-, and 6.66-fold, respectively, compared with the mock-treated control after treatment with SA (Fig. 2A, B, a). These results indicate that at least one SA-responsive element is located in the promoter region from $\sim$418 to $\sim$1, although additional cis-elements could be present in regions further upstream. After treatment with ABA, the LP1, LP3, and LP4 promoter constructs were strongly stained compared with the control, whereas the LP2 and LP5 constructs remained unstained (Fig. 2A). We observed that GUS activity increased significantly for the LP1, LP3, and LP4 fragments, whereas no significant change was detected for LP2 and LP5 (Fig. 2B, b). These results indicate that the promoter regions from $\sim$2054 to $\sim$1639 and $\sim$1284 to $\sim$418 play a positive role in the response to ABA and may contain the cis-element that responds positively to ABA treatment. Furthermore, we found that the promoter region from $\sim$1639 to $\sim$1284 may contain repressor elements that respond negatively to ABA treatment. In response to treatment with MeJA, we were unable to detect any significant staining or GUS activity in any of the \textit{CmLOX08} promoter deletion fragments in comparison with the control treatment (Fig. 2A, B, c). For H$_2$O$_2$ treatment, the changes in GUS activity were consistent with those observed following SA treatment (Fig. 2B, d). Accordingly, these findings indicate that, whereas the \textit{CmLOX08} promoter responds positively to ABA, SA, and H$_2$O$_2$, it shows no detectable response to MeJA.

**Analysis of abiotic stress-induced activity of the \textit{CmLOX08} promoter**

To examine the activity of the \textit{CmLOX08} promoter in response to environmental stress and to identify the corresponding cis-regulatory regions, tobacco plants infiltrated with \textit{Agrobacterium} harbouring \textit{CmLOX08} promoter fragments of five different deletion lengths were subjected to salt, drought, wounding, heat, and cold stresses.

For NaCl andPEG treatments, the promoter activities of LP1–LP5 were examined in leaves by incubating the detached leaves in half-strength liquid MS medium supplemented with 200 mM NaCl (salt stress treatment) or 18% PEG 6000 (drought stress treatment). pBI121 (35S)
Fig. 1 (See legend on next page.)
Fig. 1 Nucleotide sequence of the CmLOX08 promoter. The “A” of the translation initiation code “ATG” of CmLOX08 was designated as “+ 1”. Putative cis-acting elements are underlined, shadowed, colored and labeled. The horizontal arrows show their directions. See Table 1 for descriptions of the elements. The vertical arrow above the sequence indicates the start point of different deletion fragments; the blue nucleotide sequences represent special primers for amplifying deletion fragments (LP1–LP5).

Table 1 Identification of cis-acting elements in the CmLOX08 promoter using the PlantCARE and PLACE databases

