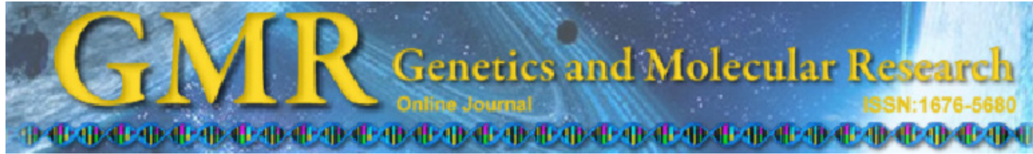

Cloning and expression analysis of the *Lonicera japonica* Thunb. chlorogenic acid synthetase gene (*LjCCoAOMT1*) in rice

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ABSTRACT. Complete coding DNA sequences of a closely related chlorogenic acid synthetase gene (*LjCCoAOMT1*) were isolated from *Lonicera japonica* Thunb. by reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). *LjCCoAOMT1* was subsequently overexpressed in *Escherichia coli* and a 25-kD protein was detected by electrophoresis and western blot analysis. High-performance liquid chromatography (HPLC) analysis showed that recombinant LjCCoAOMT1 methylates the caffeic acid substrate to generate ferulic acid. Further analysis showed that the chlorogenic acid content was significantly correlated with the expression level of *LjCCoAOMT1* in various tissues of *L. japonica* Thunb. at different developmental stages. A plant expression vector containing *LjCCoAOMT1* was constructed and *Agrobacterium*-mediated transgenic rice was successfully obtained. Light treatment analysis showed that *LjCCoAOMT1* transgenic rice was more sensitive than wild-type rice in responding to the changes in lighting conditions. Although gibberellic acid (GA3) could promote the growth of both wild-type and

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LjCCoAOMT1 transgenic rice, *LjCCoAOMT1* transgenic rice appeared to be more sensitive to GA3. Furthermore, high concentrations of GA3 significantly facilitated the growth of *LjCCoAOMT1* transgenic rice.

Key words: *Lonicera japonica* Thunb.; Chlorogenic acid synthetase; *CCoAOMT1* cloning; Expression analysis

INTRODUCTION

Lonicera japonica Thunb. (family Caprifoliaceae) are perennial arching shrubs or twining vines that are commonly found as dried buds or early-opened flowers. *L. japonica* Thunb. is used in traditional Chinese herbal medicine to remove heat and toxic materials, nourish the gall bladder and liver, and prevent viral infections. It is also commonly used to treat respiratory diseases, diarrhea, headaches, fevers, and other diseases. The main components of *L. japonica* Thunb. include chlorogenic acid (CGA), iso-chlorogenic acid, luteolin, caffeic acid, and volatile oil (Machida et al., 2002), of which CGA and iso-chlorogenic acid are the main antibacterial ingredients (Lee et al., 2001). CGA is a secondary metabolite and its concentration varies dramatically among different tissues and flowering stages of the *L. japonica* Thunb. Recently, the contents of CGA, its biosynthetic pathway, and the effective development and utilization of *L. japonica* Thunb. have become the focus of substantial research in the fields of biochemistry, molecular biology, and metabolic engineering.

