J. Plant Biol. (2009) 52:482–491
DOI 10.1007/s12374-009-9062-6

ORIGINAL RESEARCH

Identifying a Cinnamoyl Coenzyme A Reductase (CCR) Activity with 4-Coumaric Acid: Coenzyme A Ligase (4CL) Reaction Products in *Populus tomentosa*

Dong-Dong Wang · Hua Bai · Wei-Qi Chen · Hai Lu · Xiang-Ning Jiang

Received: 9 December 2008 / Revised: 7 May 2009 / Accepted: 11 May 2009 / Published online: 22 August 2009

Abstract A cinnamoyl coenzyme A reductase (CCR, EC 1.2.1.44), one of the key enzyme involved in lignin biosynthesis, was cloned from *Populus tomentosa* (Chinese white poplar). At the same time, a 4CL1 gene was cloned from *P. tomentosa*, too. The two genes were subcloned in pQE31 vector and expressed in *Escherichia coli* M15. Both of them were purified by Ni-NTA. Purified CCR protein was digested by trypsin and analyzed by HPLC-MS; the peptide segments had 27% similarity with the sequence of the CCR protein. 4CL was thought to be a neighbor enzyme of CCR in lignin biosynthesis. In this paper, a 4CL1 from *P. tomentosa* was cloned, and its enzyme reaction products were extracted for the substrates of CCR. Three 4CL1 enzyme reaction products were monitored by HPLC-MS and then the CCR enzyme reaction was detected by GC-MS. In the CCR reaction, the three corresponding aldehyde (*p*-coumaraldehyde, caffealdehyde, and coniferaldehyde) were detected and identified by Frontier3 software. The results showed that the CCR that we cloned from *P. tomentosa* had affinities with 4CL1 enzyme reaction products and a ptCCR that was cloned from aspen (Li et al., *Plant Cell Physiol* 46(7):1073–1082, 2005) only had affinity with feruloyl-CoA. The different results maybe depend on the different study method. The method of exacting 4CL enzyme products as the substrates of CCR in the paper was reliable and can be used in lignin biosynthesis network to detect the enzymes in the neighborhood that depended on the polarity of the substrates and products. This CCR gene had eight homology sequence CCR gene when a BLAST was conducted in *Populus trichocarpa* genome database. The CCR homology genes in *Populus* suggested that some CCRs may take part in the lignin biosynthesis, too. The gene family would be the hot spot in the future study.

Keywords Lignin · CCR · 4CL · HPLC-MS · GC-MS

D.-D. Wang · H. Bai · W.-Q. Chen · H. Lu · X.-N. Jiang (✉)
College of Life Sciences and Biotechnology,
Beijing Forestry University,
No 35, Qinghua Donglu, Haidian District, P.O.Box 162,
Beijing 100083, People's Republic of China
e-mail: jiangxn@bjfu.edu.cn

X.-N. Jiang
The Tree and Ornamental Plant Breeding and Biotechnology Laboratory of State Forestry Administration,
Beijing 100083, People's Republic of China

Introduction

Lignin is a complex phenylpropanoid polymer located in the cell walls conducting and supporting tissues such as vascular elements and phloem fibers, where it provides hydrophobicity and mechanical strength (Inoue et al. 1998; Kawasaki et al. 2006). They result from the hydrogenative polumerization of three cinnamyl alcohols, i.e., the *p*-coumaryl alcohol, coniferyl alcohol, and sinaply alcohol, which differ by their degree of methoxylation and give rise, respectively, to hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units in the polymer (Freudenberg 1959). One of the driving forces in the research area of lignin biosynthesis has been the pulp and paper industry for which lignin is an undesirable polymer (Baltas et al. 2005). The chemical treatment needed to remove lignin during the paper pulping process is expensive and environmentally unfriendly. Lignin also negatively affects forage digestibility (Huntley et al. 2003; Barriere et al. 2004).

4-Coumaric acid, coenzyme A ligase (4CL)[EC.6.2.1.12], the last step of phenylpropanoid metabolism, catalyzes the

reaction of the hydroxylated cinnnamic acids to their corresponding thioesters (Lee et al. 1997; Hu et al. 1998; Jones et al. 2001). The 4CL1 from *P. tomentosa*, which was kept in our lab, has the highest affinity with *p*-coumarate, followed by caffeate and ferulate (Fan et al. 2006), and only the trans-coumaric acid can be catalyzed in vivo but not *cis*, which is converted from *trans* under illuminated condition.

