Molecular Cloning and Characterization of Hydroperoxide Lyase Gene in the Leaves of Tea Plant (Camellia sinensis)

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ABSTRACT: Hydroperoxide lyase (HPL, E.C. 4.1.2.) is the major enzyme in the biosynthesis of natural volatile aldehydes and alcohols in plants, however, little was known about HPL in tea plants (Camellia sinensis). A unique cDNA fragment was isolated by suppressive subtractive hybridization (SSH) from a tea plant subjected to herbivory by tea geometrid Ectropis obliqua. This full length cDNA acquired by RACE was 1476 bp and encoded 491 amino acids. DNA and protein BLAST searches showed high homology to HPL sequences from other plants. The His-tag expression vector pET-32a(+)/CsHPL was constructed and transferred into Escherichia coli Rosetta (DE3). The expression product of recombinant CsHPL in E. coli was about 60 kDa. The enzyme activity of CsHPL was 0.20 μmol·min⁻¹·mg⁻¹. Quantitative RT-PCR analysis indicated CsHPL was strongly up-regulated in tea plants after Ectropis obliqua attack, suggesting that it may be an important candidate for defense against insects in tea plants.

KEYWORDS: Camellia sinensis, hydroperoxide lyase, Ectropis obliqua, suppressive subtractive hybridization (SSH), induced defense response

INTRODUCTION

Tea plant (Camellia sinensis), an evergreen shrub, is one of the most important economic plants in the world. Tea plants produce specific and healthy compounds such as caffeine, theanine, and catechins. Many reports exist on their content, their biosynthesis, and the healthy function of these compounds. As an evergreen woody plant, the cultivation of a tea plant takes a long time and is very susceptible to biotic (e.g., insect attack) and abiotic (cold and drought, etc.) stresses.

Plant defense mechanisms include both direct and indirect defenses. The lipoxygenase (LOX) pathway, an important indirect defense pathway, includes two biosynthetic routes, mediated by allene oxide synthase (LOX/AOS) or hydroperoxide lyase (LOX/HPL). The LOX/AOS pathway results in production of jasmonic acid (JA). The LOX/HPL pathway results in the derivation of some green leaf volatiles (GLVs), including C6 aldehydes, and alcohols and their esters. HPL is a key enzyme in the LOX/HPL pathway. In recent years, the LOX/HPL pathway has been reported to operate in insect- or pest-induced plant defense. For instance, tobacco plants (Nicotiana tabacum) release herbivore-induced volatiles (such as GLVs) exclusively at night that are highly repellent to female moths (Heliothis virescens). Moreover, Kessler and Baldwin found that volatile emissions from Nicotiana attenuata plants during attack by leaf-feeding herbivores increased egg predation rates by a generalist predator, that linalool and the complete blend decreased lepidopteran oviposition rates, and that a plant, through releasing volatiles, could reduce the number of herbivores by more than 90%.

Fatty acid hydroperoxide lyase (HPL) is an enzyme that cleaves a fatty acid hydroperoxide (HPO) derived from the activity of a lipoxygenase (LOX) into two carbonyl compounds. Depending on the substrate specificities, HPLs can be divided into three groups: 9-HPL, 13-HPL, and 9/13-HPL, which catalyze their corresponded substrates 9-HPO (9-HPOD, 9-hydroperoxy-10(E), 12(Z)-octadecadienoic acid and 9-HPOT, 9-hydroperoxy-10(E), 12(Z), 15(Z)-octadecatrienoic acid), 13-HPO (13-HPOD, 13-hydroperoxy-9(Z), 11(E)-octadecadienoic acid and 13-HPOT, 13-hydroperoxy-9(Z), 11(E), 15(Z)-octadecatrienoic acid) and 9- or 13-HPO, respectively. 9/13-HPL activity has been reported in the fruits of cucumber (Cucumis sativus), seeds and seedlings of soybean (Glycine max), and seedlings of alfalfa (Medicago sativa). However, only 13-HPO activity has been reported in watermelon (Citrus lanatus) seedlings, tea (Camellia sinensis) leaves, apple (Malus pumila) fruits, tomato (Lycopersicon esculentum) leaves and fruits, green bell pepper (Capsicum annuum) fruits, guava (Psidium guajava), and fruit and leaves of potato (Solanum tuberosum). In pear (Pyrus communis) fruits, only 9-HPL has been reported. However, two HPL genes were isolated from the leaves of grape berries (Vitis vinifera). VvHPL1 is specific for 13-HPO with higher activity and VvHPL2 could catalyze the cleavage of both 9- and 13-HPO.