CGA is a type of phenylpropanoid substance that is generated during the process of aerobic respiration. This phenylpropanoid substance is an ester of caffeic acid and quinic acid and contains multiple unstable parts such as an ester bond, an unsaturated double bond, and polyhydric phenol. Therefore, CGA is usually present in several isomeric forms, including caffeoylquinic acid (CQA), dicaffeylquinic acid (diCQA), and ferulic acid quinate. CQA and a diCQA are generated from 5-O-caffeoylquinic acid (5-CQA) and are considered to be CGA Usually (Campa et al. 2003) suggested that coumaroylquinic acid and caffeic acid are important precursors during the synthesis of CGA in coffee. Schoch et al. (2001) and Kuhnl et al. (1987) demonstrated that the generation of 5-CQA from coumaroylquinic acid is catalyzed by coumaric acid hydroxylase in *Arabidopsis* and in carrot. 5-CQA can be converted to caffeoyl coenzyme A by the hydroxylated cinnamoyl coenzyme A or by quinine acid hydroxylation of cinnamoyl transferase. 5-CQA can also be converted to ferulic coenzyme A by caffeoyl coenzyme A O-methyltransferase (CCoAOMT) (Schoch et al., 2001). CCoAOMT was initially found to be an enzyme involved in dicotyledon stress responses to pests and diseases. Later studies showed that CCoAOMT could also induce lignification reactions after the differentiation of vessel elements in zinnia (Ye et al., 1994). Introduction of antisense RNA of the gene *CCoAOMT* into alfalfa resulted in a significant reduction in lignin G, but not lignin S (Guo et al., 2001), indicating that *CCoAOMT* plays a role in the regulation of lignin G biosynthesis. After cloning *CCoAOMT1* from coffee, Maud et al. (2007) found that its overexpression was closely correlated with the biosynthesis of both lignin and CGA.

CGA can be synthesized using chemical approaches. However, direct extraction of CGA from plants is economically more efficient than chemical synthesis because of its high abundance and low cost. Therefore, genetic engineering of the genes involved in the CGA synthesis pathway would allow its yield to be enhanced without the need for difficult or costly procedures.

Molecular studies on *L. japonica* Thunb. are relatively sparse, and most have focused on the genetic diversity, collection, and comparison of germplasm resources. The key genes involved in the biosynthesis of CGA in honeysuckle have not yet been determined. In this study, we cloned a key gene (*LjCCoAOMT1*) involved in the biosynthetic pathway of *L. japonica* Thunb. CGA. Functional analysis showed that the expression of *LjCCoAOMT1* was positively correlated with CGA synthesis.

MATERIAL AND METHODS

Plant materials

Branches, leaves, and flowers of *L. japonica* Thunb. were harvested from trees located in the Hunan University medicinal garden during the flower development stage. The flowers were immediately separated according to their developmental stage: green bud, white bud, white flower, or yellow flower. The samples were frozen immediately in liquid nitrogen and then sent to the State Key Laboratory of Chemo/Biosensing and Chemometrics at Hunan University on dry ice. Upon arrival, all samples were stored at -80°C until use.

The frozen tissues of *L. japonica* Thunb. were homogenized in liquid nitrogen with a SPEX CertiPrep 6800 Freezer Mill. The powders were either used immediately for RNA extraction as described below or lyophilized for CGA analysis. For the CGA analysis, 0.5 g lyophilized powder was incubated for 1 h at 40°C in 70 mL 70% methanol. The sample was then cooled for 30 min, and the volume was made up to 100 mL. A 1-mL was filtered with a 0.2 µm filter and analyzed using high-performance liquid chromatography (HPLC) with a Macherey Nagel Nucleosil C18 column (5 mm, 4 x 250 mm) and a gradient of 8-50% acetonitrile, containing either 0.1% phosphoric acid or 0.1% formic acid. Compounds were detected at 328 nm and were characterized by their elution times and their UV absorption spectra. Identities were confirmed using CGA (5-CQA) (Sigma) standards when available. The peak areas were measured and the concentration of the compounds was calculated based on a direct comparison with a standard curve generated for 5-CQA.

RNA preparation

Samples stored at -80°C were first homogenized in a SPEX CertiPrep 6800 Freezer Mill with liquid nitrogen. Total RNA was isolated using the Trizol RNA isolation kit (Invitrogen) and then treated with DNaseI (RNase free) according to manufacturer recommendations (RNeasy Plant Minikit, Invitrogen).

