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Research article

The involvement of a herbivore-induced acyl-CoA oxidase gene, *CsACX1*, in the synthesis of jasmonic acid and its expression in flower opening in tea plant (*Camellia sinensis*)

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ABSTRACT

The biosynthesis of jasmonic acid (JA) in plant peroxisomes requires the action of acyl-CoA oxidase (ACX; EC 1.3.3.6). Multiple isoforms of ACXs have been identified in various annual herbaceous plants, but the genes encoding these enzymes in perennial woody plants are yet to be fully investigated. In this study, an ACX gene named *CsACX1* (GeneBank accession: KX650077.1) was isolated from tea plant (*Camellia sinensis* L.). *CsACX1* was predicted to consist of 664 amino acid residues. Transcriptional analysis revealed that *CsACX1* can be induced by mechanical wounding, JA application, and infestation by the tea geometrid *Ectropis obliqua* Prout and the tea green leafhopper *Empoasca* (*Matsumurasca*) *onukii* Matsuda. To further elucidate the function of *CsACX1*, it was heterologously expressed in a bacterial system and characterized. Recombinant CsACX1 showed preference for C_{12}– ~ C_{16}-CoA substrates. The constitutive expression of *CsACX1* can rescue wound-related JA biosynthesis in *Arabidopsis* mutant *acx1*. *CsACX1* was expressed in different organs, predominantly in flowers. Notably, *CsACX1* transcripts were detected up-regulated during flower opening, and the JA levels were correlated with *CsACX1* expression. All these results enrich our knowledge of the regulatory pathway involved in the JA biosynthesis in tea, and helps further understand the defense mechanism of tea plant against insects.

1. Introduction

Peroxisomal β-oxidation is the primary pathway of fatty acid degradation in plants. It not only plays an essential role during plant development and defense response to stresses, but also is involved in the synthesis of fatty acid-derived signal molecules such as jasmonic acid (JA), which is vital in the regulation of plant defense against different types of insects (Baker et al., 2006; León, 2013; Kachroo and Robin, 2013; Poelman and Kessler, 2016). In plants, JA is synthesized from linolenic acid through the octadecanoid pathway (Wasternack and Hause, 2002). Free linolenic acid is released from membrane lipids by the lipolytic enzyme, oxygenated by lipoxygenase (LOX), and subsequently converted to 12-oxo-phytodienoic acid (OPDA) by allene oxide synthase (AOS) and allene oxide cyclase (AOC) in chloroplasts (Baker et al., 2006). The pathway is completed in the peroxisomes, where OPDA is reduced by OPDA reductase (OPR), followed by conversion to JA in three cycles of β-oxidation (León, 2013). Thus, JA triggers a cascade of reactions that ultimately results in plant resistant responses, such as the accumulation of defense-related compounds and the release of volatile organic compounds that can repel herbivores and attract their natural enemies (Wu and Baldwin, 2010; Poelman and Kessler, 2016; Turlings and Erb, 2018).

Acyl-CoA oxidase (ACX, EC 1.3.3.6) is the first and the key step controlling enzyme involved in fatty acid β-oxidation; in this process, the fatty acyl-CoA is oxidized to 2-*trans*-enoyl-CoA. During this step, the flavine adenine dinucleotide (FAD)-containing ACX donates electrons directly to molecular oxygen, forming hydrogen peroxide (Arent et al., 2008). In plants, ACX is involved in the mobilization of galactolipid during foliar senescence and starvation and membrane–lipid turnover/remodeling (Hooks et al., 1999). In the organs and tissues of non-oil-rich plants, several other processes also depend on ACX, including embryo development, seed germination, seedling establishment, volatile lactone production, and the synthesis of indole-acetic acid (Baker et al., 2006; Xi et al., 2012; Li et al., 2015). ACX is necessary for wound-induced JA biosynthesis in tomato and *Arabidopsis*. For example, the antisense expression of *AtACX1* reduces wound-activated JA and the expression of JA-responsive genes in *Arabidopsis* (Castillo et al., 2004; Khan et al., 2012). The reduced expression of *LeACX1* in tomato

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Abbreviations

ACX	acyl-CoA oxidase
AOC	allene oxide cyclase
AOS	allene oxide synthase
FAD	flavine adenine dinucleotide
GC–MS	gas chromatography with mass spectrometry
JA	jasmonic acid
JA-Ile	jasmonoyl-isoleucine
LOX	lipoxygenase
OPDA	12-oxo-phytodienoic acid
OPR	OPDA reductase
PTS	peroxisomal targeting signal
qRT-PCR	quantitative real-time PCR
SA	salicylic acid
TG	tea geometrid
TLH	tea green leaf hopper

(*Lycopersicon esculentum*) correlates with defects in the vast majority of wound-induced JA production, and the mutant is compromised in its defense against tobacco hornworm (*Manduca sexta*) attack (Li et al., 2005). In various plants, ACX makes up a family of isoenzymes. In *Arabidopsis*, ACX makes up a family of six enzymes with overlapping specificity for acyl-CoA substrates of various chain lengths (Schilmiller et al., 2007; Khan et al., 2012). Five ACX genes were cloned in peach (Xi et al., 2012). Biochemical characterization has revealed that each ACX enzyme acts on targets with specific chain length, which indicates the functional diversification of ACX in plants (Khan et al., 2012). Multiple isoforms of ACX have been identified in a wide range of annual herbaceous plants. However, the genes encoding these enzymes in perennial woody plants have not received as much attention.

The tea plant, *Camellia sinensis* (L.), is an economically important evergreen horticultural crop that suffers heavily from a chewing insect - the tea geometrid (TG) *Ectropis obliqua* Prout and a piercing insect-the tea green leaf hopper (TLH) *Empoasca* (*Matsumurasca*) *onukii* Matsuda in China (Ye et al., 2014). In response to herbivore attack and mechanical damage, tea plants can accumulate significantly increased amounts of JA (Xin et al., 2016, 2017). Genes showing sequence homology to ACX have been isolated from barley (Grossi et al., 1995), *Phalaenopsis* (Do and Huang, 1997), pumpkin (Hayashi et al., 1999), *Arabidopsis* (Hooks et al., 1999; Eastmond et al., 2000), soybean (Agarwal et al., 2001), and peach (Qi et al., 2012; Xi et al., 2012). Despite the important role of JA, its biosynthesis and signaling have not been documented in tea. Thus, the discovery of genes related to the biosynthesis of JA is crucial for understanding defense mechanisms of tea against herbivores. Recently, five tea putative ACX genes have been submitted to NCBI database (GenBank accession: KX650076.1, KX650078.1, KX650075.1, KX650079.1, and KX650077.1). In this study, we cloned the full-length cDNA of *CsACX1* (GeneBank accession: KX650077.1) and studied different expression of *CsACX1* under herbivore infestation, wound, or JA treatment and in flower opening. *CsACX1* was heterologously expressed in a bacterial system and functionally characterized in vitro. Furthermore, complementation of *CsACX1* rescued the wound-induced JA level in the *Arabidopsis* mutant *acx1*. Our results confirmed that the *CsACX1* encoded enzyme participates in the biosynthesis of JA in tea and helps further elucidate the defense mechanisms of tea plants against herbivores.