| Cis-elements | Description                                                                 | Position from ATG                   | No. |
|--------------|------------------------------------------------------------------------------|-----------------------------------|-----|
| HSE          | cis-acting element involved in heat stress responsiveness                   | –1990, –1332, –1015, –709, –454    | 5   |
| GAG-motif    | part of a light responsive element                                          | –1883                             | 1   |
| Box-W1       | fungal elicitor responsive element                                         | –1859                             | 1   |
| WRKY71OS     | Core of W-box, transcriptional repressor of GA signal                       | –1863, –1182, –1063, –1023, –784, –730, –173 | 7   |
| WBOXATNPRI1  | Regulates NPR1, SA-induced                                                  | –1859, –1183, –1064               | 3   |
| ARE          | cis-acting regulatory element essential for the anaerobic induction         | –1851                             | 1   |
| TCA-element  | cis-acting element involved in salicylic acid responsiveness               | –1796, 1564, –1205, –510, –474, –82 | 6   |
| MRE          | MYB binding site involved in light responsiveness                          | –1557                             | 1   |
| TC-rich repeats | cis-acting element involved in defense and stress responsiveness          | –1548, 1384, –1262                | 3   |
| CCAATBOX1    | Act cooperatively with HSE to increase the activity of the promoter        | –1515, –1027, –1005, –228          | 4   |
| TBOXATGAPB   | Involved in light-activated transcription                                   | –1462                             | 1   |
| Box I        | light responsive element                                                    | –1367, –443                       | 2   |
| TGA-element  | auxin-responsive element                                                    | –1344, –132                       | 2   |
| CAAT-box     | common cis-acting element in promoter and enhancer regions                 | –1669, 1477, 1273, –1219, 993, –934, –778, 459, 379, 248, 150 | 11  |
| BIHD1OS      | Binding site of OsBIHD1 in disease resistance responses                     | –1178, –780, –169                 | 3   |
| PYRMDINEB    | involved in sugar repression and the regulation of gibberellin-responsive genes | –1126                           | 1   |
| MBS          | MYB binding site involved in drought-inducibility                          | –1098                             | 1   |
| MYB2AT       | Binding site for ATMYB2 that involved in the regulation of dehydration-responsive genes | –1098                         | 1   |
| IBOXCORE     | Light-responsive element                                                    | –1056, –890                       | 2   |
| TATCAOSAMY   | Gibberellin response element in sugar sensitivity of alpha-amyrase genes   | –1051                             | 1   |
| REALPHALGLHC21 | Required for phytochrome regulation                                      | –1007                             | 1   |
| TCCCC-motif  | part of a light responsive element                                         | –847                              | 1   |
| GT1GMSCAM4   | Plays a role in pathogen- and salt-induced SCaM-4 gene expression           | –822, –269                        | 2   |
| GARE-motif   | gibberellin-responsive element                                              | –400                              | 1   |
| TATA-box     | core promoter element around – 30 of transcription start                   | –1980, 1756, 1391, 1288, 1074, –805, –674, 642, 631, 580, –286 | 11  |
| GT1-motif    | light responsive element                                                    | –279                              | 1   |
| ABRE         | cis-acting element involved in the abscisic acid responsiveness             | –200                              | 1   |
| ACGTATERD1   | required for etiolation-induced expression of erd1 (early responsive to dehydration) | –201                           | 1   |
| LTR          | cis-acting element involved in low-temperature responsiveness              | –181                              | 1   |
| CGTCA-motif  | cis-acting regulatory element involved in the MeJA-responsiveness           | –136                              | 1   |
(positive control) and p121GUS (negative control) plants were also treated in parallel. In response to osmotic stress (NaCl or PEG) treatments, there was a significant increase in the inducible GUS activity of leaves harbouring the LP1, LP2, and LP4 deletion fragments, whereas no significant changes were detected for the LP3 and LP5 fragments when compared with the untreated controls (Fig. 3B, a and b). Furthermore, we observed that induced GUS activity was highest in the LP4 fragment (the promoter region from $-1047$ to $-1$), indicating that this region contains elements of a salt- or drought-inducible nature and may promote high levels of gene expression. In response to wounding treatment, the GUS activity induced by LP1, LP2, and LP4 fragments increased by $7.74$, $6.69$, and $12.77$-fold, respectively, compared with the control, whereas that induced by LP3
and LP5 remained stable (Fig. 3B, c). These results indicate that there are no wounding-responsive-elements present in the $-418$ to $-1$ region of the CmLOX08 promoter, whereas in contrast, the region from $-1047$ to $-418$ contains major cis elements that respond to wounding treatment.

When tobacco leaves were exposed to high temperature treatment, the highest level of GUS activity was detected in the LP3 deletion structure, which increased significantly (4.46-fold) compared with that of the control treatment (Fig. 4B, a). However, no significant changes in GUS activity were detected for any of the other deletion fragments, thereby indicating that the key cis elements that respond to heat reside in the $-1284$ to $-1047$ region of the promoter, and that repressor elements may exist in the $-2054$- to $-1284$-bp region of the CmLOX08 promoter.

The results of GUS activity assays under cold treatment were essentially similar to those obtained for heat treatment. Furthermore, the GUS activity of the LP4 deletion structure was 3.79-fold higher than that of the mock-treated control, whereas the other deletion structures showed no significant change compared with the control (Fig. 4B, b). Accordingly, these results indicate that the promoter region from $-1047$ to $-418$ harbours a cold-inducible cis-element and that repressor elements may be present in the $-2054$- to $-1047$-bp fragment of the CmLOX08 promoter.