Isolation of full-length cDNA encoding *Lonicera Japonica* Thunb. CCoAOMT

cDNA synthesis was performed according to manufacturer protocols (Superscript III Reverse Transcriptase Kit, Invitrogen). For each sample, 50 ng poly(dT) 18 primers (Invitrogen) were used. The cDNA samples were then diluted 100-fold in sterile water and stored at -20°C for later use. 5'-Rapid amplification of cDNA ends (RACE) and 3'-RACE were carried out using the SMARTer™ RACE cDNA Amplification Kit, following manufacturer instructions (Clontech). The 5' and 3'- RACE sequences were aligned, revealing 117 overlapping nu-

cleotides. The assembled sequence was then used to design 2 new primers, CCoAOMT-FullF and CCoAOMT-FullR (Table 1), in order to amplify the full-length cDNA of *CCoAOMT*. The polymerase chain reaction (PCR) product was cloned into the pUC18 vector.

Table 1. PCR primers used for 5'-RACE and 3'-RACE and cDNA amplification.

Primer name	Sequence
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
NUPM	AAGCAGTGGTATCAACGCAGAGT
CCoAOMT1-GSP1	GAAAACTACGAACTGGGCCTACC
CCoAOMT1-NGSP1	CCCAACTTTAACCAAGTCGATAA
CCoAOMT1-GSP2	TAATCGAAAAGGCTGGTCTTGCC
CCoAOMT1-NGSP2	GATCAAATGATCCAAGATGAA
CCoAOMT - FullF	ATGGCAACCAACGGAAACTC
CCoAOMT - FullR	ACTGATACGGCGGCATAGC
Rice-OsActin-F	CAATCGTGAGAAGATGACCC
Rice-OsActin-R	GTCCATCAGGAAGCTCGTAGC
LjACTIN-F	ATGATGCTCCCAGGGCAGTTT
LjACTIN-R	TCCATGTCATCCCAGTTGCTG
LjCCoAOMT1-DL-F	TGCTTTGCCTGTTCTTGAT
LjCCoAOMT1-DL-R	TTCCATAGGGTGTTGTCGT
LjCCoAOMT1-PCOYFP-F	GCGTCGACATGGCAACCAACGGAAACTC
LjCCoAOMT1-PCOYFP-R	AACTGCAGACTGATACGGCGGCATAGC

DNA sequence analysis

Plasmid DNA was purified using the Qiagen kit, according to manufacturer instructions. DNA samples were sequenced using the dideoxy termination method described by Sagon Biotech Co. Ltd (Shanghai, China). Computer analyses were performed using the Laser Gene software package (DNASTAR). Sequences were verified against those in the GenBank database using BLAST.

Quantitative reverse transcription PCR (qRT-PCR) experiments

Quantitative PCR, using TaqMan probes, was carried out as described by Simkin et al. (2006) with slight modifications. Specifically, a 100-fold dilution of the cDNA was used as the template, corresponding to approximately 0.25 ng original RNA. The qPCR primers and TaqMan probes were designed using the Primer Express software (Applied Biosystems) (Table 1). The amplification efficiencies of the primer/probe sets were tested with various dilutions of the corresponding plasmids and were all close to 100%. Gene expression was quantified using the *Lonicera Japonica* Thunb. ACTIN gene (GenBank: GQ241342.1) as reference.

Overexpression and purification of LjCCoAOMT polypeptides

Recombinant LjCCoAOMT proteins with histidine tags were expressed in *Escherichia coli* and affinity purified. The *LjCCoAOMT* genes were PCR-amplified using the primers CCoAOMT-PCOLDF and CCoAOMT-PCOLDR (Table 1). A *Sal*I site was added to the 5' end of the forward PCR primer just before the ATG start codon, and a *pst*I site was added to the reverse primer just after the stop codon. The PCR reactions were performed with PrimeSTAR HS DNA Polymerase (Takara) under conditions specified by the manufacturer. The

