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ORIGINAL RESEARCH PAPER

Molecular cloning and characterization of α-amylase/subtilisin inhibitor from rhizome of *Ligusticum chuanxiong*

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Abstract
Objectives To clone and characterize a novel bi-functional α-amylase/subtilisin inhibitor (*LASI*) from the rhizome of *Ligusticum chuanxiong*, a traditional Chinese medicine.
Results The LASI showed strong homology with members of the Kunitz trypsin inhibitor family. Its putative amino acid sequence has a 40 % identity with that of the α-amylase/subtilisin inhibitor from rice. *LASI* gene without signal peptide was expressed in *E. coli* Rosetta. After purification, the recombinant LASI protein was inhibitory against not only α-amylase from porcine pancreas, *Helicoverpa armigera*, *Spodoptera litura* and *Plutella xylostella*, but also subtilisin A, but not against trypsin or chymotrypsin. In addition, the expression level of *LASI* in rhizome was higher than that in leaf and *LASI* expression was enhanced by salt, chilling and drought treatment.
Conclusions This is the first member of the Kunitz-protease inhibitor family identified in traditional Chinese medicine and it might be involved in the plant defense responses against lepidopterous pests, microorganisms and abiotic stresses.

Keywords Abiotic stress · α-Amylase/Subtilisin inhibitor · Heterologous expression · Lepidopterous pest · Microorganism

Introduction

α-Amylase/subtilisin inhibitors inhibit not only mammalian α-amylase but also α-amylase from lepidopterous pests (Franco et al. 2002). For this reason, α-amylase/subtilisin inhibitors can act as a defense factor to protect plants against lepidopterous pests. Furthermore, α-amylase/subtilisin inhibitors strongly inhibit bacterial subtilisin and therefore they have been implicated in plant defense system against microorganisms. α-Amylase/subtilisin inhibitors are present in significant quantities in the seeds of plants. To date, several α-amylase/subtilisin inhibitors have been purified and characterized from a number of various plants, including rice, barley, wheat, and triticale, etc. These inhibitors are similar to soybean Kunitz trypsin-inhibitor family.

Ligusticum chuanxiong L. (Apiaceae), a herb commonly cultivated in Sichuan province of China,

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has attracted attention as its rhizome can be used for the treatment of headache, rheumatic arthralgia, cardiovascular diseases, menstrual disorders and swelling pain (Li et al. 2012). Previously, we obtained the transcriptome of *L. chuanxiong* (Song et al. 2015). Based on function annotations, a unigene (C2889) that had the highest homology with *Helianthus annuus* Kunitz-like protease inhibitor (AFL91226) was found and named as *L. chuanxiong* α-amylase/subtilisin inhibitor (*LASI*). It had a fragment of 515 bp length but it is an incomplete ORF. Since this is the first time that α-amylase/subtilisin inhibitor has been found in a rhizome, the main aim in this paper was the full-length molecular cloning of *LASI* from the rhizome and its expression in *Escherichia coli*. In addition, the in vitro activities of the recombinant LASI against α-amylase, including mammalian α-amylase and α-amylase of pests, and bacterial subtilisin were evaluated. Finally, the expression pattern of *LASI* in different organs and under various stresses was investigated. This gene might therefore be useful for the transgenic engineering to improve the resistance of plants against pests and microorganisms.

