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Research Article

Molecular cloning and characterization of a lipoxygenase gene (*CsLOX1*) from cucumber

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Abstract

Lipoxygenases (LOXs) are non-heme iron enzymes that play crucial roles in many developmental processes during plant life, and defense responses against biotic and abiotic stresses. In this study, a lipoxygenase gene (*CsLOX1*) was cloned and characterized from cucumber (*Cucumis sativus*). The coding sequence (CDS) of *CsLOX1* was 741 bp, and encoded an 878 amino-acid residue protein, which was predicted to be located in the cytoplasm. CsLOX1 contained the conserved LH2/PLAT and lipoxygenase domains, as well as the representative 38 amino acids motif [His-(X)4-His-(X)4-His-(X)17-His-(X)8-His]. Multiple sequence alignment and phylogenetic analysis indicated that CsLOX1 was closely related to other dicot 9-LOXs and posesess the essential conserved residues involved in the binding of the iron atom. Promoter analysis suggested that several development-, stress-, and hormone-related *cis*-acting regulatory elements were present in the promoter region of *CsLOX1*. The function of *CsLOX1* was assessed by overexpression it in *Arabidopsis*, and the transgenic plants were male sterile and displayed obviously increased floral shoots. These results provide some clues for revealing the biological roles of *CsLOX1* in cucumber.

Keywords: cucumber; expression pattern; lipoxygenase (LOX); transgenic *Arabidopsis*

Introduction

Lipoxygenase (LOX, EC 1.13.11.12) is a group of non-heme iron enzymes widely distributed in plants, animals, fungi, and bacteria (Yamamoto, 1992; Porta and Rocha-Sosa, 2001; Feussner and Wasternack, 2002; Brodhun and Feussner, 2011). In plants, LOXs can insert molecular oxygen into position carbon atom 9 or position carbon atom 13 of the oxygenation of polyunsaturated fatty acids (PUFAs) to produce 9-hydroperoxide octadecadi(tri)enoic acids (9-HPOD/T) and 13-hydroperoxide octadecatrienoic acid (13-HPOT) hydroperoxides, respectively, and then initiate the oxylipin biosynthesis to form a large class of biologically active compounds, such as jasmonic acid (JA), green leaf volatiles (GLVs) and other related compounds (Zhu *et al.*, 2018; Zhou *et al.*, 2019a; Viswanath *et al.*, 2020). Therefore, plant LOXs can be classified into two groups: 9-LOXs and 13-LOXs, based on the carbon atom positional specificity of substrates

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(Feussner and Wasternack, 2002; Wasternack and Feussner, 2018). Beside 9-LOXs and 13-LOXs, plant LOXs displayed a dual 9/13-LOX specificity were also identified (Wang *et al.*, 2008; Liu and Han, 2010; Padilla *et al.*, 2012; Zhu *et al.*, 2018). Furthermore, 13-LOXs can be further divided into two subgroups, type I 13-LOX and type II 13-LOX, according to their primary structure and sequence similarity (Feussner and Wasternack, 2002; Liavonchanka and Feussner, 2006).

LOXs are encoded by a multi gene family in numerous plants, and *LOX* family genes were identified in various plant species, such as *Arabidopsis* (6 genes) (Bannenberg *et al.*, 2009; Umate, 2011), pepper (8 genes) (Sarde *et al.*, 2018), radish (11 genes) (Wang *et al.*, 2019), peach (12 genes) (Guo *et al.*, 2017), rice (14 genes) (Umate, 2011), tomato (14 genes) (Upadhyay and Mattoo, 2018), apple (23 genes) (Vogt *et al.*, 2013), and pear (23 genes) (Li *et al.*, 2014). And functional characterizations of *LOX* genes indicated that they play vital roles in a variety of developmental processes during plant life, such as tuber development (Kolomiets *et al.*, 2001), nodule development (Hayashi *et al.*, 2008), sex determination (Acosta *et al.*, 2009), anther and pollen development (Caldelari *et al.*, 2011), seed germination and longevity (Huang *et al.*, 2014), leaf senescence (Hou *et al.*, 2015; Springer *et al.*, 2016), and fruit ripening (Guo *et al.*, 2017; Zhang *et al.*, 2017), Moreover, LOXs also play key roles in defense responses against biotic and abiotic environmental stresses. For example, overexpression of *TomLOXD* (*SlLOXD*) in transgenic tomato plants increased plant resistance against insect attack, pathogen infection, and high temperature stress (Hu *et al.*, 2013; Yan *et al.*, 2013). Pepper *CaLOX1* was involve in defence and cell-death responses against pathogens (Hwang and Hwang, 2010), and transgenic *Arabidopsis* plants overexpressing *CaLOX1* resulted in enhanced tolerance to osmotic, drought, and high salinity stress (Lim *et al.*, 2015). Another gene, *CaLOX2*, was involved in the JA biosynthesis and resistance of cultivated pepper to Western flower thrips (*Frankliniella occidentalis*) (Sarde *et al.*, 2019). In addition, overexpression of persimmon *DkLOX3* in *Arabidopsis* promoted resistance to drought, high salinity and osmotic stress via regulating reactive oxygen species accumulation and stress responsive genes expression (Hou *et al.*, 2015). *Arabidopsis* plants overexpressing oriental melon *CmLOX13* also exhibited enhanced resistance to drought stress via regulating abscisic acid (ABA) accumulation and stomatal closure (Xing *et al.*, 2019).