PCR products were gel purified, digested with *Sal*I and *pst*I, ligated to the *Sal*I- and *pst*I-digested expression vector pCOLD, and transformed into DH5a competent cells (Invitrogen). Plasmids containing the *LjCCoAOMT* gene were named pCOLD-CCoAOMT. The inserts of the plasmids were verified by sequencing, and the plasmids were transformed into the *E. coli* strain BL21(DE3). After the cells were grown to an optical density of 1 (at 600 nm) in 200 mL Luria-Bertani medium with 50 mg/L ampicillin, protein expression was induced with 0.2 mM isopropylthio-b-galactoside for 3 h at 37°C. The cells were then pelleted and resuspended in 25 mL lysis buffer (20 mM Na_3PO_4, pH 7.3, 150 mM NaCl, 1% Triton X-100, 2 mM ethylenediaminetetraacetic acid, 0.1% b-mercaptoethanol, 1X protease inhibitor mix). Lysis was carried out by sonication on ice for 3 cycles of 10 s each. The lysed cells were centrifuged (30 min at 10,000 *g*), and the supernatant was mixed with a sample of HIS-Sepharose 4B media (Amersham) for 2 h. This mixture was then transferred to a small chromatography column and washed 3 times with 5 mL wash buffer (420 mM Na_3PO_4, pH 7.3, 150 mM NaCl, 1X protease inhibitor mix). The HIS-tagged protein was eluted with elution buffer (50 mM Tris-HCl, pH 8, 10 mM reduced glutathione, 10% glycerol, 1X protease inhibitor mix). The recombinant proteins were then separated on 4-17% SDS-PAGE gradient gels. Protein expression was verified using Western blotting analysis with a HIS tag-specific antibody. To remove the HIS tag, the purified HIS-CCoAOMT recombinant proteins were digested with AcTEV, according to supplier recommendations (Invitrogen). Briefly, 140 mL recombinant protein elution was digested at 30°C for 2 h in a mixture of 7.5 mL TEV buffer (20X buffer, Invitrogen), 1.5 mL 0.1 mM dithiothreitol, and 2 mL AcTEV protease (20 units).

Enzyme activity measurements

The activities of LjCCoAOMT recombinants were measured using a protocol similar to that described in Inoue et al. (1998). The assay was carried out in a 200-mL reaction mixture, consisting of 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2 mM $MgCl_2$, 20 mM b-mercaptoethanol, 10% glycerol, 150 mM S-adenosyl-L-methionine, and 200 mM caffeic acid. The reaction was initiated by the addition of 40 mg protein and incubated at 30°C. At the indicated times, a 20-mL reaction mixture was removed and the reaction was stopped by the addition of 180 mL stop buffer (0.1%, formic acid, 5% acetonitrile, pH 2.5). The sample aliquots were then filtered with 0.22-mm filters and a 20-mL filtered sample was analyzed by HPLC using the same method described above for the CGA analyses.

Analysis of LjCCoAOMT1 expression in rice

Gateway cloning technology was used to build the pCAMBIA1301::LjCCoAOMT1 plant overexpression vector. The expression vector was transformed in rice using an *Agrobacterium*-mediated method similar to that used by Hiei et al. (1994). *LjCCoAOMT1* and *AsRed* gene-specific primers were used to perform PCR amplification and to test for positive transgenic plants. The red fluorescence marker in pCAMBIA1301 was used as a detection index.

T3 LjCCoAOMT1 transgenic rice and wild-type rice seedlings were grown up to the 2-leaf stage under natural light conditions: blue all day (28°C), dark all day (28°C), and sunshine (28°C, light intensity 100 µmol/m^2s). The seedlings were first placed in darkness for 5 days and were then transferred to blue light for 10 days. Seedlings were sprayed with dif-

ferent GA3 concentrations (10, 100, 200, or 400 mg/L) every 3-5 days for a total of 3 times, repeating each process 3 times per seedling. As an internal control, a 295-bp PCR fragment of the actin gene RAC1 in rice (Singh et al., 1989) was amplified from rice leaves samples, and the expression of the rice *CCoAOMT1* genes and *LjCCoAOMT1* genes were determined by quantitative PCR. Phenotypic changes in the seedlings were compared, and *LjCCoAOMT1* expression was detected by quantitative PCR.

CGA content of transgenic and wild-type rice

T3 transgenic rice and wild-type rice seedlings were grown under different concentrations of GA3 and different lighting conditions. The CGA content of leaves of transgenic and wild-type rice was detected by HPLC, following the methods described above.