Materials and methods

RNA isolation and PCR cloning

The mature rhizome of *L. chuanxiong* was collected and ground into a fine powder in liquid N_2. Total RNA was isolated from rhizome of *L. chuanxiong* following the protocol of Li et al. (2015) and treated by DNase I (1 unit μg^{-1}) to remove DNA contamination. The treated RNA was used to synthesize cDNA using oligo dT-AP primer (5′-GCT GTC AAC GAT ACG CTA CGT AAC GGC ATG ACA GTG TTT TTT TTT TTT TTT TTT-3′). The 5′- and 3′-ends were obtained by rapid amplification of cDNA ends (RACE). The first PCR for 3′-RACE was performed by Ex Taq (TaKaRa, Japan) based on 1 μl tailed cDNA template in 25 μl reaction buffer using 5′-TAC GAG GTA TGG GAG GTG GTG-3′ and 5′-GTC AAC GAT ACG CTA CGT AAC G-3′ as primers. The second PCR for 3′-RACE was carried out by Ex Taq based on 1 μl PCR products from first PCR as template in 50 μl reaction buffer using 5′-TTG TTT TCT GCC CAA CCG TAT-3′ and 5′-GTC AAC GAT ACG CTA CGT AAC G-3′ as primers. The first PCR for 5′-RACE was performed by Ex Taq (TaKaRa, Japan) based on 1 μl tailed cDNA template in 25 μl reaction buffer using 5′-GGG AAG GTT ACC GTT GTC TGT TT-3′ and oligo dT-T11 (5′-AGG ACT CAC TAT AGG GCT TTT TTT TTT TVN-3′) as primers. The second PCR for 5′-RACE was carried out by Ex Taq based on 1 μl PCR products from first PCR as template in 50 μl reaction buffer using 5′-GCA TCC CCT GAA ACA AGA GAG-3′ and 5′-GTA ATA CGA CTC ACT ATA GGG C-3′ as primers. All resulting products were subcloned into pMD19-T vectors (Takara) and sequenced (Shanghai Sangon, China). The specific primers were designed with Primer Premier 5.0 software. Multiple alignments of deduced amino acid sequences were carried out using DNAMAN software. The phylogenetic relationship of LASI was analyzed with MEGA 6.0 programs (Li et al. 2015).

Construction of expression plasmids

The protein coding region of *LASI* was amplified with 5′-primer with an *Bam*H1 site before the start codon (5′-CGC GGA TCC G *ATG* CAT CGC CTG ATG CT-3′, the *Bam*H1 site is underlined and the initiation codon is shown in italics) and 3′-primer inserting a *Eco*R1 site after the stop codon (5′-CCG GAA TTC *TCA* AAC CTT CAA GAA CAT AAC CA-3′, the *Eco*R1 site is underlined and the stop codon is shown in italics). The fragment was cloned into a pMD19-T for DNA sequencing to verify no mutation occurred. The *LASI*-coding DNA clone was subsequently isolated by digestion with *Bam*H1 and *Eco*R1 and then integrated into a purified pET28a vector (Amersham), containing a histidine tag at its *N*-terminus, digested with the same enzymes, resulting in a recombinant plasmid pET28a-*LASI*. The recombinant plasmid pET28a-*LASI* and empty vector pET28a were transformed into *E. coli* Rosetta (DE3) using standard procedures.

Protein expression and purification

Heterologous expression of the recombinant LASI protein in *E. coli* was carried out as described in our previous report (Liu et al. 2015). The transformed cells were incubated in lysogeny broth (LB) in the presence of 0.1 mg kanamycin ml^{-1} and 0.07 mg chloramphenicol ml^{-1} at 37 °C. When the OD_{600} reached 0.6, IPTG was added to 1 mM to induce the expression of

the recombinant protein. After 8 h at 37 °C, the cells were harvested by centrifugation at 5000×*g* for 20 min at 4 °C. The pellet was resuspended in 10 ml 20 mM KH_2PO_4/K_2HPO_4 buffer, pH 8.0 and disrupted ultrasonically. The resulting lysate was centrifuged (13,000×*g* for 10 min at 4 °C). The supernatant was loaded onto a Ni–NTA His binding resin previously equilibrated with buffer [20 mM Tris/HCl (pH 7.9), 150 mM NaCl] then washed with buffer [10 mM Tris/HCl (pH 7.9), 150 mM NaCl and 20 mM imidazole] and finally the LASI proteins were eluted with 200 mM imidazole, which was subsequently removed by dialysis using 0.05 mM Tris/HCl, pH 7.4 containing 10 % (v/v) glycerol. Protein concentration was measured by the Bradford method. Fifteen microgram protein extract was analyzed using 12 % SDS-PAGE gels. To confirm the homogeneity of the LASI protein, gel fragments containing Coomassie Blue-stained LASI band were digested by trypsin followed by ESI–MS/MS. Partial amino acid sequences were analyzed by *de novo* sequencing technology. The sequences derived from ESI–MS/MS were submitted to automatic alignment with LASI using DNAMAN software.