2. Materials and methods

2.1. Plant growth

The tea plant (*C. sinensis* L.) cultivar used for the experiments was ‘Longjing 43’. Tea seeds were germinated in seedling trays filled with commercially available vermiculite. Two-month old seedlings were individually planted in communal plastic pots (14 cm i.d. × 15 cm high), irrigated once every other day, and fertilized with rapeseed cake once a month. Plants were kept in a controlled climate room (26 ± 2 °C, 12 h photophase). Three-year old plants were used for the actual experiments. The seeds of *Arabidopsis* ecotype Columbia (WT) and *acx1* were ordered from ABRC (Arabidopsis Biological Resource Center) and grown in soil in a growth chamber maintained at 21 °C under 16 h of light and 8 h of dark.

2.2. Insects

E. obliqua colonies and *E.onukii* nymphs of 3–4 instars were originally obtained from tea fields at the Tea Research Institute of the Chinese Academy of Agricultural Sciences (TRICAAS, N30°10′, E120°5′) in Hangzhou, China, and maintained in the insectaries. Insects were reared by potted fresh ‘Longjing 43’ tea shoots in enclosed net cages (75 cm × 75 cm × 75 cm) and kept in a controlled climate room (26 ± 2 °C, 12 h photophase) with 80% relative humidity.

2.3. Plant treatments

2.3.1. Wound treatment

Tea plants (one per pot) were individually damaged using a needle patch containing 20 pricks on their second leaves, each with 200 pricks (W). Untreated plants were used as control (C).

2.3.2. TG and TLH treatment

Pots with a single plant were used for this experiment. For insect damage, the second leaves of individual plants were continuously infested with a third-instar larva of TG or 15 female TLH starved for 6 h. The leaves used for the experiments were covered with a fine mesh sleeve to prevent the insects from escaping. Leaves that are only covered with a fine mesh sleeve were used for controls (C). Each time point was replicated five times.

2.3.3. JA and Salicylic acid (SA) treatment

Tea plants were individually sprayed with 10 mL of JA (150 μg mL^{-1}) or SA (300 μg mL^{-1}) in 50 mM sodium phosphate buffer (titrated with 1 M citric acid to pH 8 and with 0.01% Tween-20). Control plants were sprayed with 10 mL of the buffer (BUF group).

2.4. Isolation and characterization of CsACX1

The full-length of *CsACX1* was obtained by reverse transcription-polymerase chain reaction (RT-PCR) techniques from total RNA isolated from tea leaves using the primers F1 and F2 (Table S1). The PCR-amplified fragments were cloned into the pMD19-T vector (TaKaRa, http://www.takara-bio.com/) and sequenced.

2.5. Sequence analysis

The ExPaSy (http://www.expasy.org) Translate tool was used to translate the open reading frame (ORF) sequence of *CsACX1* to protein sequence. The molecular mass and theoretical pI of CsACX1 was calculated using the online Computer pI/Mw Tool (http://cn.expasy.org/tools). CsACX1 was used to construct a phylogenetic tree by using MEGA software 7.0 through the UPGMA method with 1, 000 bootstrap replicates.

2.6. Quantitative real-time PCR (qRT-PCR)

For the qRT-PCR analysis, five independent biological samples were used. Plants were harvested at different time points after insect feeding, wounding, or chemical treatment. Total RNA was extracted as described by Xin et al. (2016). One microgram of each total RNA sample was reverse-transcribed using PrimeScript™ RT-PCR Kit (TaKaRa). The qRT-PCR assay was performed on the CFX96™ Real-Time System (Bio-Rad) using Premix Ex Taq™ Kit (TaKaRa). Tea GADPH gene was used as an internal standard for normalization. The primers used for qRT–PCR are shown in Table S1.

2.7. Heterologous expression of CsACX1 in E. coli and purification of recombinant CsACX1

Full-lengths of *CsACX1* were used as the template for a PCR-based approach to construct a vector for *CsACX1* expression with eight C-terminus histidine residues. The ORF of *CsACX1* was amplified using the oligonucleotide primers F3 and R3 (Table S1). The PCR products of *CsACX1* were ligated to the expression vector pET-32a (+) (Invitrogen, Carlsbad, CA, USA), resulting in a recombinant construct vector and transformed into *E. coli* BL21 host strain. The recombinant CsACX1 protein was inducted and purified according to the method of Zhang et al. (2017). The purified proteins were analyzed by SDS-PAGE. The purified recombinant protein fractions were concentrated using Millipore ultracentrifugation filter units, diluted to the required concentration by using ddH$_2$O, and stored at −80 °C.

2.8. Biochemical characterization of recombinant CsACX1

The enzyme activity of the recombinant CsACX1 was analyzed using short-chain substrates C_6– and C_8-CoA, medium-chain substrates C_{10}–, C_{12}–, and C_{14}-CoA, and long-chain substrates C_{16}–, C_{18}–, and C_{20}-CoA (Sigma-Aldrich) with a H_2O_2-coupled spectrophotometric assay according to the method of Zhang et al. (2017). Typical enzyme assays contained 0.5 mg of affinity-purified CsACX1 and 50 mM of acyl-CoA substrate. Protein concentrations were measured according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.9. Plant transformation

The binary Ti vector pCAMBIA1301 was used for transformation. pCAMBIA1301 contains a hygromycin phosphotransferase gene and a β-glucuronidase gene as selection markers, both of which are driven by the cauliflower mosaic virus 35S promoter (CaMV35SP). The ORF sequence of *CsACX1* was cloned using the primers F4 and R4 (Table S1), and then inserted into the pCAMBIA1301, yielding an overexpression construct, *35S*::*CsACX1* (Fig. S1). The vector was transformed into the *Arabidopsis* mutant *acx1* through *Agrobacterium*-mediated floral-dipping method (Clough and Bent, 1998). Two T1 generation transgenic lines were obtained, and T3 generations were used for analyses.

2.10. JA and jasmonoyl-isoleucine (JA-Ile) analysis

Tea flowers in different opening stages and the leaves of different wound-treated *Arabidopsis* lines were used to quantify JA or JA-Ile levels. For the wound treatment, *Arabidopsis* plants were individually damaged using a needle patch on the second leaves (containing 10 pricks), avoiding the major veins, each with 50 pricks (W). Untreated plants were used for C. JA or JA-Ile was analyzed by gas chromatography with mass spectrometry (GC–MS) by using labeled internal standards ($^{13}C_2$-JA or $^{13}C_2$-JA-Ile) according to the method of Lou and Baldwin (2003). Each treatment at each time interval was replicated 5 times.

2.11. Data analysis

Data were analyzed using Statistica 6.0 (SAS Institute). The differences of gene expression levels involving different tissues and flower stages were analyzed by one-way ANOVA; $P < 0.05$ indicates statistical significance. Duncan's multiple-range test was used to detect significant differences between groups. Differences in gene expression levels between two treatments were determined by Student's *t*-test.