RESULTS

Profile of the main CGA molecules in *Lonicera Japonica* Thunb. during flower development

The total amount of CGA decreased dramatically as the flower matured (Figure 1). For example, the amount of CGA decreased from approximately 2.95% at the green bud stage to 0.84% at the yellow flower stage. The CGA level in the leaf was lower than that in the flower of *L. japonica* Thunb. At later stages of development, the CGA level was 0.84-1.19%, which was relatively stable.

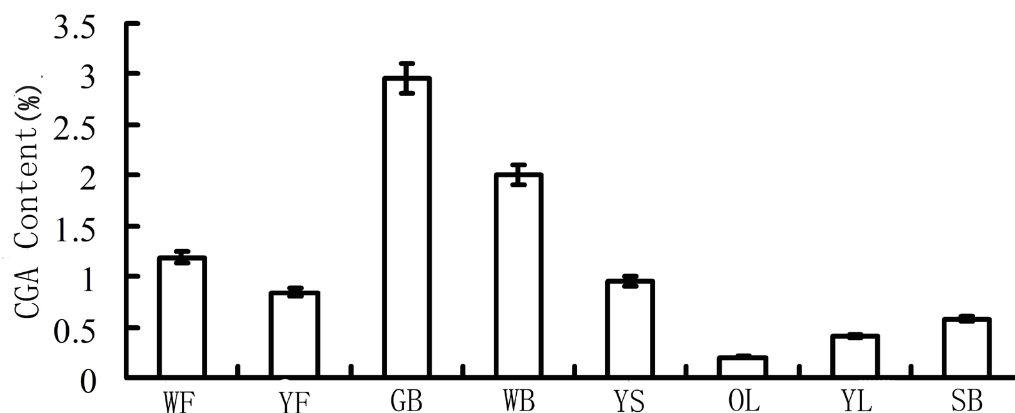

Figure 1. Different tissue CGA content in *Honeysuckles* during flower development. WF = white flower; YF = yellow flower; GB = green bud; WB = white bud; YS = young shoot; OL = old leaf; YL = young leaf; SB = stem bark.

Isolation and characterization of cDNA encoding *L. japonica* Thunb. CCoAOMT1

To explore whether the high level of CGA in *L. japonica* Thunb. is due to changes in the expression and/or activity of one or more of the enzymes involved in the phenylpropanoid pathway, and to further address the preponderance of CQA over other potential CGA mol-

ecules in *L. japonica* Thunb., we isolated and characterized cDNA encoding LjCCoAOMT1 proteins that are directly involved in the synthesis of CGA; specifically those that move CGA toward S and G lignins.

We isolated and sequenced *LjCCoAOMT1* (*L. japonica* Thunb. caffeoyl-CoA 3-O-methyltransferase 1). This sequence is 744 bp in length and encodes a 248-amino acid protein that has an estimated molecular weight of 27.94 kDa (Figure 2). The LjCCoAOMT1 recombinant protein, with an N-terminal HIS-tag, was also overexpressed in *E. coli*, as described in Material and Methods. This HIS-tag fusion protein was then purified. The purified protein preparation contained 1 major band of approximately 75.3 kDa on a Coomassie-stained SDS-PAGE gel, which corresponded to the expected size of LjCCoAOMT1 plus the HIS-tag. CCoAOMT is known to methylate caffeic acid, albeit at a lower efficiency than its preferred substrate, caffeoyl CoA (Parvathi et al., 2001). Thus, to verify the activity of the recombinant LjCCoAOMT1, we tested the capability of this protein to methylate caffeic acid to generate ferulic acid. The HPLC spectra of the control and test reactions (Figure 3) clearly showed that 2 new peaks appeared 34.0 and 40.5 min after 4 h of incubation, and these peaks were only present in test samples with the recombinant enzymes. Another small peak with a retention time of 36.5 min was only seen in the reaction with the recombinant enzyme.

