For. Stud. China, 2007, 9(3): 208–212
DOI 10.1007/s11632-007-0033-z

RESEARCH ARTICLE

# High-level expression of 4-coumarate:coenzyme A ligase gene *Pt4CL1* of *Populus tomentosa* in *E. coli*

*Fan Bing-you*[1,2] *Lu Hai*[1] *Jiang Xiang-ning*[1*]

[1]College of Biological Sciences and Biotechnology, Beijing Forestry University, Beijing 100083, P. R. China
[2]College of Agriculture, Henan University of Science and Technology, Luoyang 471003, P. R. China

**Abstract** In order to investigate the enzymatic properties of the 4CL1 of *Populus tomentosa*, the recombinant expression vector pQE31-4CL1 was constructed. The recombinant was identified by three restriction endonucleases, then the vector pQE31-4CL1 was transformed into expression host M15 (pREP4) and induced by isopropyl-*α*-D-thiogalactoside (IPTG) to express 60 kD fused protein Pt4CL1. The biologically active Pt4CL1, expressed as soluble protein, was achieved with 0.6 mmol·L$^{-1}$ IPTG induction as the expression temperature declined from 37 to 28°C. The 6×His tag facilitates affinity binding to $Ni^{2+}$-nitrolotriacetic acid (NTA) and enables one-step purification to acquire the molecular SDS-PAGE electrophoresis purity of the active 4CL1 protein by agarose coupled with $Ni^{2+}$-NTA affinity chromatography. The optimal substrate for Pt4CL1 was 4-coumarate.

**Key words** 4-coumarate:coenzyme A ligase, *Populus tomentosa*, prokaryotic expression, enzyme activity

## 1 Introduction

Lignin is a complex phenolic polymer based on cinnamyl alcohol subunits derived from phenylpropanoid metabolism and is a major plant natural product. Ubiquitous in vascular plants, lignin plays important roles in structural support, water transport and defense (Whetten and Sederoff, 1995). The 4-coumarate:coenzyme A ligase (4CL, EC 6.2.1.12) catalyzes the activation of the hydroxylated cinnamic acids to their corresponding thioesters (Lozoya et al., 1988). The central position of 4CL, linking the general with specialized branches of phenylpropanoid metabolism, led to the suggestion that 4CL could play a pivotal role in regulating the flux of the activated CoA ester intermediates into subsequent biosynthetic pathways (Lindermayr et al., 2002). Distinct 4CL isoenzymes mediate the biosynthesis of two groups of phenolic metabolites, i.e. lignin and flavonoids. Isoenzymes of 4CL in soybean (*Glycine max*) (Knobloch and Hahlbrock, 1975), petunia (*Petunia hybrida*) (Ranjeva et al., 1976), pea (*Pisum sativum*) (Wallis and Rhodes, 1977), oat (*Avena sativa*) (Knogge et al., 1981), and poplar (*Populus euramericana*) (Grand et al., 1983) displayed different substrate affinities. In order to investigate enzymatic properties of the 4CL1 of *Populus tomentosa*, the prokaryotic expression vector pQE31-4CL1 was constructed and high-level expression system was established. Soluble recombinant Pt4CL1 protein, which was active to hydroxylated cinnamic, was expressed successfully in *E. coli*. The SDS-PAGE electrophoresis purity Pt4CL1 protein was acquired by one-step metal affinity column chromatogram. Pt4CL1 can catalyze 4-coumarate, caffeate, ferulate, cinnamate, but had no activity to sinapate.

## 2 Materials and methods

### 2.1 Enzymes and reagents

Restriction endonucleases *Bam*H I, *Sma* I, *Kpn* I, $T_4$ DNA ligase and isopropyl-*α*-D-thiogalactoside (IPTG) were all from Takara (Dalian, China), DNA recovery kit from BioDev-Tech Co., Ltd (Beijing, China), $Ni^{2+}$-nitrolotriacetic acid (NTA) were from Qiagen (Germany), 4-coumarate, caffeate, ferulate, cinnamate, and sinapate were from Sigma (USA). Other analytic reagents were from domestic and foreign biology companies.