In addition to the aforementioned treatments, we also examined GUS activity in positive control tobacco leaves infected with Agrobacterium harbouring p121GUS:35S, which showed strong GUS activity (Figs. 2, 3 and 4), whereas negative control tobacco leaves infected with
*Agrobacterium* harbouring p121GUS showed no GUS activity (Additional file 2).

**Discussion**

In plants, LOX enzymes are involved in different forms of stress response, including that induced by wounding or different signalling molecules such as MeJA and SA, which are well-known modulators of defence responses [32, 33]. To investigate how *CmLOX08* gene expression could be regulated when oriental melon is subjected to different abiotic stresses, we initially cloned the 2054-bp promoter of *CmLOX08* and identified therein several cis-regulatory elements that are predicted to respond to signalling molecules and environmental stresses, based on reference to the PlantCARE and PLACE databases (Fig. 1 and Table 1). Subsequently, we performed deletion analysis of the *CmLOX08* promoter, with the aim of determining the major promoter regions that mediate the responses to signalling molecules and abiotic stresses using an *Agrobacterium*-mediated transient assay in tobacco leaves.

Our data showed that the GUS activity of all the examined *CmLOX08* promoter deletion structures was significantly induced after SA treatment (Fig. 2B, a). Furthermore, we found that, at numerous positions, the *CmLOX08* promoter contains a TCA-element, which is a cis-acting element involved in SA responsiveness [15]. Thus, the TCA motif may play a role in regulating the expression of *CmLOX08* that is similar to that observed in *GPP* from *Actinidia deliciosa* when SA acts as a signal molecule [34]. We observed that the expression level of *CmLOX08* was significantly reduced after SA treatment [29], which may due to associated transcription factors that play a negative regulatory role with respect to *CmLOX08* transcription in response to SA treatment [35, 36].

In plants, ABA is a broad-spectrum phytohormone involved in integrating various stress signal transduction...
pathways during the response to abiotic stresses [37]. The signalling pathways involved in the response to abiotic stress are mainly divided into ABA-dependent and ABA-independent signalling pathways [38–40]. These pathways can be regulated by ABREs (abscisic acid-responsive elements), DRE/CRTs (dehydration-responsive element/C repeats), or MYB and MYC recognition motifs [41, 42]. Although we detected the presence of ABRE, MYB2AT, and multiple MYB-like (T/AGTTA/T) elements spread across the entire region of the CmLOX08 promoter, we were unable to locate any DRE/CRTs elements (Fig. 1).

Furthermore, we also found that in response to ABA treatment, the GUS expression induced by the promoter deletion fragments LP1, LP3, and LP4 was significantly higher than that in the mock-treated control (Fig. 2B, b). On the basis of these observations, we can infer that the ABRE- and MYB-binding sites in the promoter of CmLOX08 may play a significant role in the response to exogenous ABA. Surprisingly, ABA treatment had no effect on the gene expression of CmLOX08 [29], and hence, further studies are needed to determine whether CmLOX08 responds to abiotic stress via an ABA-independent pathway.

Previous studies have shown that ABA, SA, MeJA, and H2O2 interact with each other under stress conditions [12]. For example, in tomato, ABA and MeJA were shown to synergistically promote expression of the PIN2 gene [43], whereas in the present study, unlike the response to ABA, GUS activity of the CmLOX08 promoter was not induced by MeJA (Fig. 2B, c), indicating that there may be no interaction between ABA and MeJA in the regulation of CmLOX08 gene expression. Interestingly, the expression of CmLOX08 was significantly down-regulated after 3 h under MeJA treatment [29]. This response may be attributable to other factors that affect the expression of CmLOX08 independently of MeJA treatment.

In contrast, we found that treatment with H2O2 resulted in a significant increase in GUS activity of the CmLOX08 promoter (Fig. 2B, d), which is consistent with the up-regulation of CmLOX08 [29]. In this regard, it is worth noting that the CmLOX08 promoter has no H2O2-inducible motifs (Fig. 1). These results indicate that certain cis-acting regulatory elements involved in H2O2 responsiveness may be present in the CmLOX08 promoter. However, the sequences of H2O2-inducible motifs need to be further confirmed.