HPLs are widely distributed in plants and produce not only main particular volatiles, but also relate to disease-resistance and pest/insect-resistance in plants. The first cDNA sequence encoding HPL was cloned from bell peppers in 1996. After that, HPL sequences from cucumber, guava, Arabidopsis, citrus, tomato, and other plants were reported. For tea plants, HPL activity was reported previously, however, cDNA cloning and expression analysis of HPL in tea plants has not been investigated up to now.

In our previous studies on the molecular response in tea to feeding by the lepidopteran pest Ectropis obliqua, a suppressive
subtractive hybridization (SSH) library was successfully constructed. A cDNA fragment similar to HPL was found in the SSH library, and differentially expressed after *Ectropis obliqua* feeding. In this study, the full length cDNA of HPL in *Camellia sinensis* (CsHPL) was amplified by RACE method, the copy number of CsHPL was determined by genomic DNA gel blotting, the in vitro catalytic function of the gene was validated using recombinant proteins expressed in *E. coli*, and the expression profile of CsHPL was investigated. These results would lay an essential foundation for further study of the defense function of Cs volatiles in response to insects attack for tea plants.

### MATERIALS AND METHODS

#### Plant Materials and Treatments.

Three-year-old clone cuttings of tea plant (*Camellia sinensis* cv. Shuchazao) were cultured with every three tea cuttings grown in one pot (30 cm diameter, 35 cm height) and grown under controlled environment at the tea plantation in Anhui Agricultural University, Hefei, China.

For insect feeding treatments, three pots with 9 tea plants were treated. Tea geometrids (*E. obliqua*) at the third larval stage were collected after 1/3 of each leaf was consumed by geometrids. To create samples of mechanical damage, tea leaves were sheared by autoclaved scissors to remove amounts of leaf tissue similar to *E. obliqua* larvae feeding. Tea leaves from nontreated plants were used as controls. The samples were collected at 0, 3, 6, 9, and 24 h after 1/3 of each leaf was consumed by geometrids or mechanical damage, immediately frozen in liquid nitrogen and stored at −80°C until use. Three replications were carried out individually.

#### cDNA Cloning.

The cDNA fragment was obtained from our previous studies on genes differentially expressed after *Ectropis obliqua* feeding, which were identified by suppression subtractive hybridization (SSH) (GenBank ID: GW342656). The cDNA was highly similar to HPLs from other plants.

The full length cDNA encoding CsHPL was acquired by Rapid Amplification of cDNA Ends PCR (RACE-PCR). Approximately 120 ng of RNA isolated from young tea leaves were used as the template in the RACE-PCR reaction for the cloning of CsHPL using the protocol described by the manufacturer (RACE CDNA Amplification Kit, Clontech Lab. Inc., USA). 3′ RACE-PCR reaction of CsHPL was conducted using the primer pair UPM (provided by kit) and GSP3 (5′-GACATCTGGCTCGCTCTCCAACTCC-3′) at first, and then by nested PCR using NUP (provided by kit) and NGPS (5′-AAGTTATCCAGAGCGAGACCGGCAAGCCA-3′). 5′ RACE-PCR amplification was carried out to amplify the 5′ end of the CsHPL gene by first using primers UPM and GSP5 (5′-GAACCGCGCTAGGCGTTAAGGCGG-3′), followed by using the nested primers NUP and NGPS (5′-AGTGGAGAGGCGAGGAGATGTC-3′). The PCR products were ligated into pMD-18T easy vector (Takara, China) and the ratio of target protein in total protein by Quantity one analysis. Meanwhile, the pellet was resuspended in distilled deionized water, boiled for 5 min, and loaded for SDS-PAGE as total crude proteins. The remaining samples from the disrupted cells were used for the recombinant CsHPL activity measure. The concentration of recombinant CsHPL was calculated by determining the total protein concentration by spectrophotometer and the ratio of target protein in total protein by Quantity one software in SDS-PAGE electrophoretogram. The substrate (2 μg 13- or 9-HPOT dissolved in EtOH) and the total proteins (40 μL) were mixed with 50 mM potassium phosphate buffer, pH 7.0 to a final volume of 1.0 mL. The recombinant CsHPL activity was assayed according to Vick (1991) by measuring the decrease in absorbance at 234 nm due to disruption of the conjugated diene chromophore of 13- or 9-HPOT.

