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ORIGINAL ARTICLE

Overexpression of the LmHQT1 gene increases chlorogenic acid production in *Lonicera macranthoides* Hand-Mazz

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Abstract *Lonicera macranthoides* Hand-Mazz contains high levels of chlorogenic acid (CGA). The CGA is synthesized via different biosynthetic pathways in various plant species, and hydroxycinnamoyl-coenzyme A quinate transferases (HQTs) are key enzymes in these routes. In this study, we isolated the *LmHQT1* gene, which encodes a protein of 447 amino acid residues with conserved HXXXD and DFGWG motifs. It is very closely homologous to *HQT* genes in *Lonicera japonica* (*LjHQT*), *Solanum lycopersicum* (*SlHQT*) and *Nicotiana sylvestris* (*NsHQT*). Quantitative reverse-transcription polymerase chain reaction showed that *LmHQT1* gene expression decreased following leaf senescence. The CGA contents displayed similar trends, suggesting a potential role of *LmHQT1* in CGA biosynthesis. To characterize its function, *LmHQT1* overexpressing plants were generated via *Agrobacterium* transformation methods established previously. Upregulation of *LmHQT1* in *L. macranthoides* was observed to elevate the CGA levels up to 60% in leaves. These findings indicated that *LmHQT1*was devoted to CGA biosynthesis in *L. macranthoides*.

Keywords *LmHQT1* · Chlorogenic acid · *Agrobacterium*-mediated transformation · *Lonicera macranthoides*

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Introduction

Lonicera macranthoides Hand-Mazz is a widely distributed plant species in southern China and has been used in traditional Chinese medicine. It is also an ingredient for functional foods and cosmetics. Dried or fresh early-stage flowers of *L. macranthoides* are used as an antitoxin, antibacterial, and antiviral reagent. Chlorogenic acid (CGA, 3-caffeoylquinic acid) is the main active ingredient in *L. macranthoides* and exhibits a strong antioxidant property when used as food (Azzini et al. 2007). Previous studies have shown that CGA is rapidly absorbed in rats that were orally administered pure CGA (Lafay et al. 2006) or plant extract (Yang et al. 2004). Statistical analyses indicated that the administration of CGA resulted in a significant decrease in blood pressure (Onakpoya et al. 2015; Zhao et al. 2012). CGA also exhibits anti-inflammatory activity in human peripheral blood mononuclear cells (Albert et al. 2002; Hammer and Birt 2014). In addition, CGA is believed to play important roles in free radical scavenging (Tamagnone et al. 1998). These findings indicate that CGA provides significant benefits to human health. There are relatively high levels of CGA produced in *L. macranthoides*, which is almost two times higher than that in *L. japonica* (Zhou and Tong 2003).

Therefore, *L. macranthoides* is considered a prime resource for natural CGA.

CGA is synthesized in various plant species, such as tomato, tobacco, eggplant, apple, pear, plum, coffee, and artichoke (Schutz et al. 2004; Tamagnone et al. 1998; Wang et al. 2003). Although major progress has been made recently in the understanding of CGA biosynthesis pathway, the key biosynthetic route of CGA remains controversial. First, the universal precursor, *p*-coumaroyl-CoA, is synthesized under the catalysis of phenylalanine ammonia-lyase (PAL), 4-coumarate CoA ligase, and cinnamate 4-hydroxylase. Subsequently, three metabolic pathways have been postulated. In the first route, hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT) catalyzes the formation of CGA from caffeoyl-CoA and quinic acid (Niggeweg et al. 2004). Caffeoyl-CoA is supplied by the combined activities of hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) and *p*-coumaroyl ester 3′ hydroxylase (C3′H) via a *p*-coumaroyl shikimate intermediate (Mahesh et al. 2007). The second proposed route is based on the synthesis of *p*-coumaroyl quinate by HCT, followed by hydroxylation via C3′H (Hoffmann et al. 2003; Zenk et al. 1980). In the third suggested route, caffeoyl glucoside serves as an activated intermediate (Villegas and Kojima 1986).To date, several genes in the above-mentioned routes have been investigated for their role in CGA biosynthesis in many species. Lepelley et al. (2007) determined the correlations between the gene expression of *HCT*, *HQT*, *C3H1* and *CCoAOMT1* and the CGA contents in different tissues at different development stages in coffee. They found that higher *HQT* expression appears to be more closely correlated with CGA accumulation. Genetic transformation results also demonstrated that PAL and HQT were key enzymes associated with CGA production in tobacco and tomato (Howles et al. 1996; Niggeweg et al. 2004). Peng et al. (2010) isolated the *LjHQT* gene and showed that the tissue distribution of *HQT* is correlated with the pattern of CGA abundance in *L. japonica*, indicating that *HQT* is an indispensable gene in CGA biosynthesis.

A survey of genetic resources of *Lonicera* identified a mutant called "Jincuilei" with several never-opening flowers. This mutant has a higher CGA content of 6.0% compared with 4.0% in the wild-type (Wang et al. 2009). The higher CGA content in "Jincuilei" indicates that this species can be used as a natural resource for CGA. Similarly, *L. macranthoides* is a plant species with high levels of CGA and may potentially serve as a prime plant model for investigations on CGA biosynthesis. According to our previous study (Chen et al. 2015), the *LmHQT1* gene (CL74326 Contig1) is a candidate gene associated with CGA biosynthesis. Therefore, functional characterization of *LmHQT1* can help us understand the CGA biosynthetic mechanism in *L. macranthoides*. In the present study, the *LmHQT1* gene was cloned and transferred into *L. macranthoides*, and positive transgenic plants were identified. The CGA contents were investigated in *LmHQT1*-overexpressing *L. macranthoides* plants. The results of this study provide novel insight into the regulation of CGA biosynthesis and can be used to develop a method for increasing CGA accumulation via genetic engineering.