3. Results

3.1. Isolation and sequence analysis of CsACX1

Transcriptome analysis shows that the putative ACX gene (GeneBank accession: KX650077.1) was upregulated following TG larvae infestation (unpublished data). In this study, we managed to clone *CsACX1* cDNA from the RNA prepared from tea leaves. The full-length clone included a putative 1995 bp ORF. Its deduced amino acid sequence reveals that *CsACX1* encodes a protein with 664 amino acid residues, predicted relative molecular mass of 74.21 kDa, and theoretical pI of 7.13. A phylogenetic tree based on translated amino acid sequences indicates that the deduced amino acid sequence of CsACX1 has a close relation to that of LeACX1A (AY817109) from tomato (*L. esculentum*) (Li et al., 2005), GmACX1-1 (AAL01887), GmACX1-2 (AAL0188) from soybean (*Glycine max*) (Agarwal et al., 2001), PpACX1 (ppa002510m) from peach (*Prunus persica*) (Xi et al., 2012; Zhang et al., 2017), and AtACX1 (NP_567513) from *Arabidopsis* (Schilmiller et al., 2007) (Fig. 1). According to sequence alignment, CsACX1 shares 85.5%, 86.9%, 83.3%, 82.4%, and 80.8% of amino acid sequence identities with them, respectively (Fig. 2). CsACX1 and the other ACXs used for the sequence assignment share several regions of high homology. A highly conserved region is the proposed flavin mononucletide binding site represented by the motif CGGHGY (Dubourdieu and Fox, 1977). This motif is not completely conserved in AtACX3 and AtACX4 from *Arabidopsis*, perhaps due to its high similarity to mitochondrial acyl-CoA dehydrogenases (Hyrb and Hogg, 1979).

The deduced amino acid sequences of CsACX1 have a carboxy-terminal tripeptide sequence ([SAC]-[KHR]-[LM]) ARL, which is a peroxisomal targeting signal of the PTS1 type (McNew and Goodman, 1996). A similar one is seen in the deduced amino acid sequences of LeACX1A (AKL), GmACX1-1 (ARL), and AtACX1 (ARL). In contrast, AtACX2, AtACX3, and AtACX4 contain an amino-terminal targeting signal of the PTS2 type (McNew and Goodman, 1996) (Fig. 2).

3.2. Wounding, herbivore infestation, and JA treatment enhance the mRNA levels of CsACX1

QRT-PCR analysis revealed that mechanical wounding, herbivore infestation, and JA treatment result in an obvious increase in the transcript levels of *CsACX1* (Fig. 3). The *CsACX1* transcript accumulated immediately (< 0.5 h) after wound treatment and peaked at 4 h (Fig. 3a). In response to herbivore infestation, the expression levels of *CsACX1* increased with the continuous infestation of TG from 2 h to 24 h (Fig. 3b) and increased at 4 h, and peaked at 12 h after TLH attack (Fig. 3c). The induction of *CsACX1* started 6 h after JA treatment, and the level of the transcript remained elevated for 24 h (Fig. 3d). SA treatment did not affect the transcript levels of *CsACX1* (Fig. 3e).

3.3. Biochemical activity analysis of recombinant CsACX1

CsACX1 encodes a presumed translation product with a molecular weight of 74.21 kDa, and the pET32a (+) vector encodes a 20.23 kDa protein, giving the molecular weight of recombinant CsACX1 of approximately 94.44 kDa. A unique band of the expected size was obtained by the SDS–PAGE of the recombinant CsACX1 (Fig. 4a), which is not found on the electrophoresis of the induced control strains

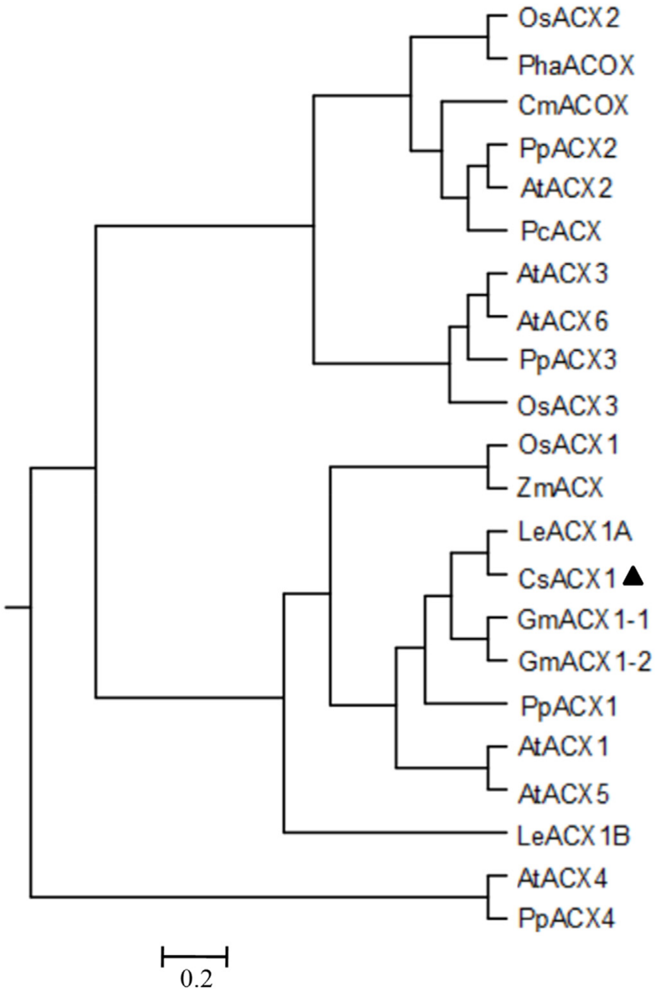

Fig. 1. Phylogenetic analysis of ACXs from tea and other plant species. The alignment of amino acid sequences of ACX was performed using ClustalW. The phylogenetic tree was constructed through the UPGMA method by using MEGA software. Scale bar represents 0.2 substitutions per site. CsACX1 are marked with black triangles. The accession numbers in the GenBank of sequences that are used to build the tree are as follows: AtACX1 (NP_567513), AtACX2 (NP_201316), AtACX3 (NP_172119), AtACX4 (NP_190752), AtACX5 (NP_181112), AtACX6 (NP_172120), GmACX1-1 (AAL01887), GmACX1-2 (AAL01888), PcACX (AAF14635), LeACX1A (AY817109), LeACX1B (AY817110), CmACOX (AAC15870), PhaACOX (AAB67883), ZmACX (AY105897), PpACX1 (ppa002510m), PpACX2 (ppa002282m), PpACX3 (ppa002439m), PpACX4 (ppa005916m), OsACX1 (AP002842), OsACX2 (DP000010), OsACX3 (AP003574), and CsACX1 (KX650077.1).

harboring the empty vector (data not shown). Recombinant CsACX1 activity was detected with the acyl-CoA substrate with differing acyl chain lengths from C_6 to C_{20}. The result shows that CsACX1 has a preference for C_{12} to C_{16} substrates and then C_{18} substrates, exhibiting a peak of activity with C_{16} acyl-CoA, but shows a little activity with short-chain (C_6) acyl-CoA and very long-chain acyl-CoA (C_{20}) (Fig. 4b). Next, with C_{16}-CoA palmitoyl coenzyme A as substrates, a linear activity was observed on five reaction time points (2, 4, 6, 8, and 10 min) (Fig. 4c). For the kinetic analysis, seven concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.2, and 1.4 mM) of the C_{16}-CoA substrate under optimum conditions were assayed to obtain the saturation curve (Fig. 4d).