In our previous study, a total of 23 *LOX* genes were identified in cucumber genome and their tissue expression patterns were examined (Liu *et al.*, 2011). Subsequently, the expression profiling of the *LOX* genes during fruit development, various abiotic stress and hormonal treatments were also determined (Yang *et al.*, 2012). And a previous report showed that the *yellow-green leaf* (*ygl1*) mutant was due to mutations in four tandem cucumber 13-LOX genes (Ding *et al.*, 2019). At the different storage temperatures, the expression of *CsLOX* genes might led to the differences in the contents of six-carbon (C6) and nine-carbon (C9) aldehydes (Yang *et al.*, 2020). However, the detailed functional analysis for cucumber *LOX* genes is still limited. In this work, a cucumber *LOX* gene (*CsLOX1*) was isolated and it was overexpressed in transgenic *Arabidopsis* plants, to investigate its potential function. Our findings indicated that *CsLOX1* plays important roles in the growth and development of cucumber.

Materials and Methods

Plant materials and growth conditions

Cucumber (*Cucumis sativus* L. cv 'Chinese long' 9930 inbred line) plants were grown in a climate chamber at a night temperature of 18 °C and day temperature of 24 °C under long-day conditions (16 h of light/8 h of dark). Flower samples of 20 main-stem nodes stages were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

Wild-type (WT, Col ecotype) and transgenic *Arabidopsis* seeds were placed 4 °C under dark conditions for dormancy breaking. And then the seeds were germinated and planted in a growth room at 24/18 °C (day/night) under long-day conditions (16 h of light/8 h of dark).

RNA extraction and cDNA synthesis

Total RNA was extracted using Trizol reagent (Tiangen Biotech, Beijing, China) according to the manufacturer's instruction. Subsequently, the integrity of the RNA samples was checked on a 1.0% agarose gel. After concentration analysis using Nanodrop 2000 (Thermo Fisher Scientific, USA), about 3 μg RNA was reverse transcribed as cDNA using the M-MLV reverse transcriptase (Invitrogen, USA) based on the manufacturer's protocol.

Cloning and sequence analysis of the CsLOX1 gene

For cloning the *CsLOX1* gene, cDNA from cucumber flower samples was prepared as the template. The specific primers CsLOX1-1F (5'-aaaaCTGCAGATGTTTGGAATTGGGAAGAACAT-3') and CsLOX1-1R (5'-aaaaTCTAGATTAGATAGAAATACTATTAGGAAT-3') were designed based on the open reading frame (ORF) sequence of *CsLOX1* (gene ID: Csa006735) in previous reports (Liu *et al.*, 2011; Yang *et al.*, 2012). The *CsLOX1* ORF was amplified with semiquantitative reverse-transcription polymerase chain reaction (RT-PCR). The PCR procedure was carried out as follows: 1 cycle at 94 °C for 5 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 59 °C, and 3 min at 72 °C, and then a final extension at 72 °C for 10 min. Then, the PCR products were inserted into the pMD18-T vector (Takara, Japan) and then sequenced (Tsingke, Beijing, China).

Sequence analysis of the CsLOX1 gene and the deduced CsLOX1 protein

The exon-intron of *CsLOX1* was analyzed by Gene Structure Display Server 2.0 (GSDS, http://gsds.cbi.pku.edu.cn/) comparing the sequences of coding sequence (CDS) and genome DNA (gDNA). The theoretical isoelectric point (pI) and molecular weight (MW) were calculated with protparam (https://web.expasy.org/protparam/). Subcellular localization of CsLOX1 was predicted by using online tools Plant-mPLoc (http://www.csbio.sjtu.edu.cn/cgi-bin/PlantmPLoc.cgi) and ProtComp Version 9.0 (http://linux1.softberry.com/berry.phtml?topic=protcomppl&group=programs&subgroup=proloc). For promoter region analysis, the 2.0-kb upstream sequences of the start codon of the *CsLOX1* gene was submitted into the PlantCARE online tool (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), and the putative development-, stress-, and hormone-related *cis*-elements were analyzed.