Tissue specific expression of LjCCoAOMT1

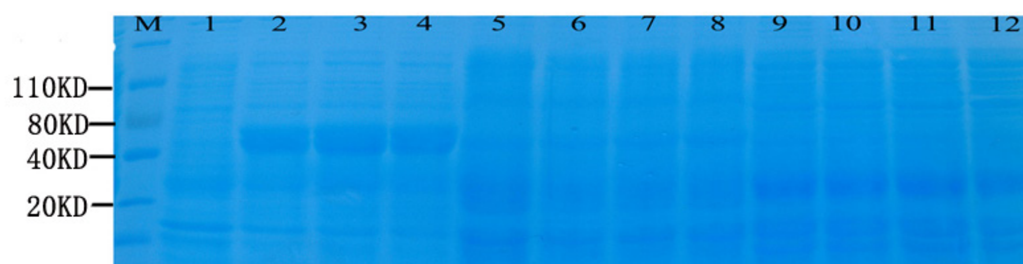

Figure 2. Effect of recombinant protein expression treated with different concentrations of IPTG. (**A**) 1, 2, 3, 4 represent recombinant protein expression treated with different concentrations of IPTG, 0 mmol/L, 0.2 mmol/L, 0.4 mmol/L, 0.8 mmol/L IPTG respectively. (**B**) 5, 6, 7, 8 represent pCOLD protein expression treated with different concentrations of IPTG, 0, 0.2, 0.4, 0.8 mM IPTG respectively. (**C**) 9, 10, 11, 12 represent *E. coli* BL21 treated with different concentrations of IPTG, 0, 0.2, 0.4, 0.8 mM IPTG, respectively.

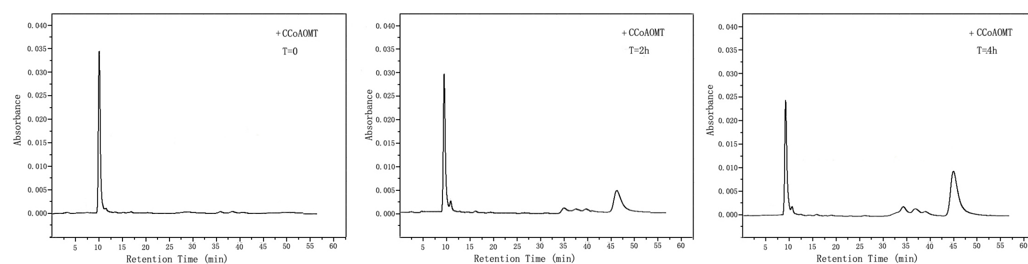

Figure 3. HPLC elution profiles of samples reacted with AcTEV digested HIS-CCoAOMT1 protein. Note: Samples were taken after T = 0, 2 and 4 h of incubation at 40°c and analyzed by HPLC.

To explore the expression profiles of *LjCCoAOMT1*, we measured the transcript levels of these genes in several *L. japonica* Thunb. tissues by qRT-PCR. Various levels of *LjCCoAOMT1* transcripts were detected in all tissues examined (Figure 4). The highest transcript level of *LjCCoAOMT* was detected in the green bud, with a template quantity (RQ) of 21.688, and was the lowest in the white bud (RQ of 0.049). The *LjCCoAOMT* transcript level was relatively high in the young stem (RQ of 6.516), but decreased to an RQ of 0.247 in the stem bark. Both old and young leaves exhibited low levels of *LjCCoAOMT* transcripts with RQ values of 0.18 and 0.119, respectively.

