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Cloning and characterization of the key 4-coumarate CoA ligase genes in *Boehmeria nivea*

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ABSTRACT

The 4-coumarate CoA ligase (4CL) is an essential enzyme, in phenylpropanoid synthesis. Its function is responsible for the formation of the hydroxycinnamate-CoA thioester, precursors of lignin and flavonoids. In this study, we isolated three 4CL genes from the ramie, named as Bn4CL1, Bn4CL2 and Bn4CL3, and they were predicted to encode three proteins of 560, 547 and 539 amino acids respectively. These genes shared 80–92% identity with other plant *4CL* homologous sequences that acquired in public databases. The amino acid sequence analysis showed that Bn4CLs contained most of the conserved active sites and two conserved motifs that called Box I and Box II of those kinds of enzymes, while Bn4CL3 has 2 bases were altered. Bn4CL1 and Bn4CL2 belong to the stable proteins and have no transmembrane region, while Bn4CL3 belong to unstable protein and has two transmembrane regions. The Bn4CL3 contains more serine amino acid residues than that in Bn4CL1 and Bn4CL2. The phylogenetic analysis suggested that the three homologous genes are in different classes that the Bn4CL1 and Bn4CL2 belonged to class I, and Bn4CL3 belonged to class II. The structural characteristics of Bn4CL proteins were analyzed by constructing the three-dimensional structure model. The results provided basic information to understand the structural characteristic of Bn4CLs. Expression patterns of the three Bn4CL genes in different developmental stages were analyzed. The expression level of Bn4CL3 was significantly different from the other two genes. In view of the above experimental results, we predicted that Bn4CL1 and Bn4CL2 may be involved in lignin synthesis in ramie, while Bn4CL3 may more likely be associated with flavonoid synthesis in ramie. The results pave the way for future work on the function of the Bn4CLs in phenylalanine metabolic pathway of ramie.

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1. Introduction

4-coumarate:CoA ligase (4CL, EC 6.2.1.12) converts 4-coumaric acid and other substituted cinnamic acids, such as caffeic, ferulic, and sinapic acids, into corresponding CoA thiolesters used for the biosynthesis of lignin and flavonoids (Beuerle and Pichersky, 2002). 4CL genes have been cloned from numerous plant species and they exist in a small gene family, with two to five members (Gui et al., 2011; Li et al., 2015; Wang et al., 2016). In other plants, such as rice and *Arabidopsis*, the 4CL family has structurally and functionally distinct members (Bjorn and Klaus, 2004). These isoforms control the relative abundance of flavonoids and various monolignols during normal development. Downregulation of *4CL* expression in *Pinus radiate* (Wagner et al., 2009), *Populus tomentosa* (Tian et al., 2013), *Panicum virgatum* (Xu et al., 2011), and hybrid poplar (*Populus tremula*×*Populus alba*) (Voelker et al., 2010) can lead to decreased lignin content, while overexpression of *4CL* in transgenic tobacco leads to increased lignin content (Rao et al., 2014). Although 4CLs have been extensively characterized in angiosperm, gymnosperm and moss species (Gao et al., 2015), characterization of 4CL in *Boehmeria nivea* (ramie) has not been studied so far.

Boehmeria nivea is a widely planted herbaceous plant in southern China that provides natural fiber, the stem of ramie can be used for generating bast fibers. Whereas lignin content in the stem can influence the fiber quality remarkably (Pandey, 2007). Thus, understanding the developmental mechanisms of stem in ramie is important to enhance ramie fiber quality and production. According to transcriptome data of *Boehmeria nivea* (Chen et al., 2014), 4CL gene were expressed in the reaction of cinnamic acid (2), p-coumaric acid (3), caffeic acid (4), ferulic acid(5), 5-hydroxyferulic acid (6) and sinapic acid (7) to generate corresponding CoA thioester in the phenylpropanoid synthesis pathyway. The CoA thioester is a precursor of the synthesis of flavonoids and lignin (Fig. 1). 4CL linked to the specific pathway of phenylpropanoid metabolism and plays a pivotal role in regulating the flow of coenzyme A ester intermediates to the subsequent biosynthetic pathway (Schneider et al., 2003; Vassão et al., 2010).