Materials and methods

Plant materials

L. macranthoides (cv Yu lei 1#), grown in a greenhouse, was used in this study. The tissues at different developmental stages, including buds, young flowers (YF, 1 day after anthesis), mature flowers (MF, 5 days after anthesis), young leaves (YL, yellow green leaves), leaves (L, green leaves) and old leaves (OL, dark green leaves) were collected from 1-year-old seedlings. All collected samples were frozen in liquid N_2 and stored at −80 °C until determination of the CGA content and qRT-PCR analysis.

Cloning the cDNA of *LmHQT1* and sequence analysis

Total RNA was extracted from leaves of *L. macranthoides* using the TRIzol reagent (Invitrogen, Germany), following the manufacturer's instructions. After *DNase*I treatment, the first cDNA strand was generated using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's protocol. The full length of the *LmHQT1* gene was amplified by PCR using the following primers: forward, 5′-ATGGGAAGTGAAGGAAGTGTGAAGA-3′ and reverse 5′-TCAGAACTCGTACAAACACTTCTCA AA-3′. The PCR product was purified, cloned into the pMD18-T vector (TaKaRa) and sequenced.

For sequence analysis, the ortholog can be searched on the website by Blast alignment (http://www.ncbi.nlm.nih.gov/BLAST/) against our nucleotide or protein datasets. Conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/) was used for functional annotation of the proteins. Multiple sequence alignment was performed using ClustalX (version 2.0.10) and the MEGA program (version 5.1) was utilized for molecular phylogenetic tree construction based on Neighbor-Joining method.

Plant transformation

Transgenic plants were obtained by *Agrobacterium tumefaciens*-mediated gene transfer system. To construct an overexpression vector, the coding sequence of *LmHQT1*

was ligated into the modified binary vector pLP100 under the CaMV35S promoter (kindly supplied by Prof. Zhengguo Li of Chongqing University), which carries the nptII selectable marker conferring kanamycin resistance. After validation by PCR amplification and sequencing of insert fragment, the plant expression vector was obtained and named as pLP100-35S-*LmHQT1*. This vector was transformed into *A. tumefaciens* strain EHA105 by using the freeze–thaw method. Plant transformation was conducted as follows: (i) Pre-culture. Young leaves of *L. macranthoides* were sterilized using 1% $HgCl_2$ for 4 min and rinsed four times with sterilized distilled water. Subsequently, they were sliced into 2 cm^2 pieces and pre-cultured on MB medium supplemented with 1.5 mg/L 2,4-dichlorophenozyacetic acid (2,4-D) and 1 mg/L kinetin (KT) for 2 days in the dark. MB medium, a modified solution based on Murashige and Skoog (MS) and B_5 medium, contains 1,400 mg/L KNO_3, 150 mg/L $CaCl_2{\cdot}2H_2O$, 1000 mg/L $Ca(C_6H_{11}O_7)_2$, 220 mg/L $Mg(NO_3)_2$, 410 mg/L KH_2PO_4, 410 mg/L NH_4NO_3, 35 mg/L $NaH_2PO_4{\cdot}H_2O$, 200 mg/L $(NH_4)_2SO_4$, 0.5 mg/L nicotinic acid, 100 mg/L inositol, 0.5 mg/L pyridoxine hydrochloride, 0.1 mg/L aneurine hydrochloride, and 2 mg/L glycine. (ii) Agrobacterium inoculation. The *A. tumefaciens* strain, EHA105, harboring the binary plasmid, pLP100-35S-*LmHQT1*, was cultured in liquid LB medium overnight at 28 °C. They were collected by centrifugation at 6000 rpm for 5 min and suspended in liquid MB medium supplemented with 1.5 mg/L 2,4-D, 1 mg/L KT, and 100 μmol/L acetosyringone (AS). The optimal cell density of *Agrobacterium* for infection was 0.4 at OD600. Explants were immersed in the bacterial suspension for 8 min under constant shaking and then co-cultured for 2 days in darkness. (iii) Shoot regeneration and kanamycin-resistance selection. After 2 days co-culture, the explants were transferred and placed on selection medium, which contains MB medium supplemented with 2.0 mg/L 6-benzyladenine (6-BA), 0.2 mg/L indolebutyric acid (IBA), 15 mg/L kanamycin (Kan), and 600 mg/L cefotaxime (Cef). The culture conditions were maintained at 25 ± 1 °C under a 12 h photoperiod with light intensity of 2000–3000 lx. The subculture period was maintained at 1-week intervals. The kanamycin-resistant calli/shoots were induced after subculture. (IV) Root induction. After about 50–70 days, the resistant adventitious shoots were transferred into 1/2 MB medium supplemented with 0.1 mg/L indole-3-acetic acid (IAA), 2.0 mg/L IBA, 100 mg/L activated carbon (AC), 15 mg/L Kan, and 600 mg/L Cef for root induction (see protocol in Fig. 1). Well-rooted explants were used for further analysis.

Transgenic lines were selected by GUS (β-glucuronidase) staining and PCR detection. In brief, the *β-gluc* gene was amplified by PCR from seven kanamycin-resistant seedlings using the following primers: forward, 5′-ACCTCTCTTTAGGCATTGGTTTC-3′ and reverse 5′-GCACACTGATACTCTTCACTCCAC-3′. To validate its accuracy, another insert sequence, which contains the 35S promoter and part of the *LmHQT1* gene, was amplified using the following primers: forward, 5′-GCTCCTAC AAATGCCATCATTGC-3′ and reverse 5′-CGAGAGCAG ACCTCA AGTACTCAT-3′.