Sequence alignment and phylogenetic analysis

The multiple sequence alignment of the deduced amino acid sequences of CsLOX1 and other plant LOX proteins were carried out using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/), and then the align results were displayed with the GeneDoc software as described previously (Zhou *et al.*, 2019b). The MEGA 7.0 software was employed to create a neighbour-joining (NJ) phylogenetic tree using the same align results with the following parameters: 1000 bootstrap replicates, poisson model, pairwise deletion.

Vector construction, Arabidopsis transformation and morphological observation

The sequencing verified pMD18-T vector carrying *CsLOX1* ORF was digested with P*st* I/X*ba* I, and then the digested fragment was inserted into the P*st* I/X*ba* I restriction sites downstream of the double 35S promoter of the pHB vector (Zhou *et al.*, 2017). The overexpression construct was named as 35S::35S::CsLOX1, and *Arabidopsis* transformation was conducted by *Agrobacterium tumefaciens*-mediated floral dip method as described previously (Zhou *et al.*, 2019b). The transgenic plants were checked with RT-PCR using the following *CsLOX1*-specific primers: 5'-AGGACCTCACTCCACCTTTG-3' and 5'-AACCGTAAGACCATCTAAACCAT-3'. The *AtTubulin4* gene is a reference gene, and its primers sequences are as follows: 5'-GCGAACAGTTCACAGCTATGTTCA-3' and 5'-GAGGGAGCCATTGACAACATCTT-3'. The reaction conditions were performed as described above. The transgenic plants exhibit increased *CsLOX1* expression were used for morphological observation.

Results and Discussion

Isolation and sequence analysis of CsLOX1 in cucumber

Using cucumber flower cDNA as the template, a 2637-bp PCR fragment was amplified with the specific CsLOX1-1F and CsLOX1-1R primers. For investigating the chromosomal localization of the *CsLOX1* gene, we carried out Blastn search against the Cucumber (Chinese Long) v3 Genome (http://cucurbitgenomics.org/organism/20). The result indicated that the Locus ID of *CsLOX1* was CsaV3_2G006380 and it was located on chromosome 2. In addition, the *CsLOX1* gene encoded a 878 amino-acid residue protein, with a pI value of 6.04, and a MW value of 99.78 kDa. The GSDS analysis showed that *CsLOX1* harbored 9 exons and 8 introns (Figure 1A). The SMART analysis showed that CsLOX1 possessed two conserved domains, PLAT (Polycystin-1, Lipoxygenase, Alpha-Toxin) or LH2 (Lipoxygenase homolog) (SMART Accession: SM000308) and lipoxygenase domain (Pfam Accession: PF00305), which were located between 40-181 and 192–861 amino acids, respectively (Figure 1B). SOPMA analysis showed that the secondary structure of CsLOX1 was composed by 39.07% alpha helix, 13.10% extended strand, 5.13% beta turn, and 42.71% random coil (Figure 1C). Plant LOX proteins are found to have various subcellular localizations, such as chloroplast, cytoplasm and vacuole. And 9-LOXs were usually found to localized in cytoplasm, while many 13-LOXs were localized in chloroplast (Upadhyay and Mattoo, 2018; Zhu et al., 2018). In this work, both of Plant-mPLoc and ProtComp subcellular prediction analysis showed that CsLOX1 was located in cytoplasm. These results indicated that CsLOX1 is a typical cytoplasmic lipoxygenase protein.