In this research we isolated the 4CL family genes from raime based on the transcriptome data and subject to bioinformatics analysis. We analyzed the putative amino acid sequences, the physicochemical properties, the transmembrane helices, the phosphorylation sites, the

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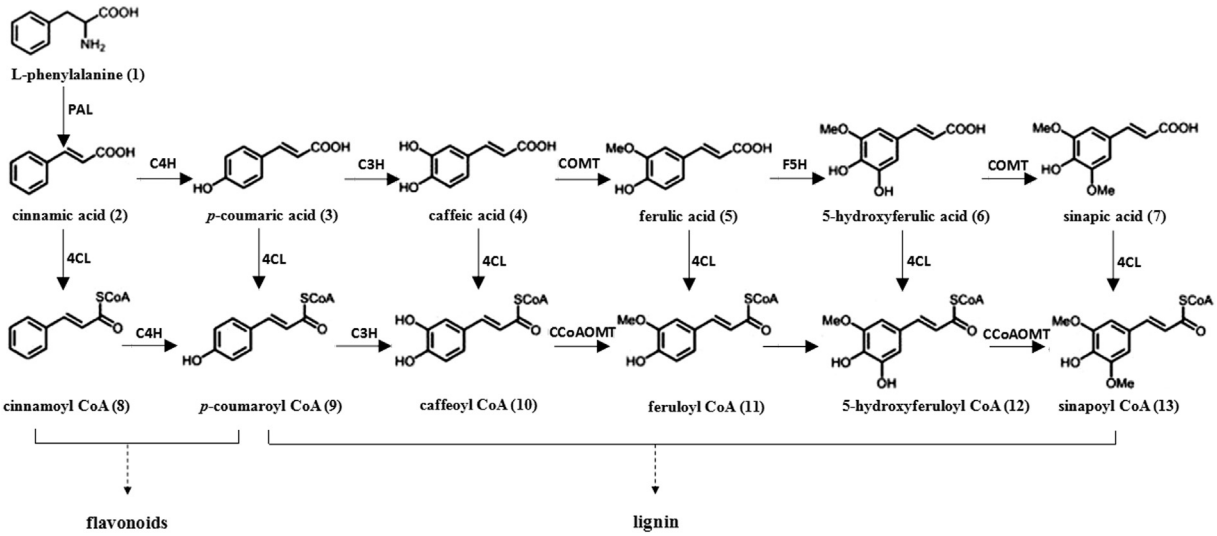

Fig. 1. The proposed synthesis pathway of polyphenol compounds in the *Boehmeria nivea*. PAL: phenylalanine ammonia lyase; C4H: cinnamate 4-hydroxylase; C3H: 4-coumarate 3-hydroxylase; COMT: caffeic acid O-methyl transferase; F5H: ferulate 5-hydroxylase; CCoAOMT: caffeoyl-CoA O-methyltransferase; 4CL: 4-coumarate:CoA ligase.

N-glycosylation sites, the phylogenetic and protein structural of these genes using VectorNTI, Ex PASy Prot Param, TMHMM2.0, NetPhos, NetNGlyc, MEGA5.1 and SWISS MODEL respectively. In addition, the real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to analyze the expression profile of 4CL mRNA in ramie during different developmental stages (initial period, mature, and late mature). We preliminary validated the characterizations of the 4CL family genes involved in the phenylpropanoid metabolism, the present findings would aid in understanding the role of 4CL in ramie.

2. Materials and methods

2.1. Ramie

Boehmeria nivea (Xiangzhu NO.3) was planted in the College of Biological and Environmental Engineering, Changsha University, Changsha, China.