In our previous studies, we found that NaCl and PEG treatments could promote an up-regulation of CmLOX08 expression levels (Additional file 3). The transient expression results obtained in the present study revealed that the GUS activities of promoter deletion fragment LP4 were the highest compared with those of other deletion fragments under NaCl and PEG treatments (Fig. 3B, a and b), which indicates that the 1047-bp (LP4) segment may contain cis-acting elements that are induced by the aforementioned abiotic stresses. Interestingly, we noted the presence of a GT1GMSCAM4 motif in LP4 (Fig. 1), which is a cis-acting element involved in salt responsiveness [44]. Therefore, collectively, these results indicate that the GT1GMSCAM4 motif may play a role in regulating the expression of CmLOX08 in response to salt stress. In contrast, we were unable to detect any drought-inducible elements in the 1047-bp (LP4) segment, indicating that this region may contain a hitherto uncharacterized element that is crucial for drought responsiveness. In our wounding treatment, GUS activity was induced by the LP1, LP2, and LP4 promoter fragments (Fig. 3B, c), with expression being significantly increased at 1.5, 3, 6, and 12 h after wounding [29]. We noted, however, that there are no recognized associated response elements in the CmLOX08 promoter. We did, nevertheless, detect a GARE motif in the 1047-bp (LP4) segment (Fig. 1), and although this motif has been recognized as a gibberellin-responsive element in the promoter region of SFR2 in Brassica oleracea, it may play an important role in the response to wounding [45]. Thus, these results tend to indicate that the promoter activity induced by mechanical damage is similar to that induced in response to exogenous stimuli [34, 40, 46]. Currently, however, we still have very limited knowledge regarding the complex interactions between promoters and transcription factors at the transcriptional level [47]. Accordingly, in order to further elucidate the regulatory mechanism of CmLOX08 in response to abiotic stress, it will be particularly important to study the transcription factors that combine with the CmLOX08 promoter under different abiotic stress treatments.

With the exception of LP3, for which GUS activity was significantly increased, we were unable to detect any GUS expression induced by the CmLOX08 promoter deletion fragments when we subjected tobacco plants to heat stress (Fig. 4B, a). Within the 1047-bp (LP4) region, there are six cis-acting elements involved in heat stress responsiveness (three HSEs and three CCAATBOX1s), which can bind heat stress transcription factors [48]. However, we unable to detect any heat-inducible elements in the 237-bp region between LP3 and LP4 (Fig. 1). Our observation that the GUS activity of LP3 was higher than that of LP4 can probably be attributed to the fact that the 237-bp region between LP3 and LP4 contains enhanced elements involved in defence and stress responsiveness (TC-rich repeats) or contains uncharacterized heat-inducible cis-acting elements [49]. Given that CmLOX08 expression is also significantly induced at various time points after heat treatment [29], we infer that HSE and CCAATBOX1 are the main heat-responsive elements and that TC-rich repeats also play an important role in the response to heat stress.

Low temperature was found to increase the GUS activity of the LP4 deletion structure (Fig. 4B, b), which may be
attributable to the presence of an LTR element that is responsive to low temperature [50]. Interestingly, low temperature significantly increased the expression of CmLOX08 after 6 h under cold stress [29]. We thus speculate that the LTR motif may play a role in regulating the expression of CmLOX08 in response to cold stress.

**Conclusion**

In this study, we cloned the promoter region of the oriental melon CmLOX08 gene and subsequently sought to identify putative cis-regulatory elements that respond to signalling molecules and abiotic stresses by reference to the PlantCARE and PLACE databases. The results of GUS histochemical staining and fluorescence assays indicated that activity of the CmLOX08 promoter is regulated by various signalling molecules and abiotic stresses, and that the promoter generally functions as a signalling molecule/stress-inducible promoter. By analysing the differing responses of CmLOX08 promoter deletion fragments of various lengths to signalling molecules and abiotic stresses, we were able to characterize the core and positive and negative regulatory regions that show responsiveness to three different signalling molecules and five types of abiotic stress, respectively. The data generated in this study will enrich the existing inducible promoter resources and provide useful information for further study of the mechanisms whereby CmLOX08 is regulated when oriental melon is subjected to different abiotic stresses.