3.4. *Constitutive expression of CsACX1 rescued wound-induced JA level in Arabidopsis mutant acx1*

The overexpression construct vector *35S*::*CsACX1* was successfully inserted into *Arabidopsis* mutant *acx1* through *Agrobacterium*-mediated floral-dipping, obtaining two T3 homozygous lines, L1 and L2. No obvious difference was observed in the growth between WT plants and transgenic lines (Fig. 5a). Fig. 5b shows that *CsACX1* was constitutively expressed in two independent transgenic lines. Next, the content of endogenous JA was measured in WT, *acx1*, and transgenic lines with or without wounding treatment to verify the function of *CsACX1*. After wounding treatment, the levels of JA drastically increased in WT and L1 and L2 transgenic plants at 1.81, 1.61, and 1.71 μg g^{-1} fw, respectively, in wounded leaves. Wound-induced JA accumulation was also observed in *acx1* plants at only 33.8%, 37.9%, and 35.8% of that in wound-treated WT, L1 and L2 plants, respectively (Fig. 5c).

3.5. *CsACX1 expression, JA and JA-Ile level in flowers under different development stage*

We used qRT-PCR to examine the expression pattern of *CsACX1* in the roots, stems, leaves, flowers, and seeds. *CsACX1* was expressed in all tissues analyzed, predominantly in the fully-opened flowers (Fig. 6). Due to the high *CsACX1* expression in flowers, we analyzed the transcript levels of *CsACX1* during flower-bud opening in five stages, which were designated as follows: S1, maturing buds but not yet green; S2, white, expanded buds with soft tips but not yet open; S3, half-opened flowers; S4, fully-opened flowers; and S5, flowers in senescence (Fig. 7a). *CsACX1* had a low expression level at S1, whereas the transcript accumulated continuously with the development of the flowers and decreased at stage 5 (Fig. 7b). The endogenous levels of JA and its derivative JA-Ile were also quantified under different development stages of tea flowers. Consistent with *CsACX1* expression, S1 presented low JA and JA-Ile levels, whereas JA and JA-Ile started to accumulate rapidly at S2—S4, then reached a peak at S4 and declined at S5 (Fig. 7c and d).

4. Discussion

JA is crucial in the regulation of plant growth, development, and defense. Thus, elucidating the regulation of JA biosynthesis in various crop plants is an important task that has not yet been fulfilled. In this study, we isolated an ACX gene *CsACX1* from the tea plants, which encoded an active ACX, and demonstrated its involvement in the biosynthesis of JA.

The phylogenetic analysis indicates that the deduced amino acid sequence of CsACX1 is relative to that of LeACX1A from tomato (Li et al., 2005) and AtACX1 from *Arabidopsis* (Schilmiller et al., 2007) (Fig. 1). LeACX1A and AtACX1 contribute to the vast majority of wound-induced JA production (Castillo et al., 2004; Li et al., 2005; Khan et al., 2012), which implies that they may have similar biological functions. The deduced amino acid sequences of CsACX1 share a proposed flavin mononucletide binding site represented by the motif CGGHGY (Dubourdieu and Fox, 1977) that is highly conserved in the ACX of known plants (Fig. 2). This motif is not completely conserved in short-chain ACX from *Arabidopsis*, perhaps due to its high similarity to mitochondrial acyl-CoA dehydrogenases (Hyrb and Hogg, 1979). CsACX1, LeACX1A, GmACX1-1, and AtACX1 contain a tripeptide sequence ([SAC]-[KHR]-[LM]) at their C-terminus (Fig. 2), which corresponds to the peroxisomal targeting signal PTS1 (McNew and Goodman, 1996) found in plant peroxisomal enzymes, such as isocitrate lyase and malate synthase (Comai et al., 1989; Graham et al., 1989; Mori et al., 1991). AtACX2, AtACX3, and AtACX4 contain a presequence at their amino terminus (Fig. 2), which correspond to the peroxisomal targeting signal PTS2 (McNew and Goodman, 1996) found in some plant peroxisomal proteins, such as malate dehydrogenase, citrate synthase, and thiolase (Kato et al., 1998). Therefore, plant ACX has either PTS1 or PTS2 with varying mechanism of import (Schaller and Stintzi, 2009).

The transcriptional analysis shows that the transcript levels of

Fig. 2. Alignment of amino acid sequences of CsACX1 and other ACX plant members. Conserved residues are shaded. The sequences were aligned by the CLUSTAL method. The putative flavin mononucleotide binding motif (CGGHGY) and the amino- and carboxy-terminal peroxisomal targeting signals are boxed with red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CsACX1 in leaves were induced by mechanical wounding, herbivore infestation, and JA treatment, but not by SA (Fig. 3). Mechanical wounding or JA treatment can mimic herbivore attacks (Diezel et al., 2009) and induce plant defense responses (Mithofer et al., 2005). Similar ACX expression patterns in response to wounding or exposure to JA/MeJA treatment were also found in other plants (Castillo et al., 2004; Li et al., 2005). In rice, *OsACX1* (the homolog of *CsACX1*) was promptly upregulated in response to wounding and MeJA treatment, whereas *OsACX2* expression remained unchanged (Kim et al., 2007). In *Arabidopsis*, mechanical wounding strongly activated the expression of *AtACX1* but not of *AtACX5*. The distinct expression patterns of ACX isogenes demonstrate their functional diversification in plants (Schilmiller et al., 2007). Mechanical wounding elicits a rapid accumulation of *CsACX1* transcript level (Fig. 3a), which is highly correlated with the rapid increase in endogenous JA levels when tea plant is subjected to mechanical wounding (Xin et al., 2017). Thus, the correlation of *CsACX1* expression and JA levels are consistent with the function of ACX in the octadecanoid metabolism in other plants (Castillo et al., 2004; Li et al., 2005). Compared with mechanical wounding, TG and TLH feeding causes lasting *CsACX1* expression levels