After that, 13-HPOT was used as substrate to determine the activity of recombinant protein pET-32a(+)//CsHPL, which were induced by IPTG for 10 h. The noninduced pET-32a(+)//CsHPL protein and pET-32a(+) vector were used as control samples. The method for determination of optimum pH and temperature for soluble CsHPL was also carried out using the substrate 13-HPOT.

#### Enzyme Activity Assay.

Bacterial cells in 1 mL of LB media were centrifuged at 13 000g for 10 min at 4°C. The pellets were resuspended in 40 mL of PBS buffer (pH 7.0) containing PMSF (1 mMol-L⁻¹). After cells were disrupted on ice by ultrasonication, both the supernatant and precipitate were collected for SDS-PAGE analysis. Meanwhile, the pellets were resuspended in distilled deionized water, boiled for 5 min, and loaded for SDS-PAGE as total crude proteins. The remaining samples from the disrupted cells were used for the recombinant CsHPL activity measure. The concentration of recombinant CsHPL was calculated by determining the total protein concentration by spectrophotometer and the ratio of target protein in total protein by Quantity one software in SDS-PAGE electrophoretogram. The substrate (2 μg 13- or 9-HPOT dissolved in EtOH) and the total proteins (40 μL) were mixed with 50 mM potassium phosphate buffer, pH 7.0 to a final volume of 1.0 mL. The recombinant CsHPL activity was assayed according to Vick (1991) by measuring the decrease in absorbance at 234 nm due to disruption of the conjugated diene chromophore of 13- or 9-HPOT.

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#### Bioinformatic and Phylogenetic Analyses.

BLAST search using CsHPL cDNA and protein sequence were performed using BLAST program at the NCBI Web site (http://www.ncbi.nlm.gov/BLAST/). The open reading frame (ORF) was found using the ORF Finder. The putative domains were identified using the InterPro database (http://www.ebi.ac.uk/interproscan/). Amino acid sequence alignments were carried out using CLUSTAL X (version 2.0) software. The phylogenetic tree was constructed using MEGA 4.0 package (http://www.megasoftware.net/mega4/mega.html) based on the neighbor-joining method with 1000 replicates.

#### Southern Blot Hybridization Analysis.

Genomic DNA was isolated from tea leaves using a CTAB extraction procedure. DNA samples (10 μg) were digested with restriction enzymes Drul, SpeⅠ, and BglⅡ (Takara, China) and separated by electrophoresis on 1% agarose gels. DNA was blotted onto a Hybond-N+ membrane (Amersham, U.K.) using a semidyrib electrolytoblet. DNA blot was hybridized at 42°C overnight in DIG Easy Hyb buffer (Roche Diagnostics, Germany) with hybridization probes that were prepared by 5′-oligonucleotide end labeling with the following sequences: 5′-ATGCGAGGCTTGGAC-TCTATG-3′. Probe labeling of DIG (DIG Probe Synthesis Kit, Roche) and membrane washing and detection of target DNA were done as described by the manufacturer.

#### Preparation of Recombinant CsHPL Protein in E. coli.

Plasmid vector pET-32a(+) (Novagen, U.S.A.) was used for producing recombinant protein. The open reading frame was amplified by PCR with pfu polymerase (Takara, China) using the cDNA generated from total RNA of *C. sinensis* leaves and gene-specific primers. A pair of forward primer and reverse primer was designed for amplifying the predicted ORF for CsHPL after analysis by ORF Finder software. 5′-CGCGGATCCATGGGCAAGGACCCGCA-3′ (forward, BamHI site underlined), 5′-CCACAGCTTTCACTTTAGTTTTCTCACAC-GGC-3′ (reverse, Hind III site underlined). Amplified DNA fragment was introduced in pMD-18T vector and thereafter transformed to E. coli DH Str, resulting in pMD-18T/CsHPL. The plasmid and pET-32a(+) were digested with the respective restriction enzymes and the CsHPL open reading frame was inserted into pET-32a(+) by ligation to form pET-32a(+)/CsHPL.