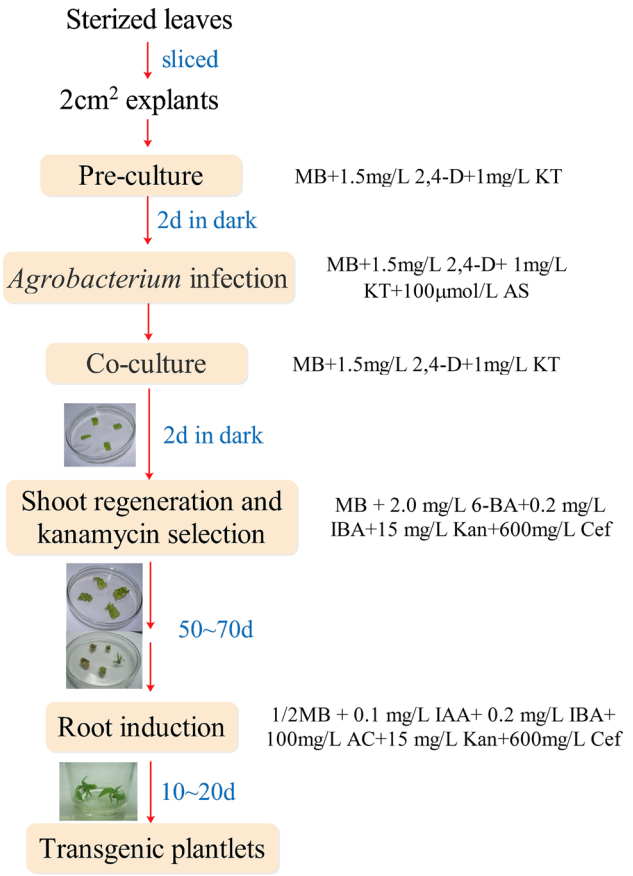

Fig. 1 Optimized protocols of *Agrobacterium tumefaciens*-mediated genetic transformation system in *Lonicera macranthoides* Hand-Mazz

Real-time quantitative PCR

Total RNA was extracted from different tissues at different developmental stages in *L. macranthoides* or transgenic lines. Total RNA isolation and cDNA synthesis were performed following the above-mentioned methods. Gene-specific primers for *LmHQT1* (forward, 5′-CCAACCA GACGAGCAAGTTA-3′ and reverse, 5′-CCATAG GTGTGGCTGTGAAC-3′) were designed by online software (https://www.genscript.com/ssl-bin/app/primer). qRT-PCR was carried out as described previously (Chen et al. 2015), and three biological replicates were performed in all qPCR experiments. Relative expression levels were calculated based on the $2^{-\Delta\Delta Ct}$ method using tubulin

(GenBank Accession No. KR233012) as a reference gene and the primers were as follows: forward, 5′-CCA CATCTGTTGTTGAGCCT-3′ and reverse, 5′-GCGCCTG CAGATATCATA GA-3′.

Determination of the CGA content by high-performance liquid chromatography (HPLC)

HPLC was employed to investigate the CGA contents from the materials including flowers and leaves at different developmental stages in *L. macranthoides*, or leaves in wild-type and transgenic lines. The detailed determination methods and data processing were carried out as described previously (Chen et al. 2015).

Results

Isolation and sequence analysis of the *LmHQT1* gene

Based on the annotated sequence of the *HQT*-homologous gene identified from transcriptome data in *L. macranthoides* (Chen et al. 2015), a 1344 bp full-length cDNA of the *HQT* gene was obtained by RT-PCR and tentatively designated as *LmHQT1* which was deposited in GenBank under accession number KR233011. The cDNA of *LmHQT1* contained a 1344 bp open reading frame (ORF), which encoded a protein of 447 amino acid residues using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, USA). Further analysis via NCBI CCD and the online tool Pfam (http://pfam.xfam.org/search/sequence) showed that the LmHQT1 protein harbored a transferase domain (Accession No. PLN02663 or pfam02458). This structural domain encompassed amino acids 1–429 and was involved in CoA-dependent acyltransferase (Fig. 2). To search homologs of *LmHQT1* in *L. macranthoides*, BLAST analysis was performed against the NCBI database. NCBI BLASTP indicated that the LmHQT1 protein was most closely homologous to the *HQT* gene in *Lonicera japonica* (*LjHQT*, ACZ52698.1). It has been also found that the *HQT* genes in *Nicotiana sylvestris* and *Solanum lycopersicum* showed high similarity to *LmHQT1*. Pairwise alignment displayed that *LmHQT1* showed 99, 72 and 71% amino acid identity with *LjHQT*, *NsHQT* (XP_009800587.1) and *SlHQT* (NP_001234850.1), respectively (Fig. 2).

Given that *LmHQT1* belongs to a plant transferase subfamily, the phylogenetic tree was constructed with 20 transferase gene sequences from several plant species. The results displayed that LmHQT1 and HCT show the highest homology. In addition, LmHQT1 exhibited close homology with hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyltransferase (HHT) and anthranilate *N*-hydroxycinnamoyl/benzoyltransferase protein (HCBT) (Fig. 3).

CGA contents in different tissues

The CGA contents in flowers and leaves at different developmental stages were quantified. The CGA content was highest in young leaves, that is, 54.01 mg/g FW (fresh weight). However, it rapidly declined during leaf aging. In addition, the CGA concentration in young leaves was higher than that in flowers at different developmental stages, and the highest levels of CGA in flowers were found in buds, followed by young flowers and mature flowers (Fig. 4a).