### 2.2 Plasmids and bacterial strains

*4CL1* cDNA gene of *P. tomentosa* (*Pt4CL1*, Genebank No. AY043495) was cloned in our laboratory and kept in plasmid pMD18T-4CL1. The plasmid pQE31, pQE40 and *E. coli* strain M15 (pREP4) were from Qiagen (Germany). *E. coli* strain JM109 was prepared in our laboratory.

### 2.3 Construction and identification of the prokaryotic expression vector of pQE31-4CL1

Empty vector pQE31 and pMD18T-4CL1 was di-

*Author for correspondence. E-mail: fanbingyou2005@163.com

gested by *Bam*H I, then the fragments of pQE31 and *Pt4CL1* were recovered with a DNA recovery kit and then ligated with $T_4$ DNA ligase at 14°C for 16 hours. The ligated products were transformed into *E. coli* JM109. The positive recombinant was characterized by three restriction endonucleases *Kpn* I, *Bam*H I and *Sma* I. The empty vector pQE31, the positive vector pQE40 coding a positive protein DHFR and the recombinant plasmid pQE31-Pt4CL1 were isolated from *E. coli* JM109 and then transformed into *E. coli* M15 (pREP4), respectively. And the transformed M15 (pREP4) was selected on LB medium containing 100 μg·mL$^{-1}$ ampicillin (Amp) and 25 μg·mL$^{-1}$ kanamycin (Kan). M15 (pREP4) transformed with pQE31, pQE40 and pQE31-Pt4CL1 were named QM31 as negative expression control, QM40 as positive expression control and QM4CL1 respectively.

### 2.4 Expression and analysis of *Pt4CL1* gene

A single QM31, QM40 and QM4CL1 colony was inoculated into LB medium containing 100 μg·mL$^{-1}$ Amp and 25 μg·mL$^{-1}$ Kan. When $OD_{600}$ reached 0.6, IPTG was added to a final concentration of 0.6 mmol·L$^{-1}$. After the culture was inducted at 37°C for 3 to 4 hours, 1.5 mL bacteria were harvested by 5,000 r·min$^{-1}$ centrifugation and the pellet was resuspended with a 2×SDS-PAGE gel-loading buffer, containing 100 mmol·L$^{-1}$ Tris-HCl (pH 6.8), 4% SDS, 0.2% Bromophenol Blue, 20% glycerol and 200 mmol·L$^{-1}$ *β*-mercaptoethanol, and was analyzed with SDS-PAGE. The concentrate gel was 4% and separate gel 10% described by Ausubel et al. (1995). After electrophoresis, the gel was stained with Coomassie Bright Blue, and then the result was observed and photographed.

### 2.5 Expression pattern of Pt4CL1 expressed at different temperature

Fifty mL bacteria expressed at 37 and 28°C was centrifuged at 5,000 r·min$^{-1}$, then the cell pellet was thawed on ice for 15 min and resuspended in lysis buffer (50 mmol·L$^{-1}$ $NaH_2PO_4$, 300 mmol·L$^{-1}$ NaCl, 10 mmol·L$^{-1}$ imidazole, pH 8.0) at 2–5 mL per gram wet weight and then the bacteria solution was sonicated on ice by a sonicator equipped with a microtip, six 10-s bursts at 200 W with a 10-s cooling period between each burst. The lysate was centrifuged at 10,000×g for 20 min at 4°C to pellet the cellular debris. The supernatant and the pellet were analyzed with SDS-PAGE.