Preparation of crude α-amylase from pests

The larvae (fourth instars) of *Helicoverpa armigera*, *Spodoptera litura* and *Plutella xylostella* were purchased from Ji Yuan Bai Yun Industry Co, China. Fifty larvae were ground into powder in liquid N_2. The powder was suspended in 10 ml 150 mM NaCl and homogenized on ice. After centrifugation for 10 min at 10,000×*g* at 4 °C, the supernatant was used as a source of α-amylase and stored at −20 °C.

Inhibitory activities of LASI

The activities of α-amylases from porcine pancreas and pests were determined using the soluble starch as substrate, as described by Yamagata et al. (1998). The assay of inhibitory activity against subtilisin A was performed according to the method of Hermosa et al. (2006). The activity of bovine trypsin and chymotrypsin was determined using the synthetic substrate *N*-benzoyl-DL-arginine-*p*-nitroaniline (BAPNA) and substrates *N*-α-glutaryl-L-phenylalanine-*p*-nitroanilide (GPNA), respectively (Teles et al. 2004). The percentage inhibition of protease and α-amylase enzyme was calculated using the following formula: $\text{Inhibition}(\%) = 100 \times [(\text{control} - \text{test})/\text{control}]$.

Expression pattern analysis of *LASI* in various organs and under different stresses

For chilling stress, *L. chuanxiong* was held at 4 °C for 0, 1, 6, 12, 24 and 48 h. For drought and salt stresses, *L. chuanxiong* were cultivated supplemented with PEG 6000 (100 mg ml^{-1}), or NaCl (200 mM) for 0, 1, 6, 12, 24 and 48 h, respectively. The total RNA of *L. chuanxiong* leaf and rhizome under different stresses were extracted and subjected to cDNA synthesis with the same method used for 5′-RACE, respectively.

Gene-specific primer pairs for real-time PCR of *LASI* (5′-TTA CGA GGT ATG GGA GGT GGT-3′ and 5′-GGG AAG GTT ACC GTT GTC TGT-3′) were designed using Primer Premier 5.0 based on the full-length cDNA. Real-time PCR was performed using SYBR Premix Ex Taq II (Takara, Japan) on LightCycler 96 system quantitative PCR machine (Roche Diagnostics, Mannheim, Germany). In each run, 1 µl cDNA template was added to 16 µl reaction buffer under the following conditions: pre-denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 62 °C for 10 s, and 72 °C for 20 s. As an internal control, level of RPL11 (5′-CTC CTT GGT AAC CCT GTG CTG A-3′ and 5′-GTG ATA CTG GAT GTT TTG GCT TTG-3′) was quantified in parallel with *LASI* gene (Song et al. 2015). Normalization and fold changes were calculated using the ΔΔCt method. Three biological repeats of each tissue were performed in the analysis.

Results and discussion

Cloning and sequence analysis of the cDNA of *LASI*

The full-length cDNA sequence (860 bp) of *LASI* contained the poly(A) tail and an ORF. There was a 91 bp 5′-untranslated sequence before the translation initiation code and a 157 bp 3′-untranslated region after the termination code. The ORF was composed of 612 bp which was deduced to encode a protein of 203 amino acid residues. The calculated molecular mass and predicted pI of the theoretical polypeptide were 22,570 Da and 5.06, respectively. The nucleotide

sequence of *LASI* cDNA has been submitted to the NCBI GenBank (accession no.KX580040). Based on the signal peptide analysis procedure using http://www.cbs.dtu.dk/services/SignalP/, the NH_2-terminal signal peptide of LASI, which contained a long stretch of hydrophobic amino acid residues, was observed and the signal peptidase processing occurred after residue Gly23, as shown in Fig. 1. Interestingly, BASI, RASI, HbASI and KTI also contained signal peptides of 22, 22, 21 and 25 amino acids respectively. However, the signal peptide of LASI had low sequence similarity with that of BASI, RASI and HbASI, but rather with STI. This might reflect the different localization between LASI and BASI, RASI, HbASI, respectively.

BLAST analysis showed that LASI had significant sequence identities (>50 %) with a number of plant Kunitz protease inhibitors. The sequence of the deduced protein shared 68, 67 and 58 % identity with the Kunitz protease inhibitor from *Cynara cardunculus*, *H. annuus* and *Theobroma cacao*, respectively. LASI was subjected to phylogenetic analysis with various ASIs in order to understand the relationship of LASI with other ASIs. It was found that LASI was close to RASI (GenBank number P29421) (Supplementary Fig. 1).