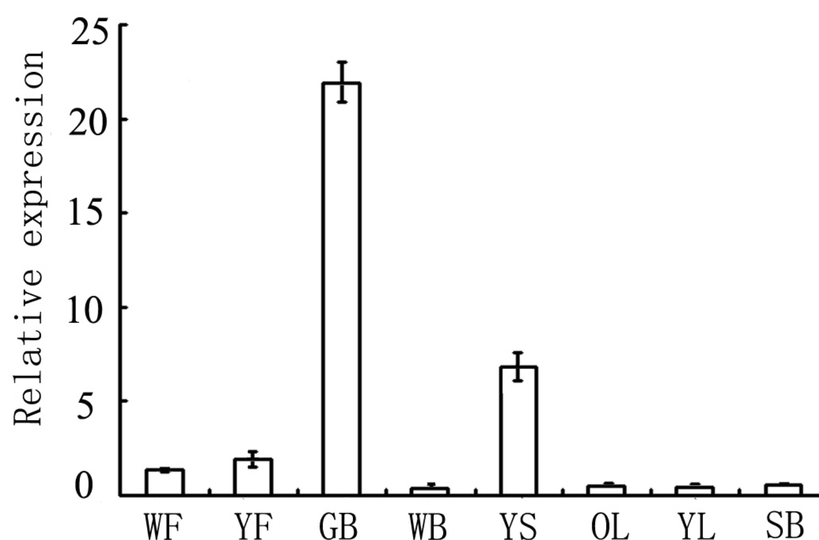

Figure 4. Different tissue q-PCR analysis and of mRNA expression of *LjCCoAOMT* in flower development in *Honeysuckles*. WF = white flower; YF = yellow flower; GB = green bud; WB = white bud; YS = young shoot; OL = old leaf; YL = young leaf; SB = stem bark.

Correlation between CGA content and LjCCoAOMT1 expression

The correlation matrix showed that *LjCCoAOMT1* expression was significantly correlated with CGA content, with a significant positive correlation coefficient of 0.773. During flower development, the transcript accumulation profile of *LjCCoAOMT1* was similar to its expression profile.

Effects of different light treatments on LjCCoAOMT1 transgenic rice

LjCCoAOMT1 transgenic rice turned yellow dramatically under dark conditions, and then turned green after exposure to blue light more quickly than did the wild-type rice, indicating that transgenic rice is more sensitive to variations in lighting conditions. The qPCR analysis further demonstrated that the relative expression quantity of transgenic rice increased more quickly under dark or blue light than it did under natural light, with RQ values 2.2 and

4.0 times higher, respectively. The expression of *CCoAOMT1* of the rice itself also increased under dark and light treatments relative to that under natural light, but the magnitude of increase was small compared to that observed for *LjCCoAOMT1*.

Effects of GA3 concentration on *LjCCoAOMT1* transgenic rice

GA3 was found to effectively promote the growth of *LjCCoAOMT1* transgenic rice and wild-type rice, although increasing the concentration of gibberellin resulted in faster growth in *LjCCoAOMT1* transgenic rice. *CCoAOMT1* expression increased along with the increase of GA3 in transgenic *LjCCoAOMT1* rice and wild-type rice, but again, the phenomenon was more apparent in the transgenic rice. When GA3 concentration increased from 200 to 400 mg/L, the expression of *CCoAOMT1* of the rice itself did not show any obvious increase, while that of the transgenic *LjCCoAOMT1* rice increased substantially, indicating that GA3 can maintain its normal physiological function under high concentrations in transgenic rice.

Comparison of CGA content between transgenic and wild-type rice

The CGA content of transgenic rice was higher than that of wild-type rice under the same experimental conditions. The order of the effects of different treatments on CGA content was as follows: GA3 > blue light > dark treatment (Table 2); however, differences were significant in all samples. This difference may have been due to factors of the experimental conditions changing only the transcription level of *LjCCoAOMT1*. However, transcription of *LjCCoAOMT1* is also closely related with the formation of lignin. In addition, the CGA content in rice leaves was originally found to be very low.

Table 2. CGA content of rice seedling under different condition.

	LjCCoAOMT1 transgenic rice				Wild type rice			
	Nature light 15 days	Dark all day 5 days	Blue light all day 10 days	GA3 (400mg/L)	Nature light 15 days	Dark all day 5 days	Blue light all day 10 days	GA3 (400mg/L)
CGA content (%)	1.31 ± 0.42	1.42 ± 0.33	1.54 ± 0.35	1.64 ± 0.34	1.34 ± 0.36	1.36 ± 0.35	1.47 ± 0.24	1.60 ± 0.45

Date are reported as means ± SD.