After confirming the cloned fragment by sequencing, the construct was transformed into the E. coli Rosetta (DE3) pLysS cells (Novagen, Germany). For comparison, only pET-32a(+) vector was also transformed into E. coli cells. Cells were grown at 37°C overnight in LB media containing ampicillin (50 μg/mL). Following centrifugation, E. coli cells were adjusted to OD600 = 0.3–0.5, and the production of recombinant protein was induced by adding IPTG to a final concentration of 0.2 mM. No IPTG induction samples were treated as controls. Incubation was continued overnight at 16°C.

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GC/MS (Fisons GC 8000 series and Fisons Instruments MD 800 Mass Lab spectrometer). The temperature program was started at the same time as the desorption: it was kept at 40°C for 2 min then raised by 10°C/min to 200°C, where it remained for 2 min. The column used was an HP-Innowax, (0.25 μm film thickness; 30 m x 0.32 mm i.d.; Hewlett-Packard, U.S.A.).

Real-Time Quantitative RT-PCR (qRT-PCR) Analysis. Total RNA was extracted from tea leaves using the RNAprep Pure Plant Kit (Tiangen, China) following the manufacturer’s instruction. cDNA was transcribed from 0.5 μg of each total RNA using a PrimeScript RT Reagent Kit (Takara, Japan). Real-time quantitative PCR (qRT-PCR) was performed using 2 μL of cDNA product and each primer of 0.4 μM in a 25 μL reaction volume with SYBR Premix Ex TaqII (Perfect Real Time; Takara) on a Mini-Opticon real-time PCR system (Bio-Rad, U.S.A.). The GAPDH (Accession No. GE651107) of tea plants was used as an internal control for transcript normalization. The specific primers used for qRT-PCR analysis were designed on the basis of the 3′ or 5′-untranslated regions (UTR) of individual genes using the Primer Premier 5.0 software (http://www.premierbiosoft.com/ primerdesign/). The primer sets for GAPDH were 5′-TTGGCATCG-TTGAAGGTCT-3′ (forward), 5′-CATGGGAACACGGAAAC-3′ (reverse); and for CsHPL were 5′-ATCCCTAACACCGCCATCG-3′ (forward), 5′-CCTTGGAACCAGAAGTAGTC-3′ (reverse). Three replicates of each PCR were run using a program: 95°C for 30 s, followed by 40 cycles (95°C for 5 s and 60°C for 30 s).

The amplification efficiencies for all genes tested in this study ranged from 95% to approximately 110%. Data were analyzed according to the threshold cycle (Ct). The relative changes in gene expression were quantified using the 2-ΔΔCt method. The indicated differences are based on Duncan’s multiple range tests using DPS software (www.chinadps.net).

Figure 1. Alignment of amino acid sequences of CsHPL (GenBank accession no. AD051747) with HPLs from other plant species. Guava, Psidium guajava (PgHPL, AF239670), bell pepper, Capsicum annum (CaHPL, U51674), Arabidopsis, Arabidopsis thaliana (AtHPL, AF087932), tomato, Lycopersicon esculentum (LeHPL, AJ239065), tobacco, Nicotiana tabacum (NiHPL, AJ414400) and alfalfa, Medicago sativa (MsHPL, AJ249246) were aligned with CLUSTALW. The A, B, C, D domains for cytochrome P450s are boxed. The conserved sequence LPxRxIPGSYGxPxxGP of CYP74 subfamily of P450 proteins is underlined. The highly conserved isoleucine in Domain A is shown with an asterisk, and the heme-binding cysteines and phenylalanines are shown with dots in Domain D.
RESULTS