Cinnamoyl-CoA reductase (CCR), the first committed enzyme of the lignin branch biosynthetic pathway, was thought to catalyze the reaction from thioesters, which were the products of 4CL to their corresponding cinnamaldehydes (Gross and Kreiten 1975; Ma 2007), which involved *p*-coumaroyl-CoA, caffeoyl-CoA, feruloyl-CoA, 5-hydroxyferuloyl-CoA, and sinapoyl-CoA. Four of them are shown in Fig. 1, and CCR protein purified from *Eucalyptus gunnii* did not have significant higher affinity for *p*-coumaroyl-CoA, caffeoyl-CoA, feruloyl-CoA, and sinapoyl-CoA; however, the K_{cat} was approximately three times higher with feruloyl-CoA (Goffner et al. 1998). However, a CCR gene was isolated from aspen (Li et al. 2005), and in the enzyme reaction, feruloyl-CoA was catalyzed as the most favorable substrate in mixed substrates. Only coniferadehyde was detected in the mix reaction with the developing xylem crude protein extracted; at the same time, in the single enzyme reaction, the caffealdehyde, coniferaldehyde, 5-hydroxyconiferaldehyde, and sinapaldehyde all were detected, but the CCR had a much higher affinity to feruloyl-CoA and no *p*-coumaraldehyde. The CCR*1* from *Arabidopsis thaliana* had similar affinity with feruloyl-CoA (higher than sinapoyl-CoA) and sinapoyl-CoA but fivefold more than *p*-coumaroyl-CoA. At the same time, *p*-coumaroyl-CoA is at the crossroad of metabolic routes leading either to flavonoids or to monolignols and sinapoylmalate [the sinapoylmalate that accumulates in cotyledons and leaves causes these organs to appear blue-green under ultraviolet (UV) illumination (Ruegger and Chapple 2001; Baltas et al. 2005; Besseau et al. 2007)], so the CCR enzyme' affinity with *p*-coumaroyl-CoA decided the quantity of flavonoid and *p*-hydroxyphenyl lignin.

The previous study all showed that the CCR enzyme have almost the same affinity with feruloyl-CoA and *p*-coumaroyl-CoA. However, recently, a CCR gene that was cloned from aspen (Li et al. 2005) was clarified almost had no affinity with *p*-coumaroyl-CoA. In our study, we found that a ptCCR, which was cloned from *P. tomentosa*, had almost the same affinity with *p*-coumaroyl-CoA and caffeoyl-CoA and feruloyl-CoA, which was extracted by 4CL1 enzyme reaction. They all showed high activity. In this paper, the products of 4CL1 (which was cloned and definitized by Lu and Fan) were extracted as the substrates of CCR. The product of the 4CL reaction were extracted by C-18 column and then monitored and confirmed by HPLC-MS. Therefore, the products of 4CL enzyme reaction were substrates for CCR, different from earlier studies, in which the substrates of CCR were synthesized via acyl *N*-hydroxysuccinimide ester. Then, the CCR enzyme reactions were monitored and identified by GC-MS and Frontier3 software. Because of the lake of standard sample of *p*-coumaraldehyde, caffealdehyde, the software Frontier 3 was used in the experiment. The GC-MS and HPLC-MS were used to monitor and quantify the reactions, and they can give us more direct structures and quantities than traditional UV spectrometry and less reaction mixture.

Results

Isolation of CCR DNA and cDNA from Poplar *P. tomentosa*

The whole sequence of *P. trichocarpa* had been sequenced and compared with the 5,000 EST sequence from the xylem cDNA library of *Populus tremula*×*P. tremuloide*, over 90% homology among the *Populus*. Based on that, two pairs of primers were designed: One is for PCR clone from the cDNA library and DNA genome, and the other is for constructing expressed vector. In this paper, a 2,396 bp DNA and a 1,017 bp RNA sequences were cloned, respectively. Here, the CCR RNA sequence was named as cptCCR. Both of them were sequenced and analyzed with DNAMAN software. Five extrons and four introns were found in the whole gene. A BLAST search was performed for the cptCCR cDNA gene in *P. trichocarpa*, the result of which showed that there are eight homologous genes in the genome of *P. trichocarpa*, and the cptCCR shared 97% with CCR-H1, the same as that of the CCR that was cloned from aspen (Li et al. 2005). It means that there was high similarity between cptCCR and the CCR from aspen.