**Methods**

**Plant materials, growth conditions, and bacterial strains**

Oriental melons (Cucumis melo var. makuwa Makino) cultivar ‘YuMeiren’, from the Yijianpu Mishijie Melon Research Institution, Changchun, China, were grown individually in a culture room at 25 ± 2 °C, under a 14-h light/10-h dark photoperiod at Shenyang Agricultural University, Shenyang, China. The young leaves of 1-month-old seedlings were harvested, immediately frozen in liquid nitrogen, and stored at −80 °C until used for cloning of the CmLOX08 promoter. Tobacco (Nicotiana benthamiana) preserved in our laboratory was raised for 6 weeks in a mix of peat, perlite, and vermiculite (2:1:1, v/v/v) at 25 °C under a 16/8 h day/night cycle followed by using for agro-infiltration. Escherichia coli strain DH5α (Tiangen Biotech, China) was used for the cloning and propagation of all recombinant plasmid vectors, and Agrobacterium tumefaciens strain GV3101 (Weidi Biotech, China) was used for tobacco leaf infiltration.

**Isolation of the CmLOX08 promoter**

Genomic DNA was isolated from young melon leaves using a NuClean Plant Genomic DNA Kit (Kangwei Biotech, China) following the manufacturer’s protocol and used for cloning of the CmLOX08 promoter. On the basis of the CmLOX08 promoter sequence obtained from the melon genome database (http://melonomics.net, accession number: MELO3C011885), we designed a pair of primers, LOX08pro-F and LOX08pro-R (Table 2), which were used to amplify the full-length genomic sequence using melon genomic DNA as a template. CmLOX08 promoter fragments were amplified using high-fidelity PrimeSTAR™ HS DNA polymerase (Takara, Japan) in a 50-μL reaction mix containing 2 μL genomic DNA and 0.2 μM concentrations of each primer. The PCR amplification conditions were as follow: 30 cycles of 10 s at 98 °C, 15 s at 54 °C, and 2.5 min at 72 °C, with a final extension at 72 °C for 10 min. The amplified fragments of approximately 2 kb in size were purified using a MiniBEST Agarose Gel DNA Extraction Kit (Takara, Japan), according to the manufacturer’s protocols, and inserted via TA-cloning into the pMD18-T vector (Takara, Japan) following the addition of poly A tails (Additional file 4). Plasmids containing inserts of the expected size, identified by plasmid PCR, were sequenced by Invitrogen (Shanghai, China). Finally, we obtained a 2054-bp fragment upstream of the translation start codon of CmLOX08, which was considered to be the full-length promoter and was designated pLOX08-pro.

**Analysis of cis-regulatory elements in the CmLOX08 promoter**

On the basis of the cloned and sequenced fragments, sequence analysis of the cis-regulatory elements of the CmLOX08 promoter was performed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/PLACE/) databases [51, 52].

| Table 2 | PCR primers used in the current study |
|---------|--------------------------------------|
| Primer name | Primer sequence (5′−3′) |
| LOX08pro-F | TATGACATTGCGGACATA |
| LOX08pro-R | TATTACGCTTGGGGAGA |
| LP1-F1 | AGCTT TATGACATTGCGGACATA |
| LP1-F2 | T TAGTACGATTGCGGACATA |
| LP2-F1 | AGCTT GGTCTCCCCGATGCATAT |
| LP2-F2 | T GTGCTCCCCGATGCATAT |
| LP3-F1 | AGCTT GTATAACATCGACTTACAAATAG |
| LP3-F2 | T GTATAACATCGACTTACAAATAG |
| LP4-F1 | AGCTT CAAAAATTTGGGTAATAAGTG |
| LP4-F2 | T CAAAAATTTGGGTAATAAGTG |
| LP5-F1 | AGCTT CAAATTTGGGTAATAAGTG |
| LP5-F2 | T CAAATTTGGGTAATAAGTG |
| LP-R1 | C TATGACGGTCTGGGAGA |
| LP-R2 | GATG TATGACGGTCTGGGAGA |

Nucleotides underlined were used for producing HindIII and BamHI restriction site.
**Construction of CmLOX08 promoter::GUS plasmids**