Isolation of cDNA Coding for HPL in *Camellia sinensis.* In our previous studies, a cDNA sequence (GenBank ID: GW342656) was isolated from tea plant and BLAST searching against the GenBank database showed high similarity to HPLs. To obtain the complete cDNA sequence corresponding to the cDNA fragment in *C. sinensis,* 5′ and 3′ RACE were performed. After sequencing, a full length HPL of 1662 bp was submitted to GenBank, with accession number HM 440156, and designated CsHPL. The CsHPL cDNA contains a 51 bp 5′-untranslated region (UTR) and a 106 bp 3′-UTR, excluding the poly(A) tail. Predicted by the Protein program in DNAStar software, open reading frame (ORF) was predicted. The deduced translation product of CsHPL consisted of 491 amino acids with a relative molecular mass of 54880 Da and a theoretical isoelectric point of 8.02.

Alignment of HPLs and Phylogenetic Relationship Analysis. The deduced amino acid sequence of CsHPL showed the highest homology with a HPL from *Psidium guajava* (71% identity),17 and 66% identity to a *Capsicum annuum* HPL.21 CsHPL contains 4 highly conserved domains (Domains A, B, C, and D) found in the Cytochrome P450 family (Figure 1). This establishes CsHPL as a new member of the CYB74B subfamily of Cyt P450s. Five putative AUG start codons (encoding Met 1, 5, 8, 9, and 15) in upstream of the deduced translation product of CsHPL were pointed out by arrows in the Lanes 3 and 4. The bands of pET-32a(+)/CsHPL were pointed out by arrows in the Lanes 3 and 4.

Figure 3. SDS-PAGE analysis of recombinant CsHPL protein expressed in *E. coli.* CsHPL was cloned into pET-32a(+) vector, and expressed in *E. coli* (Rosetta (DE3) pLysS). After IPTG-induced protein expression, total cells or cell lysates after sonication were subjected to SDS-PAGE, the gel was stained with Coomassie blue R-250. The lanes M and 1−5 are plotted as follows: M, protein molecular mass marker; 1, total crude protein from uninduced cells were transformed with pET-32a(+) vector alone; 2, total crude protein from induced cells were transformed with pET-32a(+) vector alone; 3, precipitation of total crude protein from induced cells were transformed with pET-32a(+) vector alone; 4, suspension of total crude protein from induced cells were transformed with pET-32a(+) /CsHPL after sonication; 5, total crude protein from induced cells were transformed with PET-32a(+) /CsHPL. The lanes 1a, 4a and 4b are the repetition samples of 1 and 4. The bands of pET-32a(+) /CsHPL were pointed out by arrows in the Lanes 3−4.

Genomic Southern Blotting Analysis. Southern blotting was performed to identify the number of CsHPL-related genes in *C. sinensis.* Total genomic DNA of tea leaves was digested with restriction enzymes with recognition sites (SpeI, DraI, BglII) not in the CsHPL cDNA. The cDNA of CsHPL was used as hybridization probe. One band was observed in each digestion (Figure 2), suggesting that only one copy of the CsHPL gene exists in tea genomic DNA.

Expression of CsHPL in *E. coli.* To confirm CsHPL as an HPL-encoding cDNA, recombinant protein was expressed in *E. coli* and subjected to in vitro functional enzyme assay. The putative ORF of CsHPL in *E. coli* was induced by different concentrations of IPTG (0.05−1 mM) at various temperatures ranging from 15 to 37 °C. Bacterial cultures induced to express after the recombinant pET-32a(+) /CsHPL was induced by IPTG, which coincided with the molecular weight predicted by DNAStar software.

Enzyme Activity Determination and Product Identification by GC/MS. Both the 13- and 9-HPOT substrates were used to determine the activity of recombinant CsHPL. The decrease in absorbance at 234 nm was only found when incubated with 13-HPOT, while the absorbance value was always stable when 9-HPOT was employed. The noninduced pET-32a(+) /CsHPL and pET-32a(+) vector *E. coli* samples were used as controls to evaluate the activity of recombinant CsHPL protein (Figure 4A).