### 2.6 One-step purification by metal affinity column chromatogram

One mL of the 50% $Ni^{2+}$-NTA slurry was added to 4 mL cleared lysate and the mixture was shaken gently (200 r·min$^{-1}$ on a rotary shaker) at 4°C for 60 min. The 20 mol·L$^{-1}$ imidazole in the lysis buffer suppresses the binding of nontagged contaminating proteins and leads to great purity after few wash steps. The lysate–$Ni^{2+}$-NTA mixture was loaded into a column with the bottom outlet capped. The bottom cap was removed to collect the column flow-through. The flow-through was saved for SDS-PAGE analysis. The column was washed twice with 4 mL wash buffer (50 mmol·L$^{-1}$ $NaH_2PO_4$, 300 mmol·L$^{-1}$ NaCl, 20 mmol·L$^{-1}$ imidazole, pH 8.0) and then was washed with 0.5 mL elution buffer (50 mmol·L$^{-1}$ $NaH_2PO_4$, 300 mmol·L$^{-1}$ NaCl, 250 mmol·L$^{-1}$ imidazole, pH 8.0) three times. The wash and elution fractions were collected for SDS-PAGE analysis.

### 2.7 Enzyme activity of different substrates

4CL activity was assayed by spectrophotometric monitoring of the formation of CoA esters of various cinnamate derivatives, as described by Knobloch and Hahlbrock (1975, 1977). The 4CL mixture contained 5 mmol·L$^{-1}$ ATP, 5 mmol·L$^{-1}$ $MgCl_2$, 0.3 mmol·L$^{-1}$ CoA, 0.2 mmol·L$^{-1}$ substrate and an appropriate volume of eluted Pt4CL1, in a total volume of 0.2 mL and the reaction time was 5 min. The control mixture without enzyme was used. Enzyme activity was measured by the increase in absorbance at the absorption maximum of the appropriate CoA ester.

## 3 Results

### 3.1 Construction of prokaryotic recombinant expression vector of Pt4CL1

The positive recombinant plasmid DNA was isolated with minipreps DNA extraction method. The recombinant pQE31-4CL1 whose size was bigger than the empty pQE31 was screened primarily by agrose electrophoresis. The recombinant was then identified by three restriction endonucleases *Kpn* I, *Bam*H I and *Sma* I. The *Kpn* I was applied to identify the size of the recombinant, *Bam*H I to identify the size of the insert, *Sma* I to identify the orientation of the insert. The results showed (Fig. 1) that the sizes of the recombinant pQE31-4CL1 and the insert were 5.0 and 3.4 kb respectively, which were all similar to the expected length and the orientation was correct too. It suggests that the prokaryotic expression vector of pQE31-4CL1 has been successfully constructed.

### 3.2 Expression of the recombinant plasmid induced by 0.6 mol·L$^{-1}$ IPTG

As shown in Fig. 2, after 0.6 mmol·L$^{-1}$ IPTG treatment for 4 hours, it was found that a positive 26 kD

DHFR protein and a new special 60 kD protein, all similar to the expected size, were obtained in QM40 and QM4CL1 bacteria, respectively. That is to say, the recombinant 4CL1 protein was expressed successfully in *E. coli*.

### 3.3 Effect of temperature on the expression pattern of fused protein

Bacteria of QM31 and QM40 expressed at 37°C induced by IPTG were sonicated entirety and centrifuged at 12,000 r·min$^{-1}$ for 20 min. The precipitation was resuspended in 5 mL lysis buffer for SDS-PAGE analysis. It showed that the expressed fused Pt4CL1 were composed of almost the inclusion body (Fig. 3). Eukaryotic proteins expressed intracellularly in *E. coli* were frequently expressed as insoluble inclusion bodies. The intermolecular association of hydrophobic domains during folding is believed to play a role in the formation of inclusion bodies (Mitraki and King, 1989). It was necessary to acquire the active Pt4CL1 by inclusion body denaturation and naturation (Lilie et al., 1998).

Decreasing the induction temperature can increase the soluble expression of some eukaryotic gene in *E. coli* (Baneyx, 1999). Therefore, the expression pattern of the Pt4CL1 at 28°C was studied. BandScan software analysis revealed that proportion of the recombinant Pt4CL1 protein in the total bacteria lysate was 11.8%, and soluble Pt4CL1 was 6.5% in the total soluble bacteria protein. This indicates that the recombinant Pt4CL1 was mainly expressed as soluble protein when bacteria QM4CL1 were induced at 28°C (Fig. 4).