As shown in Fig. 1, multiple alignments of LASI and Kunitz family members with known target enzyme specificity revealed that there were two regions proposed for plant Kunitz inhibitors, including the protease (including trypsin, chymotrypsin and subtilisin) inhibitory region and the α-amylase inhibitory region. The protease inhibitory region had a typical and diverse active motif (P3′-P2′-P1-P2-P3), in which the P1 residue was Lys or Arg for Kunitz trypsin inhibitor and Ala for Kunitz chymotrypsin inhibitor, respectively (Ramos et al. 2012; Liu et al. 2015). It was replaced by Asp91 in LASI, suggesting LASI had no inhibitory activity against trypsin or chymotrypsin. Interestingly, a neighboring Thr88 residue is important for enzyme inhibitor interaction involved in the binding with subtilisin. When the Thr residue of BASI was replaced by a Val residue, the inhibitory activity was lost (Micheelsen et al. 2008). Therefore, the functional diversity of protease inhibitory region

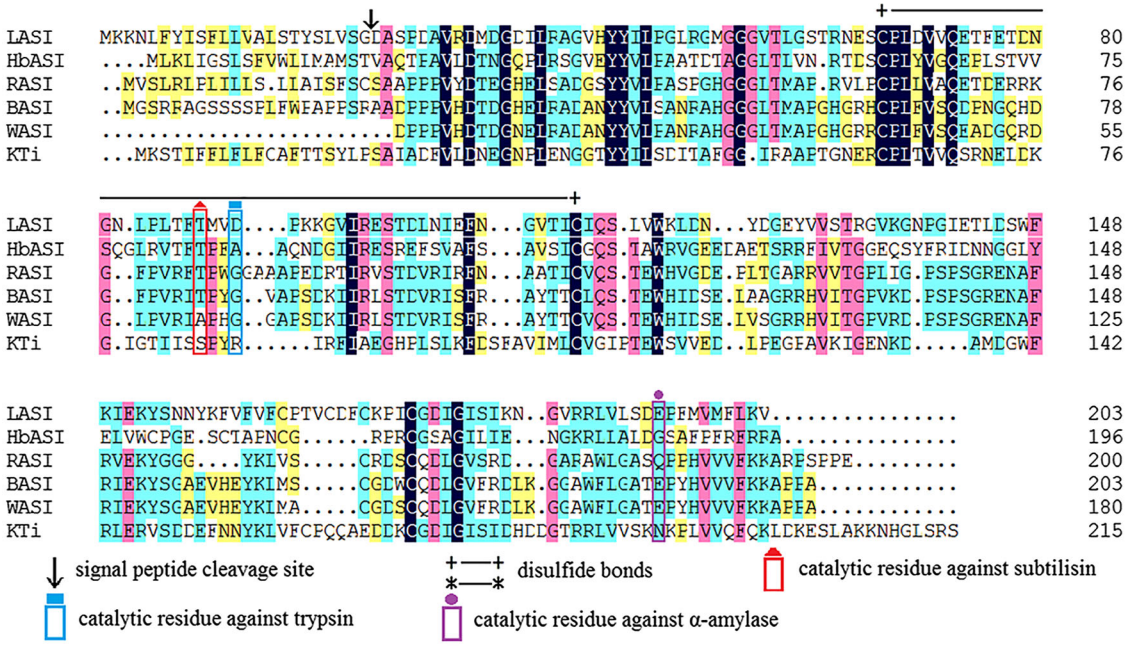

Fig. 1 Sequence alignment of LASI with other members of the α-amylase/subtilisin inhibitors and Kunitz-type trypsin inhibitor. BASI (P07596), RASI (P29421), WASI (P16347) and HbASI (KM979450) were α-amylase/subtilisin inhibitors from barley, rice, wheat and *Hevea brasiliensis*, respectively. STI (AAB23464) was Kunitz trypsin inhibitor from soybean. The accession numbers are shown in brackets. Similar and identical amino acids residues in whole sequences are outlined. The Thr88 and Glu190 residues are indicated by box and vertical arrow, respectively. Disulfide bonds are shown by +—+ and *—*

suggested that the existing Kunitz protease inhibitors might be originated from the identical ancestor gene and the diversification was created during the evolution (Dai et al. 2012). Meanwhile, LASI contained a conserved Glu194, which was believed to be crucial for inhibition of α-amylase. Moreover, LASI contained four Cys residues (Cys67, 113, 171 and 175) that might be involved to form intramolecular disulfide bonds (Fig. 1).