Expression patterns of the *LmHQT1* gene in different tissues

LmHQT1 transcripts in different flowers and leaves at different developmental stages were detected. qRT-PCR results showed that the *LmHQT1* gene was differentially expressed in different tissues at different developmental stages. In brief, *LmHQT1* was expressed at extremely high levels in young leaves and showed significantly lower levels during leaf senescence. Similarly, in flowers, *LmHQT1* displayed a relatively strong expression at the bud stage and a slight decline as the tissues aged. Compared with young leaves, *LmHQT1* was expressed at low levels in flowers at all developmental stages (Fig. 4b). Moreover, the transcripts of the *LmHQT1* gene were positively correlated with the CGA content, indicating its important role in CGA biosynthesis.

Generation and identification of *LmHQT1* transgenic plants

To characterize the function of the *LmHQT1* gene, an overexpression vector was constructed and transgenic plants were generated via *A. tumefaciens*-mediated transformation developed previously in our laboratory. Throughout the processes including resistant calli induction, shoots induction, and rooting induction, approximately 17.2% of the explants were allowed to differentiate adventitious shoots in the presence of kanamycin (Fig. 5).

To identify transgenic plants, 12 well-rooted seedlings (transgenic lines) selected randomly were used to detect insertion of the GUS gene. A specific 500 bp band was amplified from DNA of eight seedlings, indicating that the GUS fragment was integrated into the host genome successfully (Fig. 6a), which was also validated by GUS staining (Fig. 6c). An approximately 1100 bp band, which was attributed to the partial sequence of CaMV35S–*LmHQT1* gene fusion, was observed. This finding

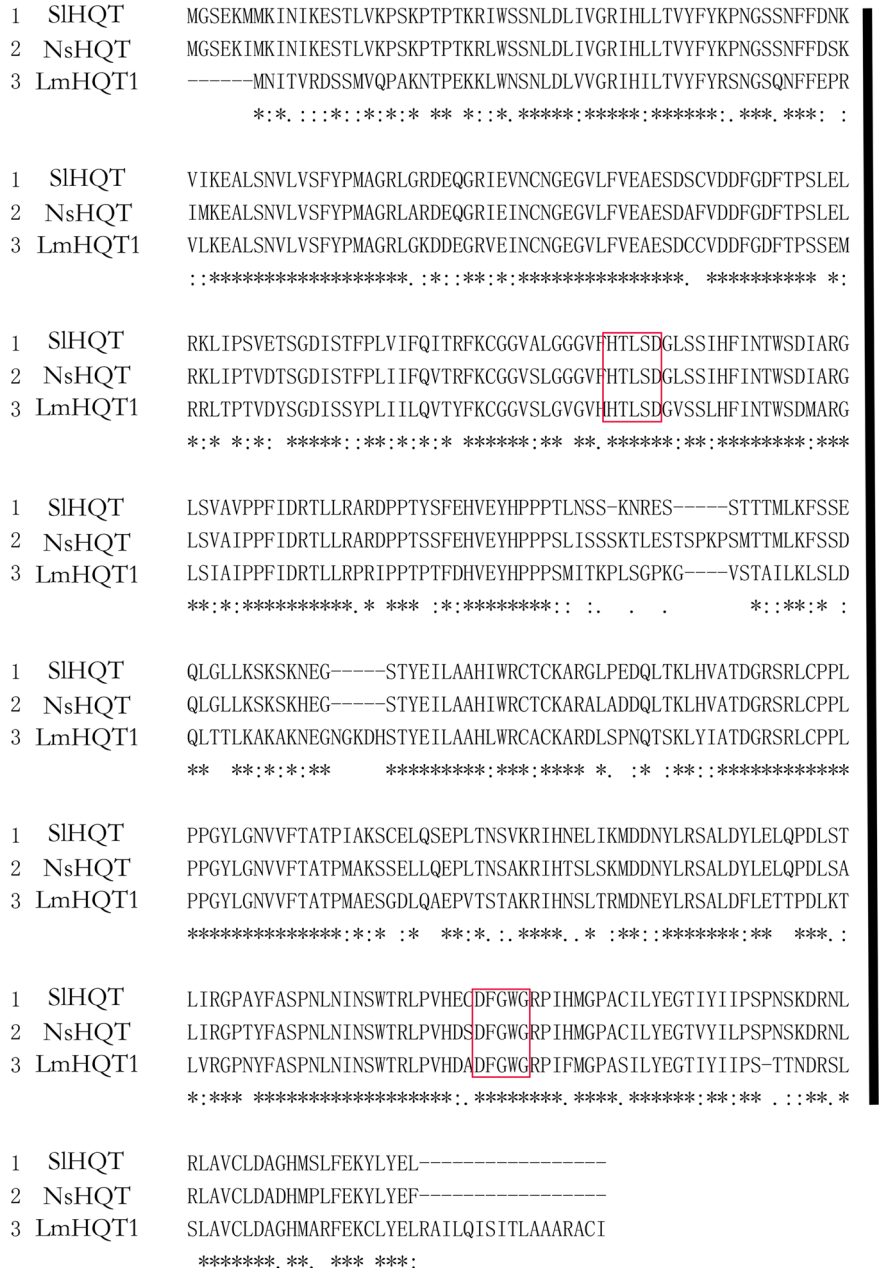

Fig. 2 Sequence analysis of *LmHQT1* in *Lonicera macranthoides*. Multiple sequence alignment of *LmHQT1* (KR233011), *SlHQT* (NP_001234850.1), and *NsHQT* (XP_009800587.1). The conserved domain (PLN02663 and pfam02458) is indicated by a *straight line*. The two conserved motifs are marked with *red frames*

suggested the integration of *LmHQT1* into the genome of the detected well-rooted seedlings (Fig. 6b).