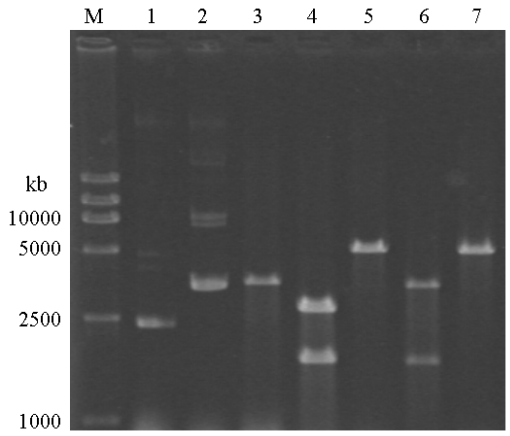


**Fig. 1** Characterization of the positive recombinant pQE31-4CL1 by enzyme digestion. Lane M: DNA Marker; Lane 1: pQE31; Lane 2: pQE31-4CL1; Lane 3: pQE31/*Bam*H I; Lane 4: pMD18-T-4CL1/*Bam*H I; Lane 5: pQE31-4CL1/*Kpn* I; Lane 6: pQE31-4CL1/ *Bam*H I; Lane 7: pQE31-4CL1/*Sma* I.

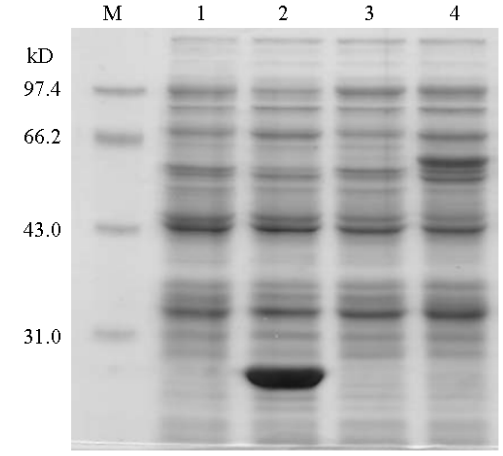


**Fig. 2** Expression of recombinant plasmid induced by 0.6 mmol·L$^{-1}$ IPTG. Lane M: Protein Marker; Lane 1: negative control QM31 not induced; Lane 2: positive control QM40 induced; Lane 3: QMCL1 not induced; Lane 4: QM4CL1 induced.

### 3.4 Purification of Pt4CL1 and the enzyme activity of different substrates

After the soluble Pt4CL1 C was bound with $Ni^{2+}$-NTA resins, loaded into a column, washed by the wash buffer, the recombinant Pt4CL1 was eluted by the elution buffer. The SDS-PAGE electrophoresis purity PtCL1 was achieved by one-step metal affinity chromatography (Fig. 5). The analysis revealed that the purity of the elution fraction reached the SDS-PAGE electrophoresis purity. The enzymatic activity of the SDS-PAGE electrophoresis purity Pt4CL1 to the five substrates was 3,949.0 pkat·mg$^{-1}$ to 4-coumarate, 2,214.0 pkat·mg$^{-1}$ to caffeic acid, 715.0 pkat·mg$^{-1}$ to ferulate, 84.9 pkat·mg$^{-1}$ to cinnamate, no activity to sinapate.

## 4 Discussion

4CL exists distinct substrate preference and specificities. For example, the 4CL purified from loblolly pine

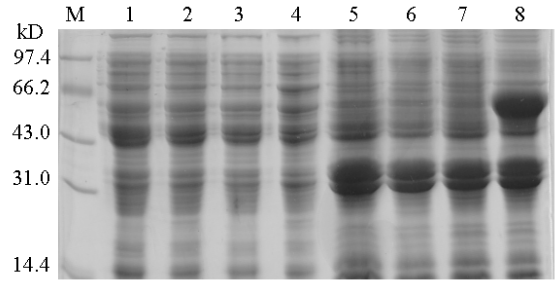


**Fig. 3** SDS-PAGE analysis of total protein, supernatant and inclusion body of QM31 and QM4CL1 induced at 37°C. Lane M: protein marker; Lane 1: supernatant of QM31 not induced; Lane 2: inclusion body of QM31 not induced; Lane 3: supernatant of QM31 induced; Lane 4: inclusion body of QM31 induced; Lane 5: supernatant of QM4CL1 not induced; Lane 6: inclusion body of QM4CL1 not induced; Lane 7: supernatant of QM4CL1 induced; Lane 8: inclusion body of QM4CL1 induced.