Expression of recombinant LASI

Due to the lack of a cleavage mechanism for the signal peptide in prokaryotic expression system, the amplification product using expressing primers did not contain the *N*-terminal signal peptide. The recombinant LASI was expressed in transformed *E. coli* Rosetta (DE3) with a molecular weight of 23 kDa (Fig. 2). Its molecular weight was higher than that of the predicted mass of 19.9 kDa due to the His-tag in the *N*-terminal fusion peptide. The recombinant LASI was loaded on Ni–NTA His binding resin and further purified by elution with 200 mM imidazole. The purified LASI showing a single band in SDS-PAGE (Fig. 2, Lane 4) was used in the enzyme inhibition assay. After trypsin digestion, three peptide fragments of LASI, *m/z* 1091.54, 1529.70 and 1855.96 with high intensity were chosen for ESI–MS/MS. These three peptides of $[M + H]^+$ *m/z* 1091.54 (GMGGGV TLGSTR), 1529.70 (LDNYDGEYVVSTR) and 1855.96 (RLVLSDQPFMVMFLK) showed identities of 100, 100 and 93.33 % with LASI, respectively, confirming the homogeneous form of the LASI protein.

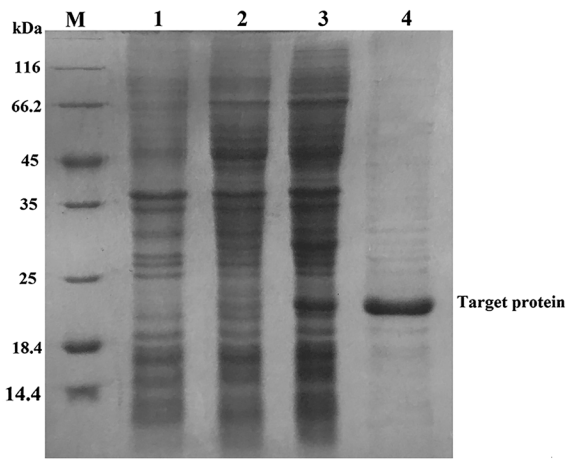

Fig. 2 Analysis of recombinant LASI expressed in *E. coli* by SDS-PAGE. *Lane "M"* indicates protein maker. *Lane 1* indicated the collected solution of *E. coli* carrying pET28a. *Lane 2 and 3* indicated the collected solution of *E. coli* carrying pET28a-*LASI* vector induced by 0 mM IPTG and 1 mM IPTG, respectively. *Lane 4* indicated the collected solution eluted with 200 mM imidazole on Ni–NTA His Bind resin

Inhibitory activity of the recombinant LASI

LASI (47 μg) inhibited subtilisin A and α-amylase from porcine pancreas, *P. xylostella*, *H. armigera* and *S. litura*, whereas trypsin and chymotrypsin were not affected (Fig. 3). The inhibition activity of LASI against subtilisin A (56 %) was higher than its inhibition activity against porcine pancreatic α-amylase (27.5 %), suggesting LASI might be an effective inhibitor against the growth of microgram. In addition, LASI could inhibit the α-amylase from *S. litura*, *H. armigera* and *P. xylostella* with inhibitory activities of 70, 61 and 31 % at 47 μg, respectively. Due to the strong inhibitory activity of LASI against subtilisn A, the inhibition activity of LASI at different molar ratios (0, 0.185, 0.369, 0.554, 0.738, 1.107 and 1.476) was also investigated. The IC_{50} molar ratio of LASI towards subtilisin A was 0.734 (Fig. 4).