After that, 13-HPOT was used as substrate to determine the activity of recombinant CsHPL, which were induced by IPTG for 10 h. The enzymatic activity of CsHPL was measured at different pH (5.0−9.0) at various temperatures ranging from 20 to 40 °C (Figure 4B, C). The optimum pH and temperature were 7.0 and 25 °C. The activity of recombinant protein pET-32a(+) /CsHPL was determined with 13-HPOT under 25 °C at a pH of 7.0.

The reaction with 13-HPOT as substrate and lysates of cells with the recombinant plasmid pET-32a(+) /CsHPL after IPTG induction was determined by Gas Chromatography (Figure 5B), and the chromatogram of control was shown in Figure 5A. Gas chromatography/mass spectrometry (GC/MS) was used to identify the metabolite produced upon incubation of 13-HPOT with lysates from bacteria that expressed the recombinant protein pET-32a(+) /CsHPL. The GC profile
induction reached 1.2 fold at 12 h. A similar trend was found in another tea variety, *Camellia sinensis* cv. Longjing 43 (data not shown). In this variety, the maximum transcript levels were found at 3 h after insect feeding and mechanical damage, and then gradually dropped back to original values in the following 21 h.

**DISCUSSION**

Fatty acid hydroperoxides produced by 13-lipoxygenase are important intermediates in the oxylipin pathway of fatty acid oxygenation in plants. One pathway for 13-HPOT metabolism is initiated by the enzyme fatty acid HPL. Aldehyde products of HPL, together with the corresponding reduced alcohols, play important roles in the odor of fruits, vegetables, and green leaves. Among them, C₆ aldehydes produced by HPL can act as phytoalexins against protozoa, bacteria, and fungi and may be signals for gene regulation. The C₁₂ oxo-acid product of HPL is the precursor of the previously identified “wound signal” known as traumatin. In our previous studies on the molecular response in tea plant to *Ectropis obliqua* feeding, a cDNA fragment from HPL was obtained through suppressive subtractive hybridization (SSH). Herein, the full length *HPL* in *Camellia sinensis* (*CsHPL*) was amplified by RACE. Molecular cloning, transcript expression profile and response, and biochemical activity of CsHPL were investigated.

*CsHPL* Southern blotting revealed a simple hybridization pattern (Figure 2), indicating that *CsHPL* may be present as a single copy in the tea genome. A similar result was reported in almond. After comparison with other plants HPLs, the deduced amino acid sequence of CsHPL was found to contain the 4 conserved domains (Domains A, B, C, and D), which are the typical structure of Cytochrome P450 family proteins. CsHPL carries the highly conserved isoleucine in the I-helix region in Domain A (asterisk, Figure 1), which is replaced by valine in *Arabidopsis* HPL (Genbank ID: AF087932). The heme-binding cysteines and phenylalanines were also found in the CsHPL Domain D (circle dot in Figure 1). A conserved sequence “LPxRxPGxGYxPxGP” indicates that CsHPL is a new member of the CYP74B subfamily of Cyt P450s (underlined in Figure 1).

Expression of *CsHPL* in *E. coli* illustrated that this cDNA encodes a functional member of the CYP74B subfamily of enzymes. CsHPL catalyzed 13- but not 9-hydroperoxides of C₁₈ fatty acid. This feature was also found in HPLs isolated from other plants, for example, bell pepper, *Arabidopsis,* and tomato. GC/MS analysis of the recombinant CsHPL lyase reaction product identified 3(Z)-hexenal as the main product.

Damage inflicted to tea leaves by either geometrid attack or mechanical damage both induced an accumulation of *CsHPL* (Figure 6). The accumulation of *CsHPL* transcripts can contribute to understanding its function in plants. After wounding, the enhanced transcription levels of *HPL* catalyzed the cleavage of polyunsaturated fatty acid hydroperoxides to aldehydes and oxoacids. These aldehydes play an important role in producing the aroma profile in tea leaves. Additionally, they have antimicrobial activity and are thought to be involved in the plant defense response against pest and pathogen attack. Further, HPL catabolites may function in a key step of the resistance mechanism of plants against some sucking insect pests or other herbivores. It will be interesting to investigate the repression in *E. obliqua,* since this lepidopteran is a key pest of tea.
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The authors declare no competing financial interest.

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