Overexpression of the *LmHQT1* gene increased CGA contents in *L. macranthoides*

qRT-PCR analysis was employed to investigate the constitutive expression of *LmHQT1* in transgenic lines. *LmHQT1* transcripts accumulated in three individual lines, which were about 6- to 20-fold higher than that in wild type (Fig. 7a). To characterize the roles of the *LmHQT1* gene in CGA biosynthesis, the CGA contents were determined in transgenic plants. Results revealed that the CGA contents significantly increased in transgenic plants, with 63.2, 74.9 and 66.0 mg/g FW in the leaves of overexpression lines 3, 4 and 5, respectively. These values were largely higher than that in wild type, which had a CGA content of 45.2 mg/g FW (Fig. 7b). Therefore, the results indicated that the *LmHQT1* gene plays an important role in CGA biosynthesis.

Discussion

Lonicera macranthoides is an exceptionally rich source of CGAs and believed to be of fundamental importance to human health because CGA is associated with antioxidant

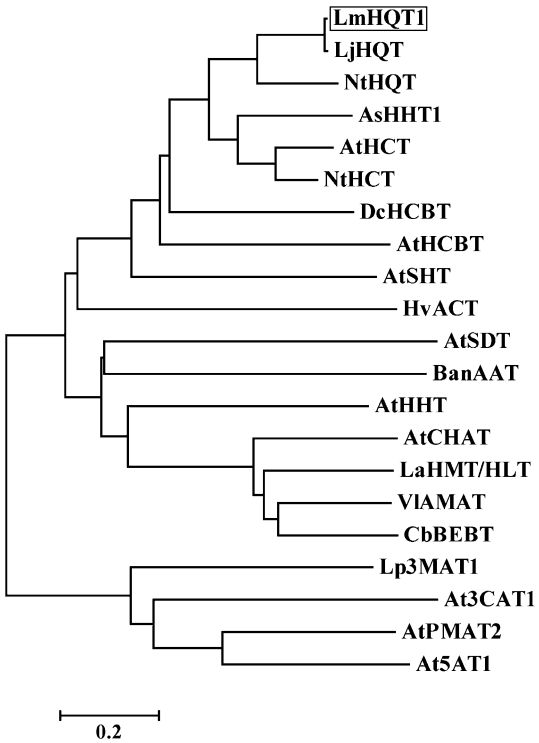

Fig. 3 *LmHQT1* belongs to plant acyltransferase family. The phylogenetic tree was constructed using MEGA software (version 5.1) based on the neighbor-joining method. Values above the branches are bootstrap percentages (1000 replicates). Abbreviations and gene ID were as follows. LmHQT1, *L. macranthoides* hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase; LjHQT (ACZ52698), *L. japonica* HQT; NtHQT (CAE46932), *N. tabacum* HQT; AtHCT (NP_199704.1), *A. thaliana* hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransferase; NtHCT(CAD47830), *N. tabacum* HCT; AtHCBT (NP_200592.1), *A. thaliana* anthranilate *N*-hydroxycinnamoyl/benzoyl transferase; DcHCBT (CAB06430), *D. caryophyllus* HCBT; AtSHT (NP_179497.1) *A. thaliana* spermidine hydroxycinnamoyl transferase; AsHHT1 (BAC78633), *A. sativa* hydroxycinnamoyl-CoA: hydroxyanthranilate *N*-hydroxycinnamoyl-transferase; AtCHAT (NP_186998.1), *A. thaliana* acetyl CoA: (*Z*)-3-hexen-1-ol acetyltransferase; VlAMAT(AAW22989), *V. labrusca* anthraniloyl-CoA: methanol acyltransferase; AtHHT (NP_568587.2), *A. thaliana* omega-hydroxypalmitate *O*-feruloyl transferase; AtSDT (NP_179932.1), *A. thaliana* spermidine disinapoyl acyltransferase; AtPMAT2 (NP_189609.1), *A. thaliana* phenolic glucoside malonyl-transferase 2; At5AT1 (NP_200924.1), *A. thaliana* anthocyanin 5-aromatic acyltransferase 1; At3CAT1(NP_171890.1), *A. thaliana* coumaroyl-CoA: anthocyanidin 3-*O*-glucoside -6″-*O*-coumaroyl-transferase 1; BanAAT (CAC09063), banana alcohol acyltransferase; HvACT (AAO73071), *H. vulgare* agmatine coumaroyltransferase; CbBEBT (AAN09796), *C. breweri* benzoyl-CoA: benzylalcohol *O*-benzoyltransferase; Lp3MAT1 (AAS77404), *L. purpureum* malonyl-CoA: flavonol 3-*O*-glucoside-6″-*O*-malonyltransferase; LaHMT/HLT (BAD89275), *Lupinus albus* (−)-13 alpha-hydroxy multiflorine/(+)-13alpha-hydroxylupanine *O*-tigloyltransferase. The LmHQT1 protein was labeled in *red frame*

activity and proved to have anti-inflammatory and anti-cancer properties (Cai et al. 2004; Chagas-Paula et al. 2011). The accumulation of CGAs is a precisely controlled process that varies considerably depending on tissues, developmental stages, and environmental conditions. Our previous study demonstrated that the CGA contents differed significantly among the tissues including leaves, stems, and flowers of *L. macranthoides* (Chen et al. 2015). In this study, the levels of CGA were extremely higher in the earliest stage tested (young leaves), but decreased sharply during aging and senescence of leaves (Fig. 4a). In addition, growth conditions exerted a remarkable effect on CGA accumulation in leaves. Clé et al. (2008) found that CGA levels rose in leaves when tomato plants were transferred from the growth room to a tunnel with higher light intensity. Moreover, the CQA content increases in response to abiotic stresses (Dixon and Paiva 1995). A previous study reported a large increase in diCQAs accumulation in the leaves when exposed to UV-C radiation in different globe artichoke genotypes (Moglia et al. 2008).