cptCCR Expression and Protein Purification in *E. coli*

The cDNA sequence was subcloned into pQE31 plasmid, and a 39602 Da protein was expressed. CCR protein was induced by IPTG at a final concentration of 1 mM, under 37°C, and quantity of expression was increased with the time; at the fourth hour, the expression of CCR was saturated in Fig. 2. The CCR protein was purified by Ni-NTA Kit (Qiagen); one clear band about 40 kDa was observed by SDS-PAGE in Fig. 3. Approximate l mg of purified CCR protein was obtained per liter of culture.

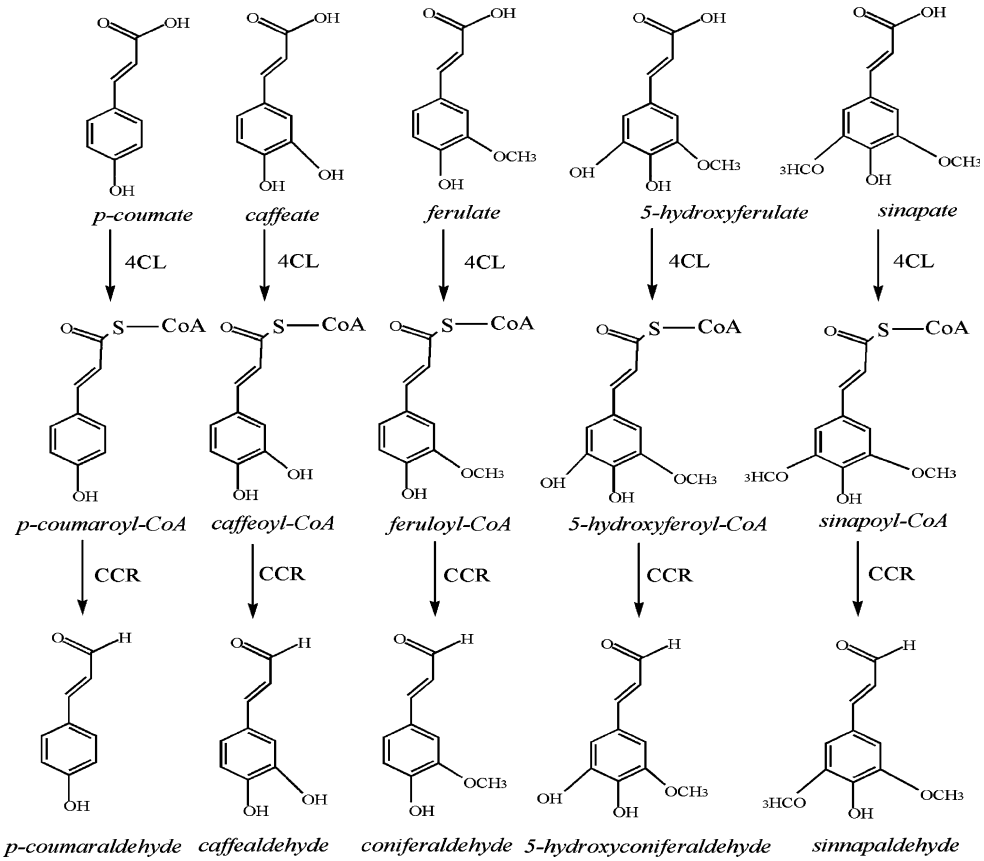


Fig. 1 The 4CL and CCR enzymes reaction in lignin biosynthesis pathway

Protein Sequence Detected by HPLC-MS

To identify the protein sequence of CCR protein, the protein was digested by trypsin and analyzed by HPLC-MS. The amino acid sequence of the CCR protein was sequenced by HPLC-MS. Then, the sequenced CCR protein was compared with the sequence translated by biosoftware from the gene sequence; there was about 27% similarity. The similarity was more than 20%, so we can say that the CCR protein expressed in the *Escherichia coli* was correct.