DISCUSSION

Because the flowering time of *L. japonica* Thunb. is relatively fast, harvesting of the flowers is concentrated over a very short period. Timely harvest of the buds is difficult, leading to complete flowering and even withering in some cases. In order to simplify this operation and save time, farmers commonly harvest all flowers at the same time, which can have a significant impact on the yield and quality of *L. japonica* Thunb. Therefore, the ability to regulate the florescence by manipulating temperature and light, and spraying hormones is of great importance for *L. japonica* Thunb. production. Hormones are extremely important regulatory factors for plant development and play an important role in regulating the biological synthesis of plant secondary metabolites. As a signaling molecule, light also plays an important role in the synthesis of plant secondary metabolites. Several studies have shown that the gene *CCoAOMT1* is closely corre-

lated with the synthesis of CGA and lignin in *L. japonica* Thunb. (Guo et al., 2001; Maud et al., 2007). Whiting et al. (1982) found that in the case of short sunlight exposure periods, treatment of wheat with intermittent red and far-red light enhanced the lignin content in the cell wall, while continuous far-red light treatment resulted in reduction in lignin content. In addition, Rogers et al. (2005) showed that transcription of lignin synthesis genes in *Arabidopsis* was affected by light, the biological clock, and hexose availability. Therefore, regulation of *CCoAOMT1* gene expression by changing lighting conditions and hormone application can effectively improve the CGA content in *L. japonica* Thunb.

In this study, we analyzed the expression characteristics of *LjCCoAOMT1* in different tissues, and the mechanisms of CGA synthesis in *L. japonica* Thunb. The results showed that *LjCCoAOMT1* played an important role in CGA synthesis in *L. japonica* Thunb. *LjCCoAOMT1* is highly expressed during the early developmental stage of *L. japonica* Thunb., indicating that the LjCCoAOMT1 enzyme is particularly important for the early synthesis of CGA. The CGA content is high at the early flowering stage, but is low by the late flowering stage, and this shift is not only regulated by *LjCCoAOMT1* expression but is also associated with the upregulation of lignin synthesis. Therefore, these results provide potential mechanisms of CGA synthesis in *L. japonica* Thunb. These results will also prove useful for studies aiming to improve lodging resistance and relevant components in rice destined for specific purposes.

Hoffman et al. (2004) showed that hy hydroxycinnamoyl-CoA shikimate/quinate hydroycinnamoyl transferase (HCT), hydroxycinnamoyl transferase (HQT), p-coumarate 3-hydroxylase (C3H), and CCoAOMT1 are key enzymes in CGA synthesis in plants. Schoch et al. (2001) and Abdulrazzak et al. (2006) showed that C3H could catalyze hydroxylation at the 3' position of coumaric acid. The substrates of C3H are quinic acid or shikimic acid. Raes et al. (2003) showed that both HCT and HQT could catalyze coumaroyl, quinic, and shikimic acids, leading to the production of caffeoyl-CoA. Hoffmann et al. (2003) first cloned the gene *HCT* from tobacco, and showed that the HCT recombinant could catalyze the production of coumaroyl, quinic acid, or shikimic acid ester. In further studies, Hoffmann et al. (2004) also demonstrated that HCT could catalyze the hydrolysis of CGA, leading to the production of quinic acid and caffeoyl-CoA. Zhang and Chinnappa (1997) showed that CCoAOMT1 could catalyze caffeoyl-CoA to generate ferulic acid coenzyme A. Zhong et al. (1998) and Ferrer et al. (2005) independently confirmed that CCoAOMT1 also catalyzes the methylation of 5'-hydroxyl-feruloyl-CoA, leading to the generation of mustard coenzyme A. Therefore, LjCCoAOMT1 appears to be just one of the key enzymes involved in the biosynthesis of CGA in *L. japonica* Thunb. Because CGA synthesis in *L. japonica* Thunb. also requires the expression and regulation of HCT, HQT, and C3H, the interaction between LjCCoAOMT1 and other relevant enzymes needs to be further investigated.

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