2.2. RNA isolation and cDNA synthesis

The total RNA was extracted from the stem of ramie using the SV Total RNA Separation and purification system (Promega, WI, USA) following the manufacturer's instruction. The RNA integrity was detected by 1% agarose gel electrophoresis, and the RNA concentration was determined using an Eppendorf BioPhotometer UV/VIS Spectrophotometer (Hamburg, Germany). A PrimeScript™ II 1st strand cDNA Syntyhesis kit (TaKaRa, Tokyo, Japan) was used to generate the first strand cDNA from 5 μg of total RNA following the manufacturer's protocol.

2.3. Isolation and annotation of 4CL genes in Boehmeria nivea

Based on the transcriptome data of *Boehmeria nivea* (Chen et al., 2014), the annotated unigenes of 4CL has total of 27 through the analysis of the KEGG, their length were from 202 bp to 2303 bp, which had similar sequences with other plants, such as *Oryza sativa*, *Arabidopsis thaliana* and *Populus trichocarpa*. We removed the unigenes that had no Nr annotations and the Nr annotation scores less than 100, and the remaining unigenes were in accordance with the length comparison with the CDS region of the known 4CL gene in the GenBank. The comparison showed that CL3398, CL2518 and CL905 were highly homologous with known 4CL genes and were used as core fragments for subsequent analysis. Three partial sequences of 4-coumarate: CoA ligase in ramie were screened out include CL3398, CL2518 and CL905, and primers were designed and synthesized as follows. CL3398: forward primer, 5′-CAGGTGTGAAGATTGTAGAAG-3′; reverse primer, 5′-TGTGACCATCAAGGAAAAA-3′; CL2518: forward primer, 5′-CCCTTCCTA ACTCCAAACTAATAC-3′; reverse primer, 5′-TACCTTTCCAACACCAACA AC-3′; CL905: forward primer, 5′-TGTGCTGTGCCATAACTGTCTTCTT-3′; reverse primer, 5′-CGTCATATTTTGGAGCGAACTTTCT-3′. The PCR system comprised 13.6 μL of sterile water, 2.0 μL of 10 × Ex buffer, 1.6 μL of 10 mmol/L deoxynucleotide triphosphate mix, 1.0 μL of cDNA, 0.8 μL of forward primer, 0.8 μL of reverse primer, and 0.2 μL of Takara Ex Taq Hot Start (TaKaRa, Tokyo, Japan). The PCR conditions were set as follows: 95 °C for 5 min, followed by 30 cycles of 30 s at 95 °C, 30 s at 46 °C (referring to CL3398 and CL2518, CL905 replace by 57 °C) and 1 min 30 s at 72 °C, then 10 min at 72 °C. The PCR products were purified from 1% agarosegel using the Gel extraction kit (Aidlab, BeiJing, China) and cloned into the pMD18-T vector (TaKaRa). Inserts were further sequenced for confirmation (Invitrogen, Shanghai, China). Then the VectorNTI11.5.1 software was used to search for the ORF of *4CLs* in ramie and predicted their amino acids.

2.4. Bioinformatics analysis of 4CL genes in Boehmeria nivea

The Basic Local Alignment Search Tool (BLAST) software from National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) was used to identify the conserved domains and analyze the relevant amino acid sequence. The amino acids sequence alignment by DNAMAN. The physicochemical properties that include amino acid numbers, molecular weight (Mr), theoretical pI, chemical formula, total number of negatively charged residues (Asp + Glu), total number of positively charged residues (Arg + Lys), instability index and grand average of hydropathicity (GRAVY) of 4CLs in ramie were predicted by using Ex PASy Prot Param tool online software (http://web.expasy.org/protparam/). The transmembrane helices of *4CLs* in ramie were predicted by using online software TMHMM2.0 (http://www.cbs.dtu.dk/servi-ces/TMHMM/). The programs NetPhos 3.1a (http://www.cbs.dtu.dk/services/Net Phos) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNglyc/) were used to identify phosphorylation and N-glycosylation sites of 4CLs in ramie. Deduced amino acids of 4-coumarate: CoA ligase genes were collected from the NCBI database as references. The corresponding phylogenetic trees

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