Identify the Products of 4CL by HPLC-MS

To examine the activity of CCR and its corresponding upper enzyme 4CL, the 4CL enzyme reaction products were made and extracted from 4CL enzyme reaction. The

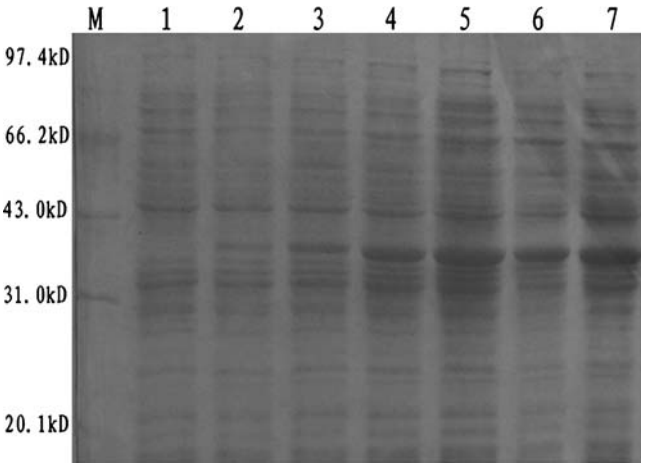


Fig. 2 The overexpression of CCR gene in *E. coli*. The overexpression of CCR gene in *E. coli*. *M* protein maker, *1* uninduced with IPTG; *2–7* induced with IPTG as the time from 10 min to 4 h

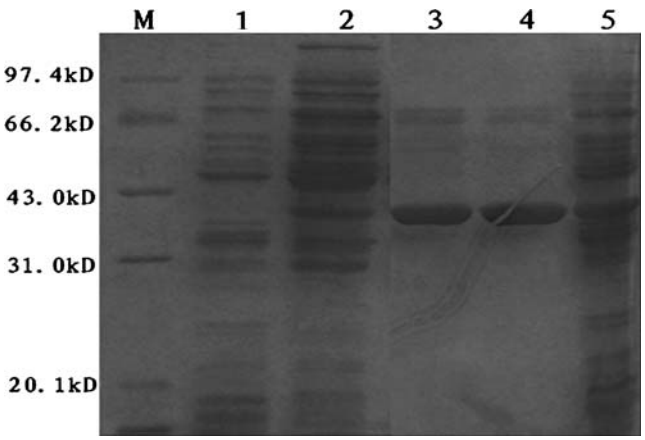


Fig. 3 Purification of CCR protein from *E. coli*. *M* protein marker, *1* uninduced with IPTG, *2* supernatant, *3* elution, *4* elution, *5* induced 4 h