The CGA biosynthetic pathway has been proposed. HQT is considered to be one of the important enzymes for CGA synthesis in plants, which can act directly on caffeoyl CoA and quinic acid to generate CGA or synthesize *p*-coumaroyl quinate from *p*-coumaroyl CoA and quinic acid, and then convert to CGA via C3′H (Niggeweg et al. 2004).

HCT/HQT enzymes are encoded by a gene family composed of at least seven members in *L. macranthoides* (Chen et al. 2015).Of these members, *LmHQT1* was cloned in the present study. The predicted protein showed a high level of identity to its tobacco and tomato orthologs (Fig. 2). We found that *LmHQT1* belongs to the plant acyltransferase family, which has two conserved peptide motifs, including HXXXD and DFGWG (Hoffmann et al. 2003; St-Pierre et al. 1998). It is also observed that all three *HQT* genes used in Fig. 2 contained the same sequence (HTLSD) in the highly conserved HXXXD motif (Fig. 2), whereas an asparagine in place of a threonine was found in the conserved HTLSD box of the *HQT* gene in coffee (Lepelley et al. 2007). Phylogenetic analysis of the acyltransferase family indicated that *LmHQT1* displayed a high level of similarity to HCBT (Fig. 3), which is a related enzyme of HCT and belongs to the subgroup D in *Arabidopsis*. Hydroxycinnamoyl groups can be transferred to acceptors generated from the shikimate pathway such as shikimate, quinate, and anthranilate via the two proteins (Hoffmann et al. 2003). Thus, the results provide fundamental information on characterizing the functions of *LmHQT1*.

In recent years, numerous studies have focused on the regulation of the CGA biosynthesis pathway at the transcription level. Reports revealed a relationship between *HQT* gene expressions and CGA levels in many plant species. DiCQAs, isomers of CGA, were observed to be in higher amounts at the early stage of grain development and then in decline as development progressed. Correspondingly, the transcripts of the *CcHQT* gene displayed the

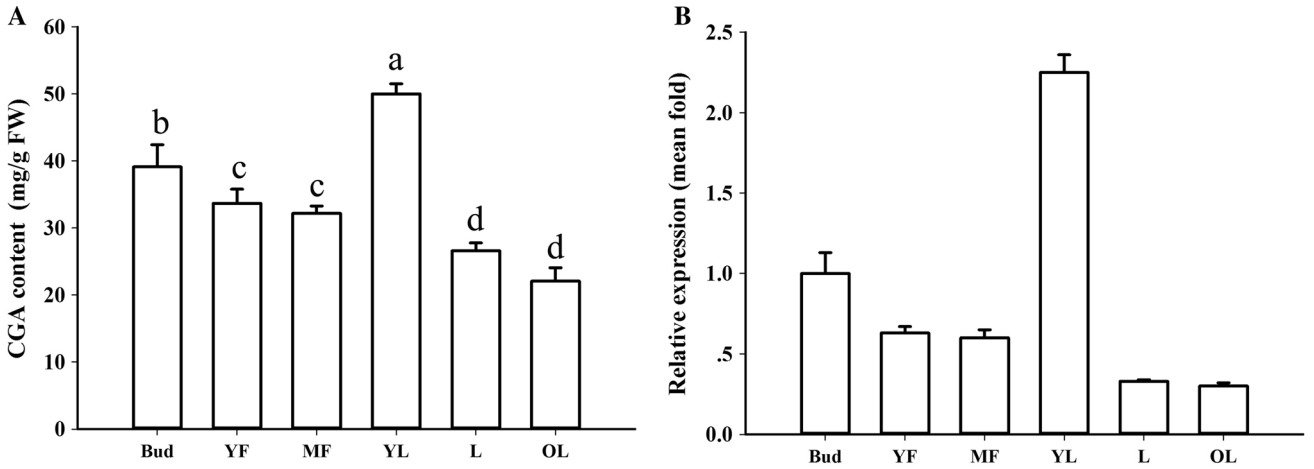

Fig. 4 The CGA contents and expression levels of *LmHQT1* in different tissues of *Lonicera macranthoides*. **a** CGA contents in floral bud (Bud), young flower (YF), mature flower (MF), young leaf (YL), leaf (L) and old leaf (OL) were determined by HPLC. Three replications were performed for each examination, Duncan's multiple range test was used to analyze the significance, and the different lowercase letters indicate significant ($P < 0.05$) differences between samples. **b** QRT-PCR was employed to analyze the expression patterns of *LmHQT1* in different tissues including Bud, YF, MF, YL, L and OL