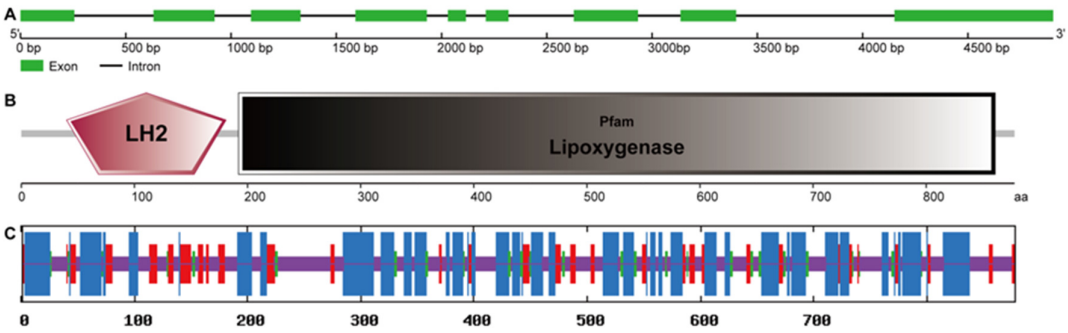

Figure 1. Sequence analysis of *CsLOX1* and its deduced amino acid sequences
(A) Exon–intron structure of the *CsLOX1* gene. (B) SMART analysis of the conserved domains of CsLOX1. (C) SOPMA analysis of the secondary structure of CsLOX1. The blue, red, green, and purple regions indicate of alpha helix, extended strand, beta turn, and random coil, respectively.

Characterization and phylogenetic analysis of CsLOX1 with other LOX proteins

To characterize the features of CsLOX1, a multiple alignment analysis was performed based on the amino acid sequences of the putative CsLOX1 and other plant LOX proteins, including olive (*Olea europaea*) Oep2LOX1 (Padilla *et al.*, 2012), maize (*Zea mays*) ZmLOX12 (Christensen *et al.*, 2014), rice (*Oryza sativa*) OsLOX2 (Huang et al., 2014), persimmon (*Diospyros kaki*) DkLOX3 and DkLOX4 (Hou *et al.*, 2015; Meng *et al.*, 2016), agarwood (*Aquilaria sinensis*) AsLOX1 (Liao *et al.*, 2015), finger millet (*Eleusine coracana*) EcLOX (Kotapati *et al.*, 2016), melon (*Cucumis melo*) CmLOX09 (Ju *et al.*, 2018) and CmLOX13 (Xing *et al.*, 2019; Cao *et al.*, 2016), pepper (*Capsicum annuum*) CaLOX1 (Hwang and Hwang, 2010; Lim *et al.*, 2015) and CaLOX2 (Sarde *et al.*, 2019). The alignment analysis results revealed that CsLOX1 shared relatively high identities to these LOX proteins, ranged from 41.80% (CmLOX13) to 62.27% (CaLOX1) (Figure 2). All of these LOX proteins shared the conserved regions such as LH2/PLAT domain, lipoxygenase domain, and highly conserved C-terminal motif. In addition, all of these LOX proteins contained the representative 38 amino acids motif [His-(X)4-His-(X)4-His-(X)17-His-(X)8-His] for enzyme stability and activity, which was provide binding sites for non-haeme iron-containing dioxygenases (Shaban *et al.*, 2018). In addition, three His

(H), one Asn (N) and one Ile (I) residues involved in the binding of the iron atom in the active site, were also present in CsLOX1 and LOX proteins from other plants (Padilla *et al.*, 2012; Christensen *et al.*, 2014; Kotapati *et al.*, 2016). Previous reports showed that Val (V) residue in the conserved motif may determine 9-LOX regio-specific activity, while Phe (F) residue indicative of LOX enzymes with 13-LOX activity (Liavonchanka and Feussner, 2006; Kotapati *et al.*, 2016). However, CsLOX1 have a His (H) at position 597 (Figure 2), which is different from the characterization of 9-LOX, suggesting that CsLOX1 may have special roles in cucumber. And previous studies showed that several 9-LOX proteins also had no Val residue at this positions (Christensen *et al.*, 2014; Li *et al.*, 2012). Similar to other LOX proteins, the conserved Ala (A) residue at position 582 was also detected in CsLOX1, which is required for substrate orientation and the S-stereo-specificity (Padilla *et al.*, 2012; Kotapati *et al.*, 2015). And the Arg (R) residue at position 747 can function in interacting with the carboxyl group of the fatty acid and required for the inverse substrate orientation in plant LOXs (Kotapati *et al.*, 2015). Moreover, CsLOX1 harbores two conservative motifs which have been shown to be essential for substrate (GWSTDEEFAREMLAG) and oxygen binding (ASALHAAVNFGQY) (Figure 2).

To further study the phylogenetic relationships between CsLOX1 and other plant LOX proteins, a phylogenetic tree was created by aligning LOX protein sequences from various plant species. Our phylogenetic analysis showed that these LOXs can be divided into two groups: 9-LOX and 13-LOX, and CsLOX1 was fall into the 9-LOX group with other dicot 9-LOXs (Figure 3), demonstrating that CsLOX1 is a 9-LOX.