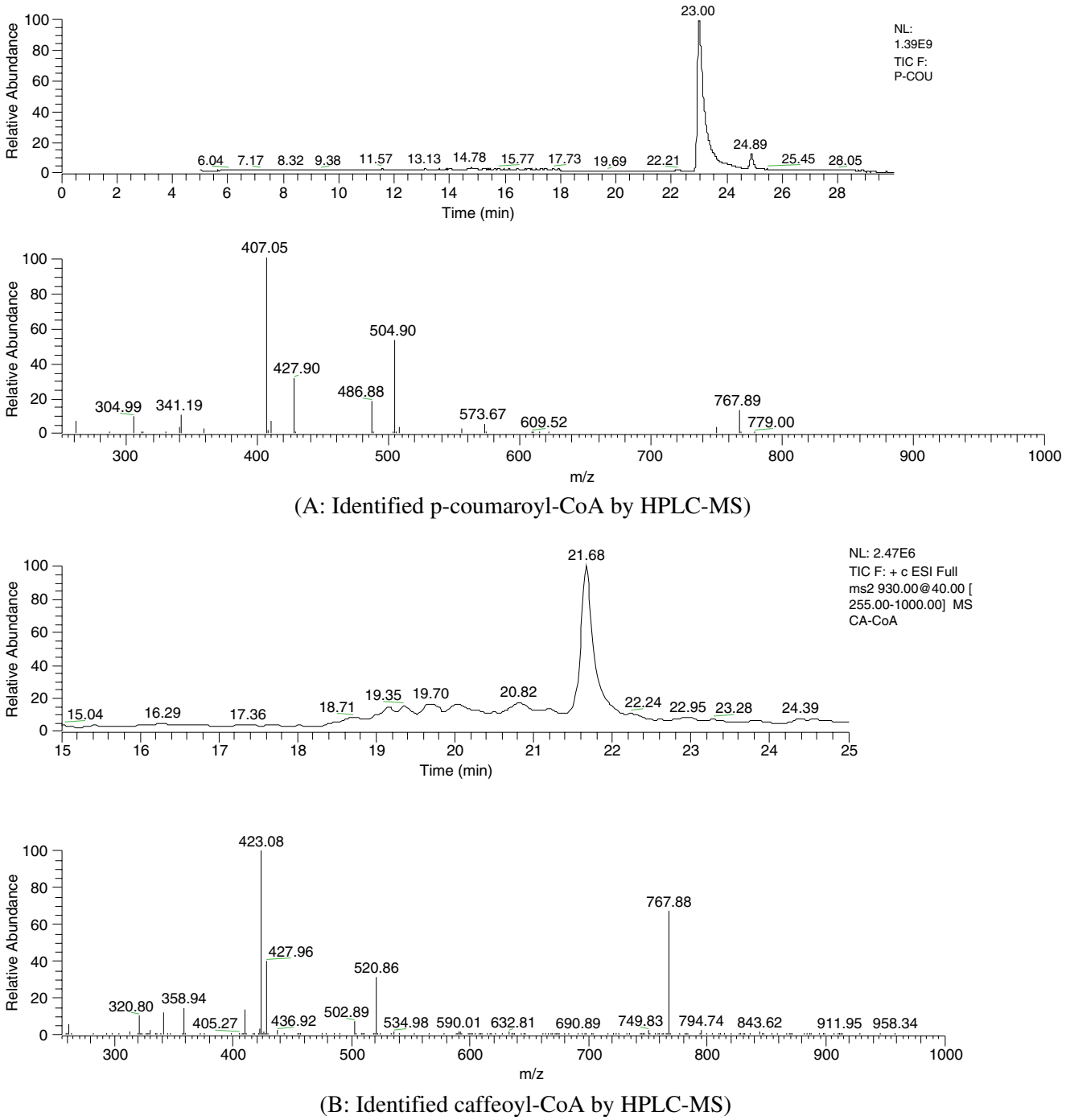
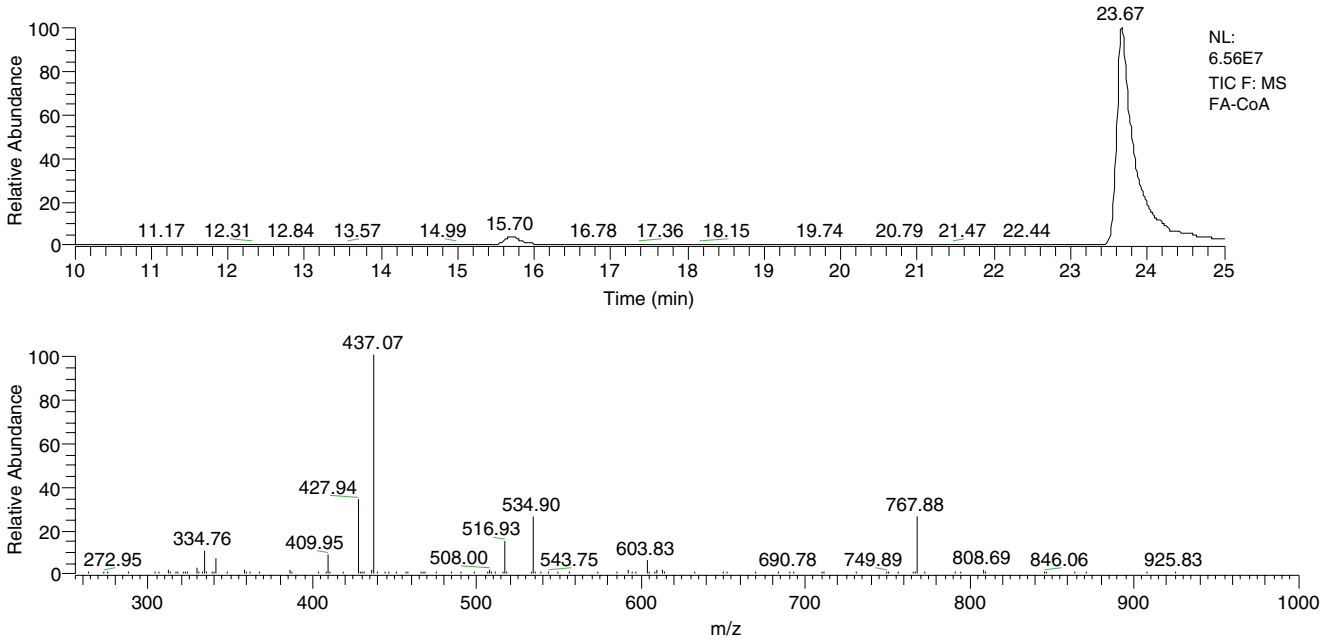