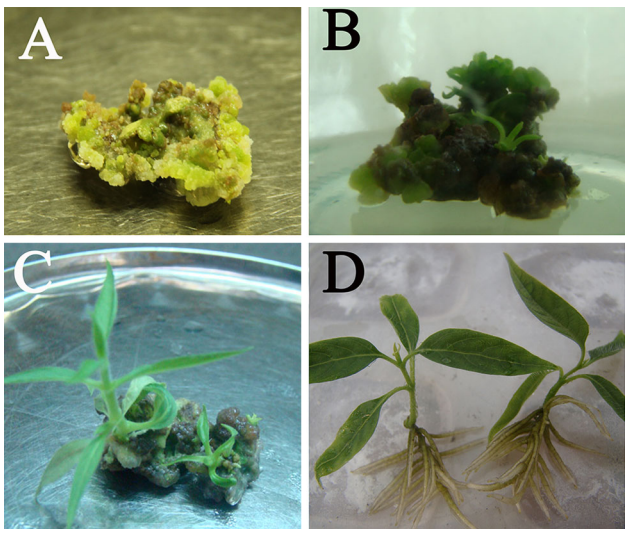

Fig. 5 Transgenic plants generated by *Agrobacterium tumefaciens*-mediated transformation. **a** Callus induction for 4 weeks under selective callus-inducing medium. **b** Shoot induction for 2 weeks after transferring to selective shoot-inducing medium. **c** Shoot induction for 6 weeks cultured on selective shoot-inducing medium. **d** Root induction for 2 weeks after shoots were transferred into selective root-inducing medium

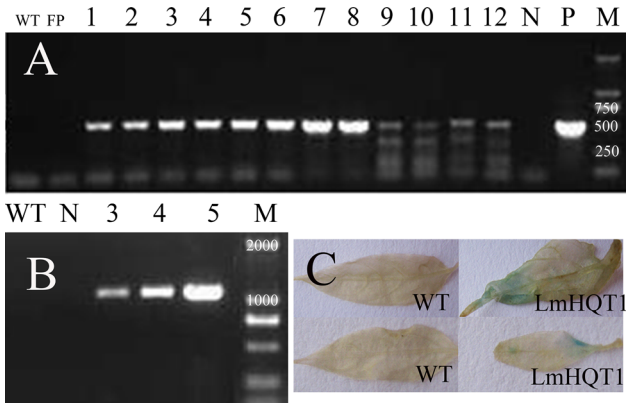

Fig. 6 Identification of transgenic plants of *Lonicera macranthoides*. **a** PCR detection of GUS gene in candidate transgenic plants and controls. WT indicates wild-type plants, FP represents the seedlings with no roots on the selective medium, 1–12 indicate the 12 well-rooted seedlings on the selective medium, N is the negative control and P is the positive control which using plasmid of *pLP100-35S-LmHQT1* as template in PCR reaction. **b** PCR amplification of the inserted target gene. WT indicates wild-type plants, N is the negative control, and 3, 4, and 5 are the three randomly selected positive transgenic plants used in the amplification of the GUS gene. **c** GUS staining of transgenic plants in *Lonicera macranthoides*, and *arrows pointing at the blue parts* indicate sites of GUS activity

highest levels at the small green stage and then decreased as the grain matured, thereby indicating an association between *CcHQT* gene expression and CGA accumulation during grain development in *Coffea canephora* (Lepelley et al. 2007). Our previous study also showed positive correlations between the gene expressions of five *HCT/HQT* homologs and CGA contents in different tissues of *L. macranthoides* (Chen et al. 2015). In the present work, we found that the *LmHQT1* gene was widely expressed and displayed a large variation in expression among different tissues or developmental stages. The transcript levels of *LmHQT1* were highest in the young leaves and then fell sharply during senescence. Similarly, CGA accumulation decreased during the aging process of leaves (Fig. 4b), suggesting a potentially important role of *LmHQT1* in CGA biosynthesis. However, Lepelley et al. (2007) reported that no significant variations in *HQT* transcripts at four different stages of leaf development in coffee. We believe that this

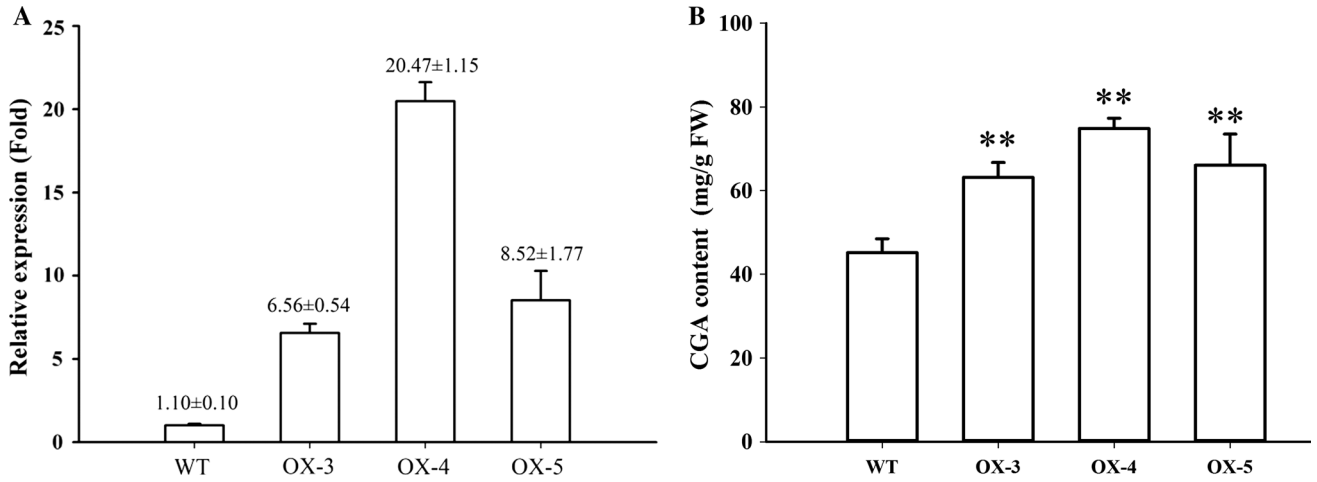

Fig. 7 The transcript levels of *LmHQT1* and CGA contents in *LmHQT1* overexpression plants in *Lonicera macranthoides*. **a** Gene expressions of *LmHQT1* were performed by qRT-PCR. WT indicates wild-type materials, while OX-3, OX-4, and OX-5 represent three well-rooted explants on selective medium. Materials for each replication were harvested from three independent explants, and three independent replications were performed for each sample. **b** CGA contents were determined using HPLC. Student's *t* test was used to determine significance, and *two asterisks* (**) indicate significant ($P \leq 0.01$) differences