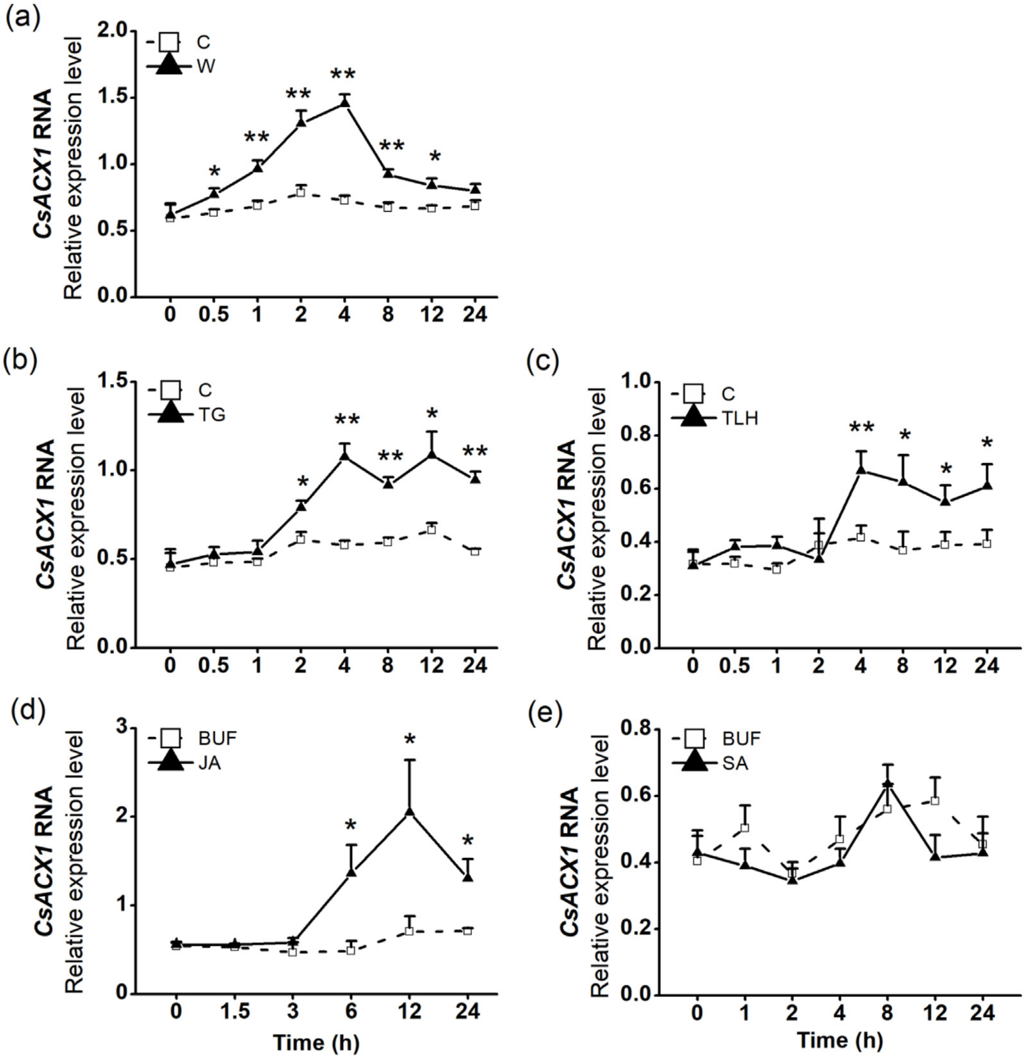

Fig. 3. *CsACX1* expression in tea leaves after different treatments. Mean transcript levels (+SE, $n = 5$) of CsACX1 in tea leaves that were mechanically wounded (a), infested by TG (b) or TLH (c), or treated with JA (d) or SA (e). Transcript levels were analyzed by quantitative RT-PCR. Asterisks indicate significant differences in transcript levels between treatments and controls (C) ($*p < 0.05$; $**p < 0.01$, Student's t tests).

in tea leaves (Fig. 3b and c). On one hand, the continuous mechanical wounding caused by insect herbivory can be one explanation. Notably, TLH can cause greater damage in plants than aphids and other leafhoppers because TLH is a cell rupture feeder and not a salivary sheath feeder (Jin et al., 2012). On the other hand, the longer expression of *CsACX1* may be related to the herbivore-specific elicitors from the regurgitant derived from the salivary system of feeding insects, such as certain enzymes (e.g., β-glucosidase) and fatty acid amino acid conjugates (e.g., volicitin) (Arimura et al., 2005; Yang et al., 2013). Further research should explore the function of the regurgitant on *CsACX1* expression. As a chemical elicitor, JA positively induces the expression of genes tested for JA biosynthesis, such as LOX, AOS, AOC, and OPR (Turner et al., 2002; Liu and Han, 2010; Xin et al., 2017). As expected, JA application can also significantly enhance the expression levels of *CsACX1* (Fig. 3d). Given that herbivore infestation and mechanical wounding increase JA levels and the transcript level of *CsACX1* (Fig. 3; Xin et al., 2016), JA appears to be synthesized in response to wound/herbivore stress cues, and the JA product of this pathway provides a positive feedback loop for the amplification of the signal to enhance plant defense responses (Stintzi et al., 2001; Sasaki et al., 2001).

The first step of β-oxidation is catalyzed by a small family of ACX enzymes that differ in the preference of acyl-CoA substrates with different lengths (Li et al., 2015; Baker et al., 2006). The in vitro enzyme assay data presented here showed CsACX1 has a relatively broad substrate specificity, exhibits activity with medium-to long-chain fatty acyl-CoAs, which is equal for C_{12}– to C_{16}-CoA, and exhibits a peak of activity with C_{16} acyl-CoAs (Fig. 4), indicating CsACX1 enzyme is being generally involved in the β-oxidation of fatty acids. Similar to that of LeACX1A, AtACX1, and two ACXs from soybean (Hooks et al., 1999; Eastmond et al., 2000; Agarwal et al., 2001; Li et al., 2005), these medium-to long-chain subfamily of ACXs also play a prominent role in JA biosynthesis (Li et al., 2005). The similarity in their enzyme properties is perhaps a reflection of the similarities in their nucleotide and amino acid sequences (Figs. 1 and 2). In tomato, LeACX1A and related enzymes possess structural features that facilitate the metabolism of C_{18} cyclopentanoid fatty acids and exhibit a preference for C_{18} cyclopentanoid-CoAs over C_{14}-CoA (Li et al., 2005). The phylogenetic tree indicates that the deduced amino acid sequence of CsACX1 is relative to that of LeACX1A (Identity: 85.5%) (Fig. 2), which implies that CsACX1 may have similar biological functions of facilitating the metabolism of C_{18} cyclopentanoid fatty acids. Thus, CsACX1 is a good candidate enzyme to catalyze the JA biosynthesis in tea. As expected, the over-expression of *CsACX1* rescued the wound-induced JA level in the *Arabidopsis* mutant *acx1* (Fig. 5).

CsACX1 was expressed in all analyzed tissues, predominantly, in flowers (Fig. 6), indicating that β-oxidation is a consistent basic