For functional validation of the CmLOX08 promoter, five 5′-deletion fragments encompassing different lengths of the CmLOX08 promoter (−2054 bp, −1639 bp, −1284 bp, −1047 bp, and −418 bp to −1 bp), and containing HindIII and BamHI restriction sites, were amplified using five sets of specific PCR primers (Table 2) and two PCR reactions according to a method described in the literature [53]. Using this method, the fact that the inserted fragment might have the same restriction site as the vector used in the subsequent step was not a relevant consideration. Using the pLOX08-pro plasmid as template DNA, the 2054-bp CmLOX08 promoter fragments containing either HindIII or BamHI restriction sites were amplified using the first (LP1-F1 and LP-R1) and second (LP1-F2 and LP-R2) pair of primers, respectively, to eventually generate two different PCR fragments. Similarly, two different PCR fragments for the other four deletion fragments of the CmLOX08 promoter (1639, 1284, 1047, and 418 bp) were acquired using the same primer pairs (Table 2). PCR reactions were performed with high-fidelity PrimeSTAR™ HS DNA Polymerase (Takara, Japan) according to the manufacturer’s instructions, using the same PCR conditions as described above. The PCR products were purified using a MiniBEST Agarose Gel DNA Extraction Kit (Takara, Japan) and ligating the blunt ends using T4 DNA ligase linearized vector with T4 DNA polymerase (Takara, Japan) and ligating the blunt ends using T4 DNA ligase (Takara, Japan) according to the manufacturer’s instructions.

**Agrobacterium-mediated transient expression assay**

Agrobacterium-mediated transient expression assays were performed as described previously [54, 55]. A single colony of Agrobacterium strain GV3101 harbouring one of the five different recombinant binary vectors was inoculated into 2 mL YEB medium supplemented with 50 μg mL⁻¹ rifampicin and 50 μg mL⁻¹ kanamycin and grown at 28 °C and 200 rpm for 24 h. Thereafter, 1 mL of the resulting culture was transferred into 50 mL YEB medium containing 50 μg mL⁻¹ rifampicin, 50 μg mL⁻¹ kanamycin, 10 mM ethanesulfonic acid (pH 5.7 MES), and 20 μM acetosyringone (AS) and grown at 28 °C and 200 rpm for 24 h. The Agrobacterium cells were harvested by centrifugation at 5000 rpm for 10 min at room temperature, and following the removal of supernatant, the pelleted cells were resuspended in infiltration medium (10 mM pH 5.7 MES, 10 mM MgCl₂, and 150 μM AS). The cell suspension was diluted to OD₆₀₀ = 0.8 using infiltration medium, and then incubated at room temperature for 3 h in the dark. The Agrobacterium suspensions harbouring recombinant binary vectors were then injected into the abaxial surfaces of the leaves of 6-week-old tobacco plants using a 1-mL needleless syringe. The inoculated tobacco plants were subsequently maintained in a growth cabinet under a 16/8 h day/night cycle at 25 °C for 48 h.

**Signalling molecule and abiotic stress treatments**

For analysis of the cis-regulatory element of the CmLOX08 promoter, agro-infiltrated tobacco plants were treated with SA, ABA, MeJA, and H₂O₂ to characterize promoter induction in response to signalling molecule treatments. For SA, ABA, and H₂O₂ treatments, the tobacco plants were sprayed with 1 mM SA, 0.1 mM ABA, or 10 mM H₂O₂ in distilled water, respectively, whereas control plants were sprayed with distilled water. For MeJA treatment, the tobacco plants were sprayed with 0.1 mM MeJA dissolved in 10% ethanol and control plants were sprayed with 10% ethanol.

We also exposed the agro-infiltrated tobacco plants to a variety of abiotic stresses, namely, low and high temperatures, mechanical wounding, salinity, and drought, to characterize the extent to which promoter activation occurs in response to these stresses. For the low and high temperature treatments, the plants were maintained in a growth cabinet at 4 °C or 42 °C, respectively. For the wounding treatment, the tobacco leaves (an area of approx. 20 cm²) were pricked 200 times with the needle.
of a 10-mL syringe. Control tobacco plants were placed in a growth cabinet at 25 °C without any treatment. For salinity and drought stress treatments, we followed the methods described by Hou et al. [24]. Leaf discs obtained from infiltrated plants were floated on half-strength liquid MS medium supplemented with either 200 mM NaCl (salt stress treatment) or 18% (w/v) PEG 6000 (drought stress treatment) for 24 h. Infiltrated leaves incubated in half-strength liquid MS medium were considered a control.