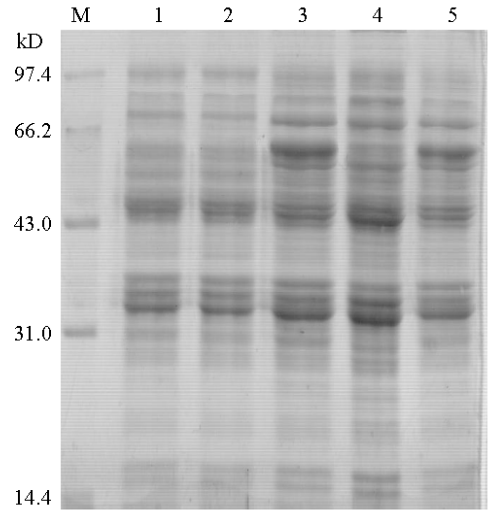


**Fig. 4** Soluble recombinant Pt4CL1 expressed at 28°C. Lane M: marker; Lane 1: total protein of QM31 induced; Lane 2: total protein of QM4CL1 not induced; Lane 3: total protein of QM4CL1 induced; Lane 4: supernatant of QM31induced; Lane 5: supernatant of QM3-4CL1induced.

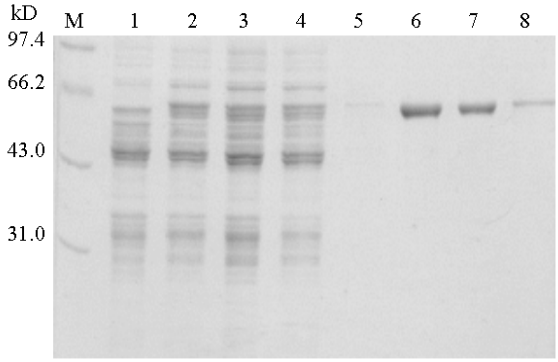


**Fig. 5** One-step purification of recombinant 4CL1 by metal affinity chromatography. Lane M: protein marker; Lane 1: total protein of pQE31 induced; Lane 2: supernatant of pQE31-4CL1induced; Lane 3: the flow-through; Lane 4: wash fractions I; Lane 5: wash fractions II; Lane 6: elution protein I; Lane 7: elution protein II; Lane 8: elution protein III.

(*Pinus taeda*) catazyled 4-coumaric acid, caffeic acid, ferulic acid and cinnamic acid as substrates, but it had no detectable activity to sinapic acid (Voo et al., 1995). And the 4CL1 and 4CL2 of hybrid poplar (*P. trichocarpa*×*P. deltoides*) exhibited no detectable activity to sinapic acid too (Allina et al., 1998). However, 4CL1 of soybean (Lindermayr et al., 2002), 4CL2 and 4CL3 of black locust (*Robinia pseudoacacia)* all displayed activity toward sinapate (Hamada et al., 2004).

To study enzymatic properties of Pt4CL1, prokaryotic expression vector pQE31-4CL1 was constructed and the high level expression of *Pt4CL*1 gene in *E. coli* was established. SDS-PAGE analysis showed that the Pt4CL1 was almost expressed as insoluble inclusion body when QM4CL1 was induced and expressed at 37°C. Though a small amount of biological active protein could often be recovered from inclusion bodies (Lilie et al., 1998), the extraction procedures require expensive and time-consuming protein solubilization and refolding. In this experiment when QM4CL1 was induced and expressed at 28°C, the recombinant Pt4CL1 was mainly expressed as biological active soluble protein. Through one-step metal affinity chromatography the SDS-PAGE purity Pt4CL1 protein was obtained. The successful expression and purity of the soluble Pt4CL1 offer an opportunity to study enzymatic characteristics of the Pt4CL1 protein and its structure by crystallization. Further research will be focused on its enzymatic properties characterization and crystallization system in order to clarify the relation of the structure and function of Pt4CL1 protein.