Cis-element analysis of CsLOX1

In consideration of the *cis*-acting regulatory elements in the promoter region is important for understanding the expression patterns of genes (Zhou *et al.*, 2020), we investigated the distribution of putative development-, stress-, and hormone-related *cis*-elements in the 2.0-kb upstream sequences of the start codon of the *CsLOX1* gene using PlantCARE. As shown in Figure 4, one ABA responsiveness element (ABRE) and three ethylene-responsive elements (ERE) were present in the promoter region of *CsLOX1*, which were correlated with the expression of *CsLOX1* in response to ABA and ethylene. In a previous study, *CsLOX1* was observably induced by exogenous ABA and 1-aminocyclopropane-l-carboxylic acid (ACC; precursor of ethylene), and its mRNA accumulations were peaked at 12 h after treatment (almost 40-fold) by ABA and 6 h after treatment (almost 17-fold) by ACC (Yang *et al.*, 2012). In addition, two types of *cis*-elements in stress responsive, ARE and TC-rich repeats, which were take part in anaerobic induction, defense and stress responsiveness, respectively, were found in promoter region of *CsLOX1* (Figure 4). And *CsLOX1* was also significantly induced by NaCl and KCl treatments (Yang *et al.*, 2012). When plants were subjected to abiotic stresses, endogenous ABA levels were increased to help plants adapt to the stress conditions (Zhou *et al.*, 2020; Vishwakarma *et al.*, 2017). And some *LOX* genes can enhanced abiotic stresses resistance by modulating the ABA-responsive genes and promoting the endogenous ABA levels, such as *CaLOX1* (Lim *et al.*, 2015), *DkLOX3* (Hou *et al.*, 2015), and *CmLOX13* (Xing *et al.*, 2019). Therefore, *CsLOX1* may also play a role in resistance to one or more stresses via ABA-dependent pathway. Previous reports showed that plant *LOX* genes can be regulated by circadian rhythms (Nemchenko *et al.*, 2006; Zhu *et al.*, 2018). In this study, *CsLOX1* promoter also harbored one circadian *cis*-element related to circadian control, suggesting that *CsLOX1* might be involved in circadian rhythms regulation in cucumber.