LASI was a weak inhibitor of α-amylase from porcine pancreas, and relative strong inhibitor of pest α-amylases, especially α-amylases from *H. armigera*

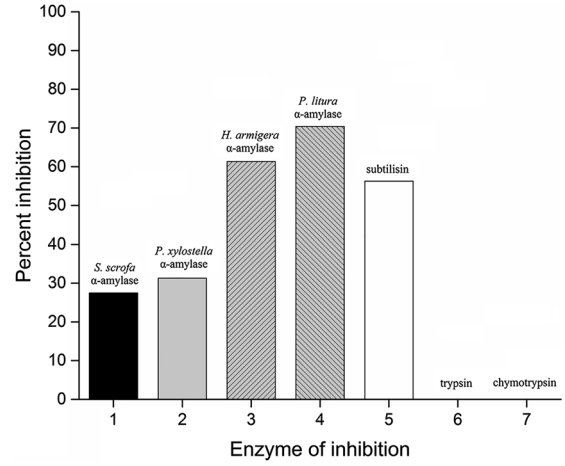

Fig. 3 Inhibitory activity of the recombinant LASI at 47 μg. *1–4* indicated α-amylase (30 μg) from porcine pancreas, *P. xylostella, H. armigera*, and *S. litura*, respectively. *5–7* indicated subtilisin A (20 μg), trypsin (20 μg), and chymotrypsin (20 μg), respectively

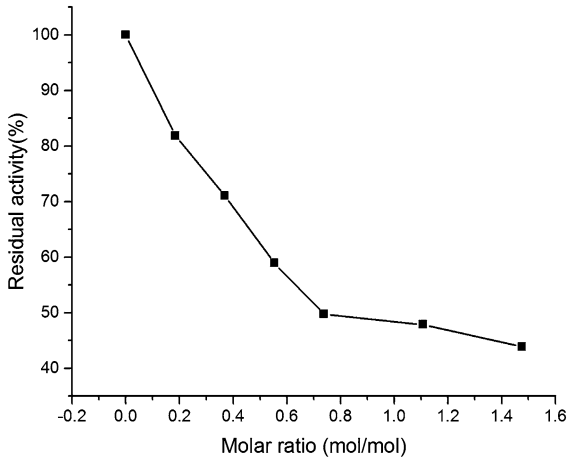

Fig. 4 Inhibition efficiency of the recombinant LASI against subtilisn A at various molar ratios

and *S. litura*. Although other inhibitors also showed strict target enzyme specificity and recognized only one of isozymes or enzymes from different species (Franco et al. 2002; Bønsager et al. 2005; Bunyatang et al. 2016), the reason was uncertain. The crystal structure of the complex between barley α-amylase 2 and BASI showed that Glu168 in BASI, which formed a hydrogen-bond to one of the Ca^{2+}-coordinated water molecules, has been regarded as one important residue for the α-amylase inhibitory activity (Nielsen et al. 2003, 2004). The fact that the corresponding amino acid in LASI is Glu might explain why LASI was an effective inhibitor against α-amylases from various pests. The substantial inhibition of α-amylase from three lepidopterous pests suggested that LASI might affect the growth and/or survival of these pests when incorporated into their diet. In the future, the biological function of the LASI to inhibit the growth of *H. armigera* and *S. litura* was necessary to investigate.

Expression pattern of *LASI* in various organs and under different stresses

Firstly, the expression pattern of *LASI* in rhizome and leaf were analyzed by qRT-PCR using RPL11 as reference gene (Fig. 5a). The result of *LASI* gene transcript in various organs indicated that most abundant of *LASI* transcript was found in the rhizome, some were detected in leaf tissues. Since amylases in plants are involved in mobilization of starch reserves that are transported as sugars and utilized in the formation and development of organs, the accumulation of *LASI*