difference was due to plant species. In addition, *HQT* transcripts can be modulated in response to abiotic stress. Clè et al. (2008) reported the involvement of the HQT gene in phenolic acids accumulation under UV stress in tomato leaves. Comino et al. (2009) demonstrated that the transcription of the *HQT* gene increased under UV-C treatment in globe artichoke. UV-C application leads to large increases in leaf DCQs (isomers of CGA) in globe artichoke (Moglia et al. 2008), indicating the central role of the *HQT* gene in CGA production in globe artichoke.

To identify the key genes associated with the CGA biosynthetic pathway, genetic transformation was performed. This technique has been recently applied in many species. Niggeweg et al. (2004) demonstrated that recombinant tobacco HQT shows higher affinity for quinate than shikimate. Downregulation of *HQT* gene in tomato resulted in a 98% reduction of the CGA content in leaves, suggesting that HQT was the principal route for CGA accumulation in *Solanaceous* species. Moreover, overexpression of *HQT* in tomato was observed to elevate the CGA levels up to 85% in leaves, and the transgenic plants displayed enhanced resistance to oxidative stress and bacterial pathogen. Sonnante et al. (2010) identified two novel *HQT* genes (*HQT1* and *HQT2*) in artichoke. They noted that both of the genes showed much higher affinity for quinate over shikimate; hence quinate was used as an acyl acceptor in preference to shikimate. Moreover, *HQT1* transcription was positively correlated with the CGA content, and overexpression of *HQT1* in *Nicotiana* causes plants to accumulate higher amounts of CGA and cynarin (1,3-dicaffeoylquinic acid), suggesting that *HQT1* is devoted to CGA synthesis. In the present study, to characterize the role of *LmHQT1* in CGA production, the recombinant protein was successfully transformed into *L. macranthoides* based on an *Agrobacterium*-mediated transformation system established in our previous work (Fig. 1). This technique is a highly efficient and credible method for the genetic transformation of *L. macranthoides*. It has generated transgenic plantlets within 3 months and about 17% of explants can be well rooted on kanamycin selection medium (Fig. 5).The results of PCR analysis and GUS staining also demonstrated that the ORF of *LmHQT1* was integrated into *L. macranthoides* plants (Fig. 6). qRT-PCR analysis showed that the transcript levels of *LmHQT1* extremely increased in different transgenic lines by about 6- to 20-fold than that in wild types (Fig. 7a). Accordingly, the CGA contents in leaves of *LmHQT1* overexpression lines increased by 40–60% compared with those of wild types (Fig. 7b). Our results confirmed the crucial role of *LmHQT1* on the CGA biosynthetic pathway in *L. macranthoides*. However, Clé et al. (2008) considered that HQT activity was not rate limiting for CGA production in tomato under certain conditions, such as high light intensity rooms. Relatively, the 1.4- to 1.6-fold increase in CGA levels that we achieved in *L. macranthoides* was lower than that in tomato (up to twofold) (Niggeweg et al. 2004), thereby indicating that the difference in the regulation of *HQT* on CGA biosynthesis may be due to plant species. The overexpression of PAL in tobacco can lead to much higher levels of CGA (up to threefold) (Howles et al. 1996). However, PAL is the first step in the phenylpropanoid biosynthesis pathway, and its overexpression elevates other intermediate secondary metabolites. By contrast, HQT is the final step in CGA biosynthesis and only affects the amounts of CGA and its isomers. Therefore, our strategy to increase CGA level was considered

more efficient. HCT showed very close homology to HQT. The reports of Comino et al. (2007) suggested that HCT may contribute to CGA biosynthesis in artichoke. However, HCT was observed to be insignificant to CGA accumulation in tomato (Niggeweg et al. 2004) and *Arabidopsis* (Hoffmann et al. 2003), but played a central role in lignin synthesis in tobacco (Hoffmann et al. 2004). Based on this controversial issue, further studies are necessary to characterize the role of *HCT* in CGA biosynthesis in *L. macranthoides.* Transcription factors were very recently found to regulate CGA synthesis. Li et al. (2015) reported that the constitutive expression of *AtMYB11* and *AtMYB12* in tobacco results in the increase in CGA levels by up to fivefold and twofold, respectively. Moreover, the gene expressions of *HQT* and *HCT* were upregulated in *AtMYB11*-overexpressing transgenic plants, indicating that *HQT* and *HCT* may be regulated by*AtMYB11*in tobacco plants. Our previous study also showed the coexpression of several transcription factors and *HCT/HQT* homologs in different tissues of *L. macranthoides* (unpublished data). Subsequent research could focus on the characterization of transcription factors that regulate CGA production.

Author contribution statement ZXC and NT designed this work. ZXC, NT and GHL performed the experiment. ZXC and YQL prepared the original draft the manuscript. NT and ZQX revised the manuscript. All authors read and approved the final manuscript.

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