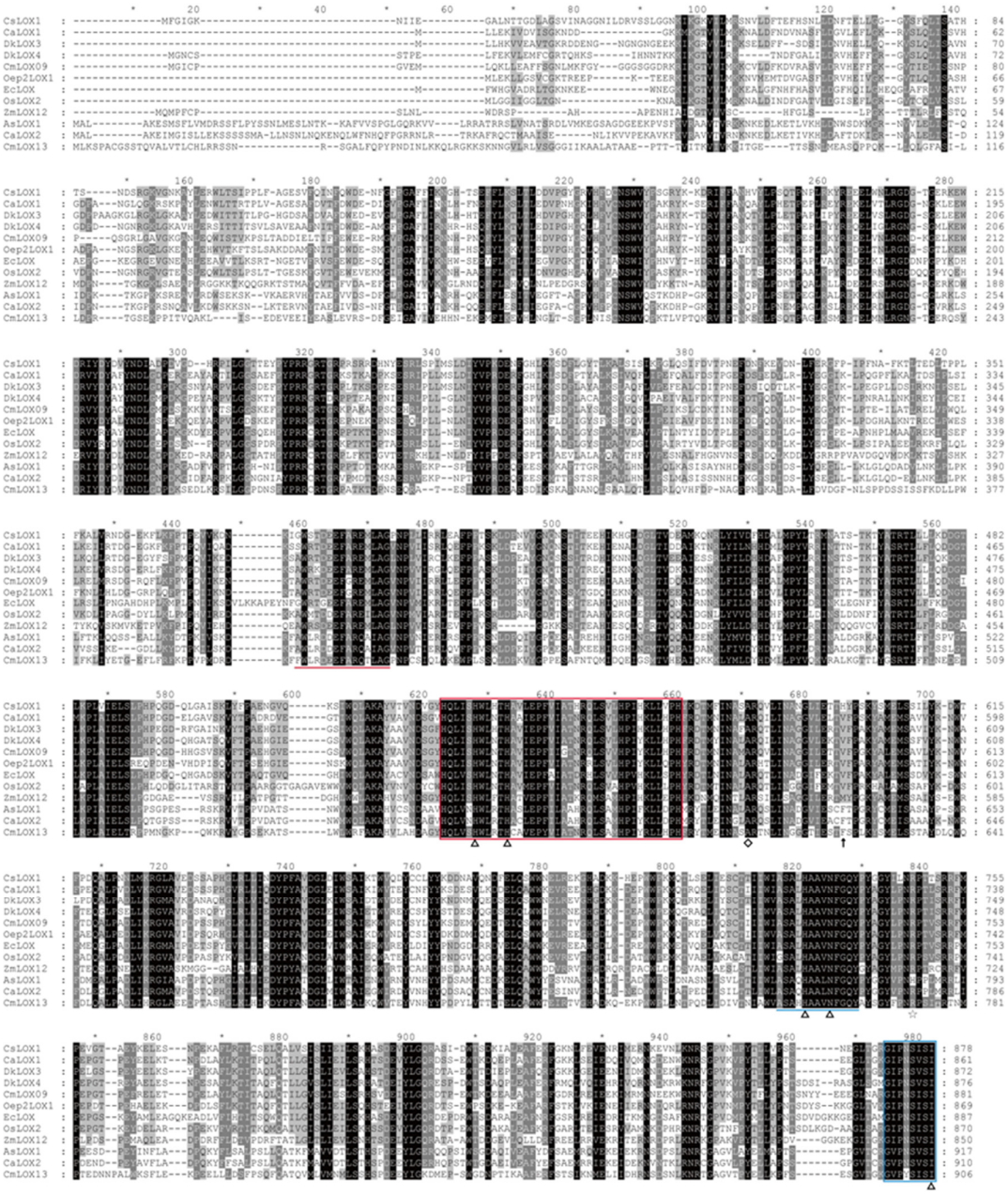

Figure 2. Alignment of the deduced amino acid sequences of CsLOX1 and LOX proteins from other plants

These sequences were aligned using Clustal Omega and visualized in the GeneDoc software. The representative 38 amino acids motif [His-(X)4-His-(X)4-His-(X)17-His-(X)8-His] and highly conserved C-terminal motif are boxed with red and blue, respectivley. Two conserved motifs required for substrate (GWSTDEEFAREMLAG) and oxygen binding (ASALHAAVNFGQY) were underlined with red and blue, respectivley. The five conserved residues (His539, His544, His730, Asn734, and Ile878) essential for the binding of the iron atom in the active site of the LOXs enzymes are indicated by triangles. The Val (V) or Phe (F) residues indicative of LOX enzymes with 9-LOX or 13-LOX activities are marked by arrow. The Ala582 that required for substrate orientation and the S-stereo-specificity is marked by rhombus, and the Arg747 determinant for the inverse substrate orientation in plant LOXs is marked by pentacle. The details of CsLOX1 and other plant LOX proteins were listed in Table S1.

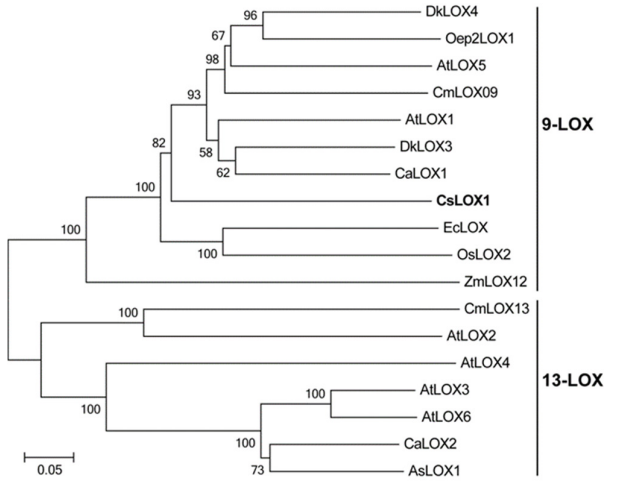

Figure 3. Phylogenetic relationships of CsLOX1 and other plant LOX proteins. LOX proteins from various plants were aligned with Clustal Omega, and the align results were employed to generate the phylogenetic tree with MEGA 7.0 using NJ method

The bootstrap test was set as 1000 replicates. The details of CsLOX1 and other plant LOX proteins were listed in Table S1. CsLOX1 is bolded.