The leaves of the tobacco plants subjected to signalling molecule and abiotic stress treatments and their controls were used for GUS analysis after 24 h. All experiments were repeated three times.

**Histochemical staining and fluorometric assays for detecting GUS activity**

Histochemical staining was performed in accordance with the procedures described by Jefferson [56]. Tobacco leaves were punched with a hole punch to obtain leaf discs with a diameter of 1 cm and these were incubated in GSU staining solution [50 mM sodium phosphate buffer (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, 1 mM X-Gluc (Sangon, Shanghai, China), and 0.1% Triton X-100] at 37 °C for 24 h. After staining, the tissues were bleached with 70% ethanol and photographed using a scanner.

Transient expression of GUS activity in the treated tobacco leaves was measured as described previously [57]. Tobacco leaf tissue (0.15 g) was homogenized in an extraction buffer [50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.1% (w/v) sodium dodecylsulfate, and 0.1% β-mercaptoethanol] at 4 °C, and centrifuged at 12,000×g for 15 min at 4 °C. Aliquots (100 μL) of the resulting supernatant were mixed with 600 μL GUS assay solution [1 mM methyl-4-umbelliferyl-

![Fig. 5](image-url) Transient transformation of tobacco plants with CmLOX08 promoter::GUS constructs. a Schematic representation of CmLOX08 promoter::GUS vector constructs. NPT II, neomycin phosphotransferase II gene; nos ter, nopaline synthase terminator; GUS, β-glucosidase gene. RB and LB, left and right T-DNA borders. The insertion position of the CmLOX08 promoter in the vector is indicated with restriction enzyme sites (HindIII and BamHI). b Schematic representation of the different 5′ deletion CmLOX08 promoter constructs used to assay GUS activity in tobacco leaves. These constructs are based on the pBI121 vector. The main cis-elements are represented with different patterns.
Statistical analysis
Data are expressed as the mean values ± standard deviation (SD) of three independent experiments and were analysed using SPSS statistical software (IBM SPSS statistics 18.0, Chinese version) using an independent sample t test. A P value ≤0.05 was considered significant. Charts presenting data were generated using Origin software (version 8.0).

Additional files

Additional file 1: Alignment of the nucleotide sequences of CmlLOX08-pro and GelLOX08-pro. CmlLOX08-pro promoter: oriental melon (Cucumis melo var. makuwa Makino); GelLOX08-pro promoter: the melon (Cucumis melon L.) genome database. Identical and dissimilar nucleotides are shown on a background of blue and gray, respectively. The two primers LOX08pro-F and LOX08pro-R which cloned the CmlLOX08 promoter are indicated by arrows. The translation initiation codon (ATG) is framed and marked for “+ - 1”. (PDF 498 kb)

Additional file 2: GUS histochemical staining of the p121GUS tobacco leaves as negative control. (PDF 125 kb)

Additional file 3: Expressions of CmLOX08 at various time points under 50 mM NaCl (a) and drought (b) treatments. (PDF 148 kb)

Additional file 4: PCR amplification of CmlLOX08 full length promoter. Lane M: DL5000 DNA Marker, lane 1 to 5: CmlLOX08 full length promoter fragment. (PDF 123 kb)

Additional file 5: The five different length recombinant vectors were verified by plasmid PCR. Lane M: DL5000 DNA Marker. Lane 1 to 4: LP1 (2054 bp); lane 5 to 8: LP2 (1639 bp); lane 9 to 12: LP3 (1284 bp); lane 13 to 16: LP4 (1047 bp); lane 17 to 20: LP5 (418 bp). (PDF 126 kb)

Abbreviations
ABA: Abscisic acid; AS: Acetosyringone; GUS: β-Glucuronidase; H₂O₂: Hydrogen peroxide; JA: Jasmonic acid; LOX: Lipoxygenase; MeJA: Methyl jasmonate; MES: Ethanesulfonic acid; MgCl₂: Magnesium chloride; PUFA: Polyunsaturated fatty acid; SA: Salicylic acid

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Availability of data and materials
The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
CWL contributed to the experimental design, oriental melon planting and sampling, tobacco planting and experimenting, data processing and result analysis and writing. GG, SC and QX contributed to tobacco sampling and experimenting. HQ defined the work objectives and technical approach, and contributed to the experimental design, result analysis and writing. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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