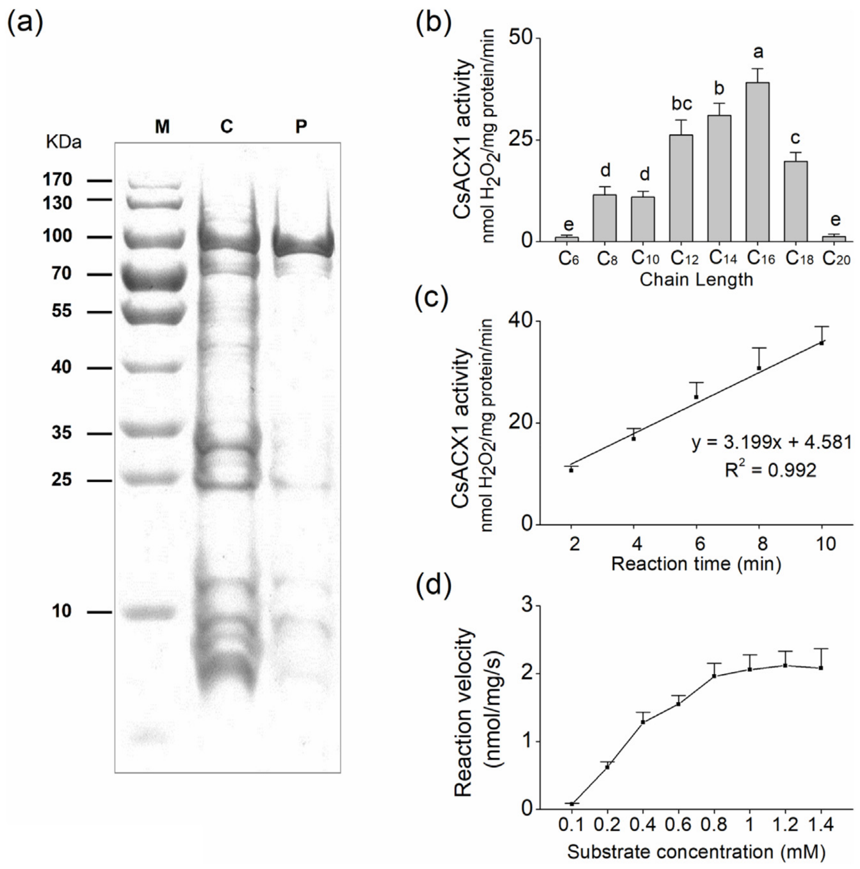

Fig. 4. Biochemical activity analysis of recombinant CsACX1. (a) SDS–PAGE analysis of recombinant CsACX1. CsACX1 was expressed as His-tagged protein in *E. coli* and was purified by ion-exchange and Ni^{2+}-affinity chromatography. **M** molecular marker; **C** crude enzyme; **P** purified enzyme. (b) Mean levels (+SE, *n* = 5) of recombinant CsACX1 protein activities under different substrates. Activities are given in nmol of H_2O_2 produced per minute per mg protein. The abscissa represents the number of carbon atoms in the acyl chains of the substrates used. (c) Mean levels (+SE, *n* = 5) of recombinant CsACX1 protein activities with C_{16}-CoA palmitoyl CoA as the substrate under different reaction times. (d) Mean levels (+SE, *n* = 5) of recombinant CsACX1 protein reaction velocity with C_{16}-CoA palmitoyl CoA as the substrate under different substrate concentrations.

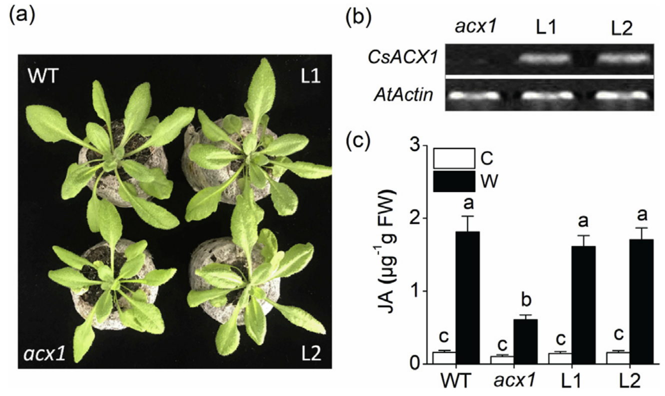

Fig. 5. Complementation experimental analysis of wound-induced JA levels in the *acx1* mutant by *CsACX1*. (a) Growth phenotypes of WT, *acx1*, and L1 and L2 lines in 25-day old plants. (b) *CsACX1* expression in *acx1* and L1 and L2 lines as confirmed by RT-PCR. (c) Mean levels (+SE; *n* = 5) of endogenous JA content in *Arabidopsis* WT, *acx1*, L1 and L2 plants with and without wounding treatment. The aerial part of 25-day old plants was used for the measurement. The letters indicate significant differences among JA levels in *Arabidopsis* WT and different lines ($P < 0.05$; Duncan's multiple-range test).

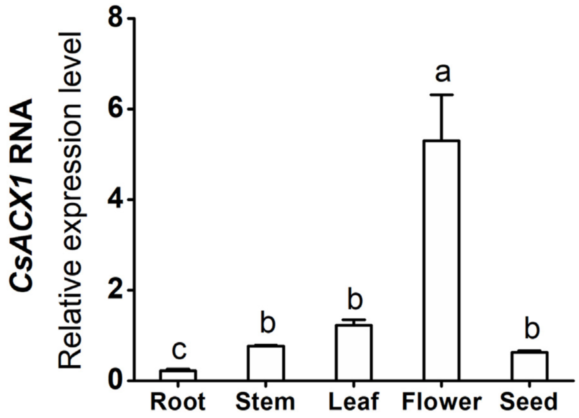

Fig. 6. Expression analysis of *CsACX1* in tea plant tissues. Total RNAs from tea leaves, stems, flowers, roots, and seeds were used for qRT-PCR. The values are expressed as mean + SE of five biological replicates. The letters indicate significant differences among different tissues ($P < 0.05$; Duncan's multiple-range test).

function in all the cells of higher plants (Schilmiller et al., 2007). β-oxidation in non-fatty tissues may be involved in energy generation, turnover of membrane lipids, and removal of toxic fatty acids (Baker et al., 2006; Yang and Ohlrogge, 2009). Due to the high ACX gene expression in flowers, which requires more lipids and energy, we analyzed the transcript levels of *CsACX1* during flower-bud opening. *CsACX1* transcripts were upregulated during flower-bud opening, but sharply decreased during petal senescence (Fig. 7b). Remarkably, *CsACX1* expression during natural opening of tea flowers had a similar pattern to that of JA and JA-Ile levels (Fig. 7c and d). The increased *CsACX1* expression and JA levels during tea flower opening may have two causes. First, physiological and biochemical analyses show that a large amount of lipids have been accumulated and oxidized during flower opening, and lipid oxidation is partially due to increased ACX activity (Thompson et al., 1998). Second, JA pathway was reported to regulate flower development, pollinator advertisement traits and floral defenses in plants (Han et al., 2007; Stitz et al., 2014; Yuan and Zhang, 2015; Li et al., 2017). However, the JA and JA-Ile levels measured in tea flowers are obvious lower than those in other plants, such as tobacco *Nicotiana attenuata* (Li et al., 2017). The low level of JAs may be due to the influence of PVPP which was used to remove PPO in tea.

In conclusion, an ACX gene *CsACX1* was cloned from the tea plant.