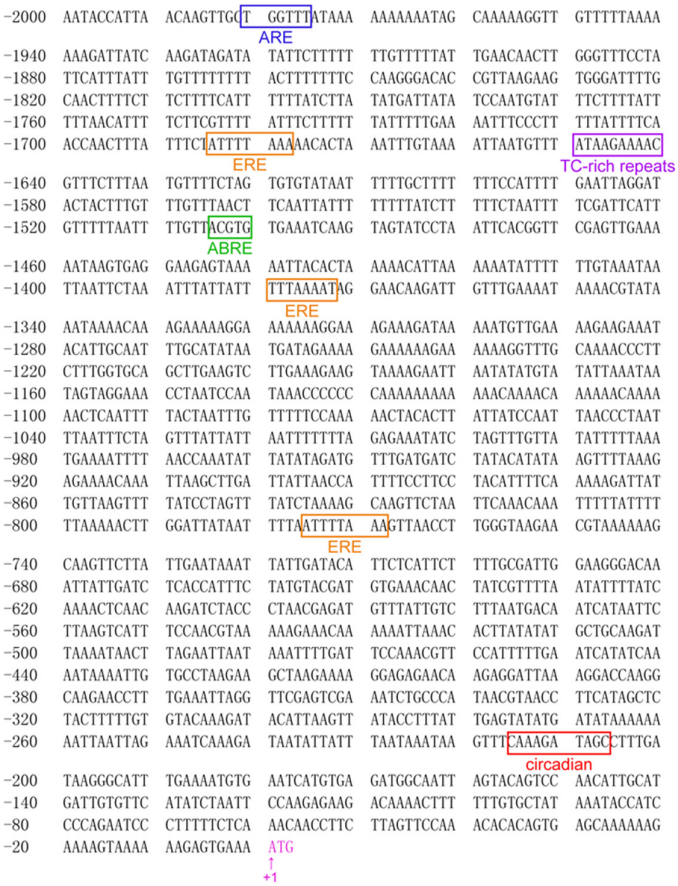

Figure 4. *Cis*-acting regulatory element analysis of *CsLOX1*

Putative *cis*-elements in the promoter region of *CsLOX1* was boxed and colored. The "A" of the start codon "ATG" of *CsLOX1* was designated as + 1 position.

Overexpression of CsLOX1 causes male sterility in transgenic Arabidopsis plants

Previous studies revealed *CsLOX1* have a constitutive expression pattern, but its expression was preferentially detected in ovaries and early developing fruits, implying its particular role in fruit development immediately after anthesis (Liu *et al.*, 2011; Yang *et al.*, 2012). To further uncover its role in cucumber, the overexpression vector 35S::35S::CsLOX1 was constructed by inserting the ORF of *CsLOX1* into the pHB vector, under the control of double CaMV 35S promoter (Figure 5A). The overexpression construct 35S::35S::CsLOX1 was transformed into *Arabidopsis*. Three transgenic lines (OE1, OE2, and OE3) were chosen for further study, and RT-PCR showed that *CsLOX1* was highly expressed in these transgenic plants while no expression was detected in WT plants (Figure 5B).

To study how the overexpression of *CsLOX1* in transgenic plants, we investigate the phenotypes of the transgenic *Arabidopsis* plants. At stage 13, the transgenic plants exhibited green anthers, and the filaments were much slenderer compared to the WT plants (Figure 6A-C), suggesting that the stamen development was impaired in transgenic plants. And all seeds of these transgenic plants are fully sterile. However, it has no impact on the development of other floral organs, such as pistils, sepals, and petals. Hence, the complete male sterility of transgenic *Arabidopsis* plants was induced by the overexpression of *CsLOX1*. In a previous study, *Arabidopsis* single *lox3* and *lox4* mutants were found to be fertile, while *lox3 lox4* double mutants were male sterile (Caldelari *et al.*, 2011). In addition, it was observed that the transgenic *Arabidopsis* plants displayed obviously increased floral shoots compared to the WT plants (Figure 6D-E), and these phenotypes were also observed in *lox3 lox4* plants (Caldelari *et al.*, 2011). Therefore, overexpression of *CsLOX1* may impair the endogenous JA production and then inhibit the expression of *Arabidopsis LOX* genes. Therefore, the role of *CsLOX1* may affect plant growth and development, such as male fertility, shoot growth and fruit development.

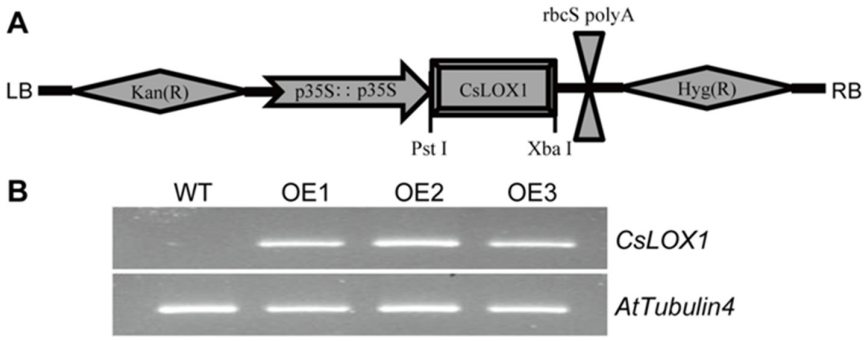

Figure 5. Analysis of transgenic *Arabidopsis* lines overexpressing the *CsLOX1* gene
(A) Schematic diagram of the T-DNA region of 35S::35S::CsLOX1 overexpression construct. (B) RT-PCR analysis of expression of *CsLOX1* in WT and transgenic *Arabidopsis* plants. WT, wild-type *Arabidopsis* plants. OE1–3, 35S::35S::CsLOX1 transgenic *Arabidopsis* lines. *AtTubulin4* gene was used as the internal control.