## Acknowledgement

This study was financially supported by the Major State Basic Research Development Program of China (973 Program) (G1999016005).

## References

Allina S M, Pri-Hadash A, Theilmann D A, Ellis B E, Douglas C J. 1998. 4-coumarate:coenzyme A ligase in hybrid poplar[1]. Properties of native enzyme, cDNA cloning, and analysis of recombinant enzymes. Plant Physiol., 116: 743–754

Ausubel F M, Brent R, Kingston R E, Moore D D, Seidman J G, Smith J A, Struhl K. 1995. Short Protocols in Molecular Biology. 3rd edn. New York: John Wiley and Sons Inc

Baneyx F. 1999. Recombinant protein expression in *Escherichia coli*. Curr. Opin. Biotechnol., 10(5): 411–420

Grand C, Boudet A, Boudet A M. 1983. Isoenzymes of hydroxycinnamate:CoA ligase from poplar stems, properties and tissue distribution. Planta, 158: 225–229

Hamada K, Nishida T, Yamauchi K, Fukushima K, Kondo R, Tsutsumi Y. 2004. 4-coumarate:coenzyme A ligase in black locust (*Robinia pseudoacacia*) catalyses the conversion of sinapate to sinapoly-CoA. J. Plant Res., 117(4): 303–310

Knobloch K H, Hahlbrock K. 1975. Isoenzymes of p-coumarate: CoA ligase from cell suspension cultures of *Glycine max*. Eur. J. Bichem., 52(2): 311–320

Knobloch K H, Hahlbrock K. 1977. 4-coumarate:CoA ligase from cell suspension cultures of *Petroselinum hortense* Hoffm. Partial purification, substrate specificity, and further properties. Arch. Biochem. Biophys., 184(1): 237–248

Knogge W, Beulen C, Weissenböck G. 1981. Distribution of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase in oat primary leaves. Z. Naturforsch., 36c: 389–395

Lilie H, Schwarz E, Rudolph R. 1998. Advances in refolding of proteins produced in *E. coli*. Curr. Opin. Biotechnol., 9: 497–501

Lindermayr C, Möllers B, Fliegmann J, Uhlmann A, Lottspeich F, Meimberg H, Ebel J. 2002. Divergent members of a soy-

bean (*Glycine max* L.) 4-coumarate:coenzyme A ligase gene family. Primary structures, catalytic properties, and differential expression. Eur. J. Bichem., 269(4): 1,304–1,315

Lozoya E, Hoffmann H, Douglas C, Schuiz W, Scheel D, Hahlbrock K. 1988. Primary structures and catalytic properties of isoenzymes encoded by the two 4-coumarate: CoA ligase genes in parsley. Eur. J. Biochem., 176(3): 661–667

Mitraki A, King J. 1989. Protein folding intermediates and inclusion body formation. Bio/Technology, 7: 690–697

Ranjeva R, Boudet A, Faggiox R. 1976. Phenolic metabolism in petunia tissues. IV. Properties of p-coumarate: coenzyme Aligase isoenzymes. Biochimie, 58: 1,255–1,262

Voo K S, Whettern R W, O'Malley D M, Sederoff R R. 1995. 4-coumarate:coenzyme a ligase from loblolly pine xylem. Isolation, characterization, and complementary DNA cloning. Plant Physiol., 108(1): 85–97

Wallis P J, Rhodes M J C. 1977. Multiple forms of hydroxycinnamate:CoA ligase in etiolated pea seedling. Phytochemistry, 16: 1,891–1,894

Whetten R, Sederoff R. 1995. Lignin biosynthesis. Plant Cell, 7: 1,001–1,003

(Received March 1, 2007 Accepted June 12, 2007)