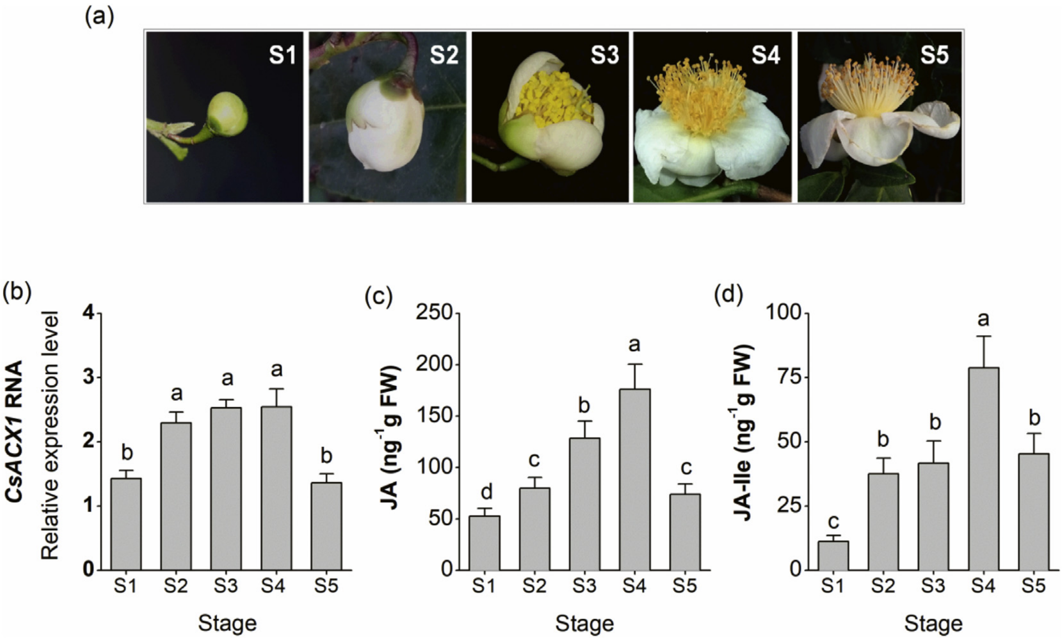

Fig. 7. Relative *CsACX1* expression, JA, and JA-Ile levels in tea flowers at different development stages. (a) The tea-flower-opening development stages. S1, maturing buds but not yet green; S2, white, expanded buds with soft tips but not yet open; S3, half-opened flowers; S4, fully-opened flowers; and S5, flowers in senescence. (b) Mean relative expression levels (+SE; $n = 5$) of *CsACX1* at different development stages. (b,c) Mean concentration (+SE; $n = 5$) of JA (b) and (c) JA-Ile levels at different development stages. The letters indicate significant differences among different stages ($P < 0.05$; Duncan's multiple-range test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CsACX1 is a herbivore-induced gene that plays an important role in the JA biosynthesis in tea. Our results will help in understanding the role of ACX in stress responses and tea–insect interactions at the molecular level. *CsACX1* can be useful in breeding or biotechnological purposes.

CRediT authorship contribution statement

Zhaojun Xin: Methodology, Writing – original draft. **Shenglong Chen:** Formal analysis. **Lingang Ge:** Formal analysis. **Xiwang Li:** Formal analysis. **Xiaoling Sun:** Methodology, Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.11.035.

References

Agarwal, A.K., Qi, Y., Bhat, D.G., Woerner, B.M., Brown, S.M., 2001. Gene isolation and characterization of two acyl CoA oxidases from soybean with broad substrate specificities and enhanced expression in the growing seedling axis. Plant Mol. Biol. 47, 519–531.

Arent, S., Pye, V.E., Henriksen, A., 2008. Structure and function of plant acyl-CoA oxidases. Plant Physiol. Biochem. (Paris) 46, 292–301.

Arimura, G., Kost, C., Boland, W., 2005. Herbivore-induced, indirect plant defences. Biochim. Biophys. Acta 1734, 91–111.

Baker, A., Graham, I.A., Holdsworth, M., Smith, S.M., Theodoulou, F.L., 2006. Chewing the fat: β-oxidation in signalling and development. Trends Plant Sci. 11, 124–132.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Castillo, M.C., Martinez, C., Buchala, A., Metraux, J.P., Leon, J., 2004. Gene-specific involvement of beta-oxidation in wound-activated responses in *Arabidopsis*. Plant Physiol. 135, 85–94.

Clough, S.J., Bent, A.F., 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. 16, 735–743.

Comai, L., Baden, C.S., Harada, J.J., 1989. Deduced sequence of a malate synthase polypeptide encoded by a subclass of the gene family. J. Biol. Chem. 264, 2778–2782.

Diezel, C., von Dahl, C.C., Gaquerel, E., Baldwin, I.T., 2009. Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. Plant Physiol. 150, 1576–1586.

Do, Y.Y., Huang, P.L., 1997. Gene structure of paco1, a petal senescence-related gene from Phalaenopsis encoding a peroxisomal acyl-coA oxidase homolog. Biochem. Mol. Biol. Int. 41, 609–618.

Dubourdieu, M., Fox, J.L., 1977. Amino acid sequence of *Desulfovibrio vulgaris* flavodoxin. J. Biol. Chem. 252, 1453–1463.

Eastmond, P.J., Hooks, M.A., Williams, D., Lange, P., Bechtold, N., Sarrobert, C., Nussaume, L., Graham, I.A., 2000. Promoter trapping of a novel medium-chain acyl-CoA oxidase, which is induced transcriptionally during *Arabidopsis* seed germination. J. Biol. Chem. 275, 34375–34381.

Graham, I.A., Smith, L.M., Brown, J.W., Leaver, C.J., Smith, S.M., 1989. The malate synthase gene of cucumber. Plant Mol. Biol. 13, 673–684.

Grossi, M., Gulli, M., Stanca, A.M., Cattivelli, L., 1995. Characterization of two barley genes that respond rapidly to dehydration stress. Plant Sci. 105, 71–80.

Han, B., Zhou, P., Cui, L., Fu, J., 2007. Characterization of the key aromatic constituents in tea flowers of elite Chinese tea cultivars. Int. J. Tea Sci. 6, 31–36.

Hayashi, H., De Bellis, L., Ciurli, A., Kondo, M., Hayashi, M., Nishimura, M., 1999. A novel acyl-CoA oxidase that can oxidize short-chain acyl-CoA in plant peroxisomes. J. Biol. Chem. 274, 12715–12721.

Hooks, M.A., Kellas, F., Graham, I.A., 1999. Long-chain acyl-CoA oxidases of *Arabidopsis*. Plant J. 20, 1–13.

Hyrb, D.J., Hogg, J.F., 1979. Chain length specificities of peroxisomal and mitochondrial β-oxidation in rat liver. Biochem. Biophys. Res. Commun. 87, 1200–1206.

Jin, S., Chen, Z.M., Backus, E.A., Sun, X.L., Xiao, B., 2012. Characterization of EPG waveforms for the tea green leafhopper, *Empoasca vitis* Göthe (Hemiptera: Cicadellidae), on tea plants and their correlation with stylet activities. J. Insect Physiol. 58, 1235–1244.

Kachroo, A., Robin, G.P., 2013. Systemic signaling during plant defense. Curr. Opin. Plant Biol. 16, 527–533.

Kato, A., Takeda, Y.Y., Hayashi, M., Kondo, M., Hara-Nishimura, I., Nishimura, M., 1998. Glyoxysomal malate dehydrogenase in pumpkin: cloning of a cDNA and functional analysis of its presequence. Plant Cell Physiol. 39, 186–195.

Khan, B.R., Adham, A.R., Zolman, B.K., 2012. Peroxisomal Acyl-CoA oxidase 4 activity differs between *Arabidopsis* accessions. Plant Mol. Biol. 78, 45–58.