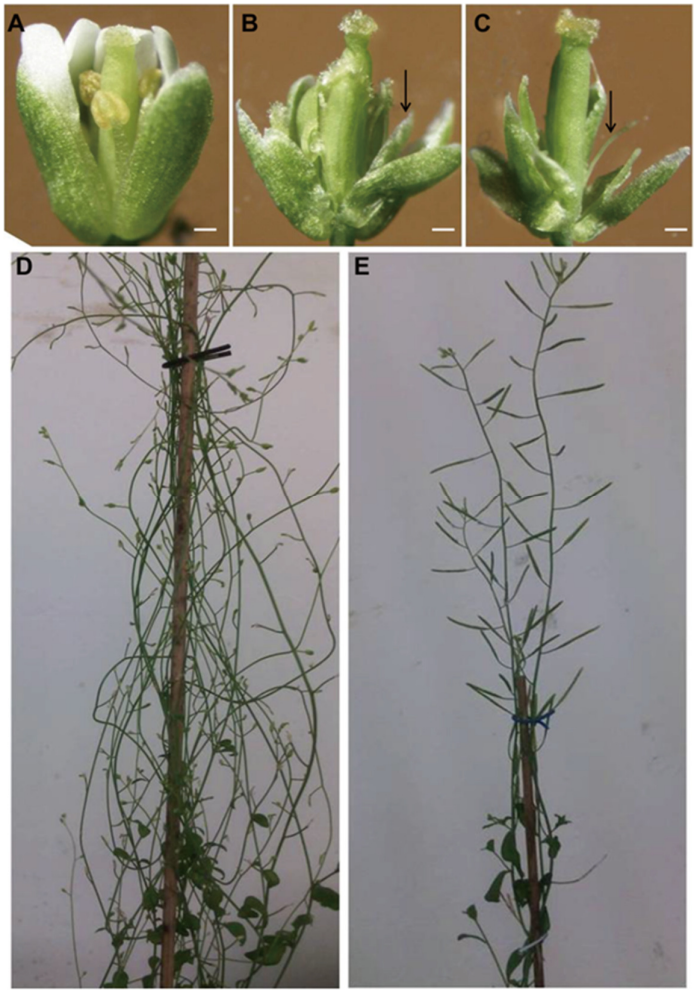

Figure 6. Comparison of the phenotypes of 35S::35S::CsLOX1 transgenic *Arabidopsis* plants
(A, E) WT *Arabidopsis* plants and (B-D) 35S::35S::CsLOX1 plants. (A) The flower buds of WT plant at stage 12. (B) Green anthers (arrow) at stage 13. (C) Filaments become slenderer than those of WT plants (arrow). (D) One 35S::35S::CsLOX1 transgenic plant at stage 17. (E) One WT plant at stage 17.

Conclusions

In this work, a cucumber *LOX* gene (*CsLOX1*) was isolated and its features were characterized by bioinformatics approaches. This gene encodes a typical cytoplasmic 9-LOX, and possesses the typical LH2/PLAT and lipoxygenase domains, as well as essential conserved residues. And transgenic analysis in *Arabidopsis* was carried out to investigate the possible functions of *CsLOX1*. Overexpression of *CsLOX1* in *Arabidopsis* induced a phenotype of male sterility that resulted from the failure of stamen development. In addition, the transgenic plants displayed obviously increased floral shoots compared to the WT plants. Our findings provide a basis for revealing of the biological roles of *CsLOX1* in cucumber.

Authors' Contributions

Conceptualization: SL; Data curation: YZ and SL; Formal analysis: YZ, MX, WL and SL; Funding acquisition: SL; Investigation: YZ, MX, WL, LC, YY and SL; Methodology: YZ, MX, WL, LC, YY and SL; Resources: YZ and SL; Software: YZ, WL and SL; Visualization; Writing - original draft: YZ and SL; Writing - review and editing: YZ, YY and SL. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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