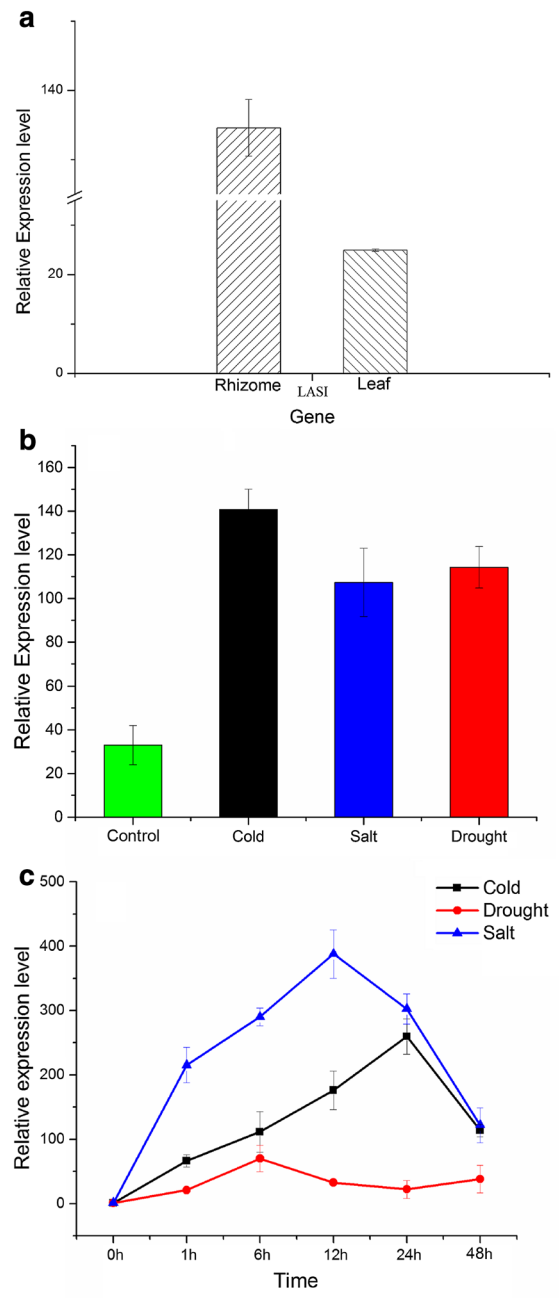

Fig. 5 Analysis of RT-PCR for *LASI* transcripts of *L. chuanxiong* in various organs and under different stresses. Each value is the mean of three replicates, and error bars are indicated as SDs. **a** Expression patterns of *LASI* in leaf and rhizome. **b** Expression patterns of *LASI* in leaf under cold, drought and salt stresses. **c** Expression patterns of *LASI* in rhizome at 48 h under cold, drought and salt stresses

transcript in normal state might control the synthesis of sugar. In addition, rhizome of plant played important role in the interaction with environmental agents,

including abiotic agents and biotic agents. Due to its high expression level in rhizome, *LASI* was reasonably regarded to be involved in the stress-response process.

Furthermore, to understand the metabolism related to *LASI* under stresses, *L. chuanxiong* was treated with different stresses and then used to analyze the expression pattern of *LASI*. As shown in Fig. 5b, the expression level of *LASI* in leaf was unstable under drought, salt and chilling treatment. The *LASI* gene expression went up rapidly and reached the maximum expression of 70 at 6 h. The expression level of *LASI* went up before 12 h and then went down under salt stress, whereas the expression level went up before 24 h and then went down under chilling stress. Furthermore, the expression level of *LASI* in rhizome at 48 h under stress was relative stable compared with that in leaf. The expression level of *LASI* in rhizome increased 4.3, 3.5 and 3.3 fold at 48 h compared with the control plant under chilling, drought and salt, respectively (Fig. 5c). Therefore, the increased expression level of *LASI* under salt, chilling treatment suggested that LASI protein were necessary for reduction of the plant damage under abiotic stresses by inhibiting the endogenous amylase in plants.

Conclusions

To date, six types of proteinaceous ASIs have been found in plants as described by the similarity in sequence and three-dimensional structure (Svensson et al. 2004). BASI, WASI and RASI, which are composed of 176–181 amino acids and show high similar sequence with that of Kunitz trypsin inhibitor, were attributed as the fourth group (Kunitz-like ASIs). In this paper, we report for the first time the cloning and expression of *LASI* gene from the rhizome of *L. chuanxiong*. According to the BLAST result, LASI could be attributed to the Kunitz-like ASIs. LASI possessed two conserved regions matched to those in Kunitz-like ASIs. The recombinant LASI protein expressed in *E. coli* without signal peptide showed strongly inhibitory activity towards subtilisin A. Moreover, it also strongly inhibited the α-amylase from *H. armigera* and *S. litura*. The *LASI* gene expression patterns revealed that LASI was induced by abiotic stresses and involved in the defense of the plant. Hopefully, *LASI* might be used to construct transgenic plants against lepidopterous pests, micrograms and abiotic stresses in the future.

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Supporting information Supplementary Fig. 1—Phylogenetic tree of LASI and other α-amylase/subtilisin inhibitors constructed by neighbor-joining algorithm. The numbers at the nodes indicated the bootstrap values. The NCBI protein database accession numbers were shown in brackets.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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