Kim, M.C., Kim, T.H., Park, J.H., Moon, B.Y., Lee, C.H., Cho, S.H., 2007. Expression of rice acyl-CoA oxidase isoenzymes in response to wounding. J. Plant Physiol. 164, 665–668.

León, J., 2013. Role of plant peroxisomes in the production of jasmonic acid-based signals. Subcell. Biochem. 69, 299–313.

Li, C., Schilmiller, A.L., Liu, G., Lee, G.I., Jayanty, S., Sageman, C., Vrebalov, J., Giovannoni, J.J., Yagi, K., Kobayashi, Y., Howe, G.A., 2005. Role of β-oxidation in jasmonate biosynthesis and systemic wound signaling in tomato. Plant Cell 17, 971–986.

Li, R., Wang, M., Wang, Y., Schuman, M.C., Weinhold, A., Schäfer, M., Jiménez-Alemán, G.H., Barthel, A., Baldwin, I.T., 2017. Flower-specific jasmonate signaling regulates constitutive floral defenses in wild tobacco. P. Natl. Acad. Sci. USA 114, E7205–E7214.

Li, X.W., Jiang, J., Zhang, L.P., Yu, Y., Ye, Z.W., Wang, X.M., Zhou, J.Y., Chai, M.L., Zhang, H.Q., Arús, P., Jia, H.J., Gao, Z.S., 2015. Identification of volatile and softening-related genes using digital gene expression profiles in melting peach. Tree Genet. Genomes 11, 71.

Liu, S., Han, B., 2010. Differential expression pattern of an acidic 9/13-lipoxygenase in flower opening and senescence and in leaf response to phloem feeders in the tea plant. BMC Plant Biol. 10, 228.

Lou, Y., Baldwin, I.T., 2003. *Manduca sexta* recognition and resistance among allopolyploid Nicotiana host plants. Proc. Natl. Acad. Sci. U.S.A. 100, 14581–14586.

McNew, J.A., Goodman, J.M., 1996. The targeting and assembly of peroxisomal proteins: some old rules do not apply. Trends Biochem. Sci. 21, 54–58.

Mithofer, A., Wanner, G., Boland, W., 2005. Effects of feeding *Spodoptera littoralis* on Lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. Plant Physiol. 137, 1160–1168.

Mori, H., Takeda, Y.Y., Hara, N.I., Nishimura, M., 1991. Pumpkin malate synthase: cloning and sequencing of the cDNA and Northern blot analysis. Eur. J. Biochem. 197, 331–336.

Poelman, E.H., Kessler, A., 2016. Keystone herbivores and the evolution of plant defenses. Trends Plant Sci. 21, 477–485.

Qi, Y.J., Zhang, X., Yang, X., Gao, Z.S., Jia, H.J., 2012. Molecular cloning and sequence analysis of Acyl-CoA Oxidase gene from 'Hujingmilu' peach fruit. Sci. Agric. Sin. 45, 1758–1765.

Sasaki, Y., Asamizu, E., Shibata, D., Nakamura, Y., Kaneko, T., Awai, K., Amagai, M., Kuwata, C., Tsugane, T., Masuda, T., Shimada, H., Takamiya, K., Ohta, H., Tabata, S., 2001. Monitoring of methyl jasmonate-responsive genes in Arabidopsis by cDNA macroarray: self-activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signaling pathways. DNA Res. 8, 153–161.

Schaller, A., Stintzi, A., 2009. Enzymes in jasmonate biosynthesis - structure, function, regulation. Phytochemistry 70, 1532–1538.

Schilmiller, A.L., Koo, A.J.K., Howe, G.A., 2007. Functional diversification of acyl-Coenzyme A oxidases in jasmonic acid biosynthesis and action. Plant Physiol. 143, 812–824.

Stintzi, A., Weber, H., Reymond, P., Browse, J., Farmer, E.E., 2001. Plant defense in the absence of jasmonic acid: the role of cyclopentenones. Proc. Natl. Acad. Sci. U.S.A. 98, 12837–12842.

Stitz, M., Hartl, M., Baldwin, I.T., Gaquerel, E., 2014. Jasmonoyl-L-isoleucine coordinates metabolic networks required for anthesis and floral attractant emission in wild tobacco (*Nicotiana attenuata*). Plant Cell 26, 3964–3983.

Thompson, J.E., Froese, C.D., Madey, E., Smith, M.D., Hong, Y.W., 1998. Lipid metabolism during plant senescence. Prog. Lipid Res. 37, 119–141.

Turlings, T.C.J., Erb, M., 2018. Tritrophic interactions mediated by herbivore-induced plant volatiles: mechanisms, ecological relevance, and application potential. Annu. Rev. Entomol. 63, 433–452.

Turner, J.G., Ellis, C., Devoto, A., 2002. The jasmonate signal pathway. Plant Cell 14, S153–S164.

Wasternack, C., Hause, B., 2002. Jasmonates and octadecanoids: signals in plant stress responses and development. Prog. Nucleic Acid Res. Mol. Biol. 72, 165–221.

Wu, J.Q., Baldwin, I.T., 2010. New insights into plant responses to the attack from insect herbivores. Annu. Rev. Genet. 44, 1–24.

Xi, W.P., Zhang, B., Liang, L., Shen, J.Y., Wei, W.W., Xu, C.J., Allan, A.C., Ferguson, I.B., Chen, K.S., 2012. Postharvest temperature influences volatile lactone production via regulation of acyl-CoA oxidases in peach fruit. Plant Cell Environ. 35, 534–545.

Xin, Z., Li, X., Li, J., Chen, Z., Sun, X., 2016. Application of chemical elicitor (Z)-3-hexenol enhances direct and indirect plant defenses against tea geometrid *Ectropis obliqua*. BioControl 61, 1–12.

Xin, Z., Zhang, J., Ge, L., Lei, S., Han, J., Zhang, X., Li, X., Sun, X., 2017. A putative 12-oxophytodienoate reductase gene *CsOPR3* from *Camellia sinensis*, is involved in wound and herbivore infestation responses. Gene 615, 18–24.

Yang, Z., Ohlrogge, J.B., 2009. Turnover of fatty acids during natural senescence of *Arabidopsis,* Brachypodium, and switchgrass and in *Arabidopsis* β-oxidation mutants. Plant Physiol. 150, 1981–1989.

Yang, Z.W., Duan, X.N., Jin, S., Li, X.W., Chen, Z.M., Ren, B.Z., Sun, X.L., 2013. Regurgitant derived from the tea geometrid *Ectropis obliqua* suppresses wound-induced polyphenol oxidases activity in tea plants. J. Chem. Ecol. 39, 744–751.

Ye, G.Y., Xiao, Q., Chen, M., 2014. Tea: biological control of insect and mite pests in China. Biol. Control 68, 73–91.

Yuan, Z., Zhang, D., 2015. Roles of jasmonate signalling in plant inflorescence and flower development. Curr. Opin. Plant Biol. 27, 44–51.

Zhang, L., Li, H., Gao, L., Qi, Y., Fu, W., Li, X., Zhou, X., Gao, Q., Gao, Z., Jia, H., 2017. Acyl-CoA oxidase 1 is involved in γ-decalactone release from peach (*Prunus persica*) fruit. Plant Cell Rep. 36, 829–842.