Fig. 4 The 4CL enzyme reaction products were identified by HPLC-MS

4CL enzyme was cloned from *P. tomentosa*, too. Figure 4 showed the products of 4CL enzyme reactions and the corresponding CoA esters identified by HPLC-MS; the upper panel was their chromatogram and lower panel was their spectrum graph. Subpanels a–c showed *p*-coumaroyl-CoA, caffeoyl-CoA, and feruloyl-CoA, respectively. Feruloyl-CoA was set at example m/z: 944→768, 768→428, 944→535,944→437. 768 and 428 were the special peaks of the CoA, so they existed all in Fig. 4a–c. The difference among special peaks of the three compounds was the difference of the corresponding acids.

CCR Catalytic Activity Characterized by GC-MS

Three substrates that were made from 4CL was identified and used in CCR enzyme reaction system, and the result



(C: Identified feruloyl-CoA by HPLC-MS)

Fig. 4 (continued)

was detected by GC-MS. In Fig. 5, the trimethylsilylation of CCR products with BSTFA are shown. When coniferaldehyde was silylated, the its molecular weight was changed into 250. In the Fig. 6c, the bottom picture was the spectrum picture, and the special peak of 250 could be seen, which was matched with the coniferaldehyde spectrum picture in the library of the GC-MS. However, there are no *p*-coumaraldehyde and caffealdehyde trimethylsilylated structure spectrum picture in the GC-MS library, but according to the molecular weight and the difference peak of the results between the controls' and active enzyme reactions, we can make sure that those were the product of CCR enzyme reactions. Figure 6a and b shows *p*-coumaraldehyde and caffealdehyde, respectively; the upper picture was the control, the middle was the enzyme reaction, and the bottom was the spectrum picture of the corresponding middle. The special peak was absent in the control, and molecular weight was just the same as the product that was doped out. Because of the lake of the standard sample of *p*-coumaraldehyde and caffealdehyde, the software Frontier 3 was employed here. The software was set as the EI source, which was used in the GC-MS, and the software gave out ten fragments of the three aldehydes differently. The prognosticate result showed that the coniferaldehyde standard sample matched it with 66%, and the other two were 70%. Figure 5 shows the trimethylsilylation of three aldehydes with ailylating reagent BSTFA; after the reaction, the MW of three aldehydes were changed, and they all could be found in the Fig. 6. Based on above-mentioned evidences, we identified that the CCR in this paper has affinities with the three product of 4CL enzyme reaction.

Discussion

In this paper, a CCR gene was cloned from *P. tomentosa* (Chinese white poplar) and then expressed in *E. coli* M15. It was purified by Ni-NTA kit. The advantage of using the products of 4CL enzyme reaction as the substrates of CCR is that it can truly clarify the activity of CCR, which was thought as the next enzyme to 4CL in the lignin biosynthesis. In this study, the 4Cl and CCR both came from the same plant material *P. tomentosa*. The 4CL protein was expressed and purified in *E. coli* M15, too. This point could not be gained by the chemical synthesis of thioesters. It was the same as the enzyme reactions inside the plant. GC-MS and HPLC-MS were employed to quantify and monitor the two reactions, so the structures and quantity were made sure to be more credible than traditional UV spectrometry. Because of the leak of the standard sample of *p*-coumaraldehyde and caffealdehyde, the Frontier3 software was employed here; it is a combination between the bioinformatics and experimental biology. In this

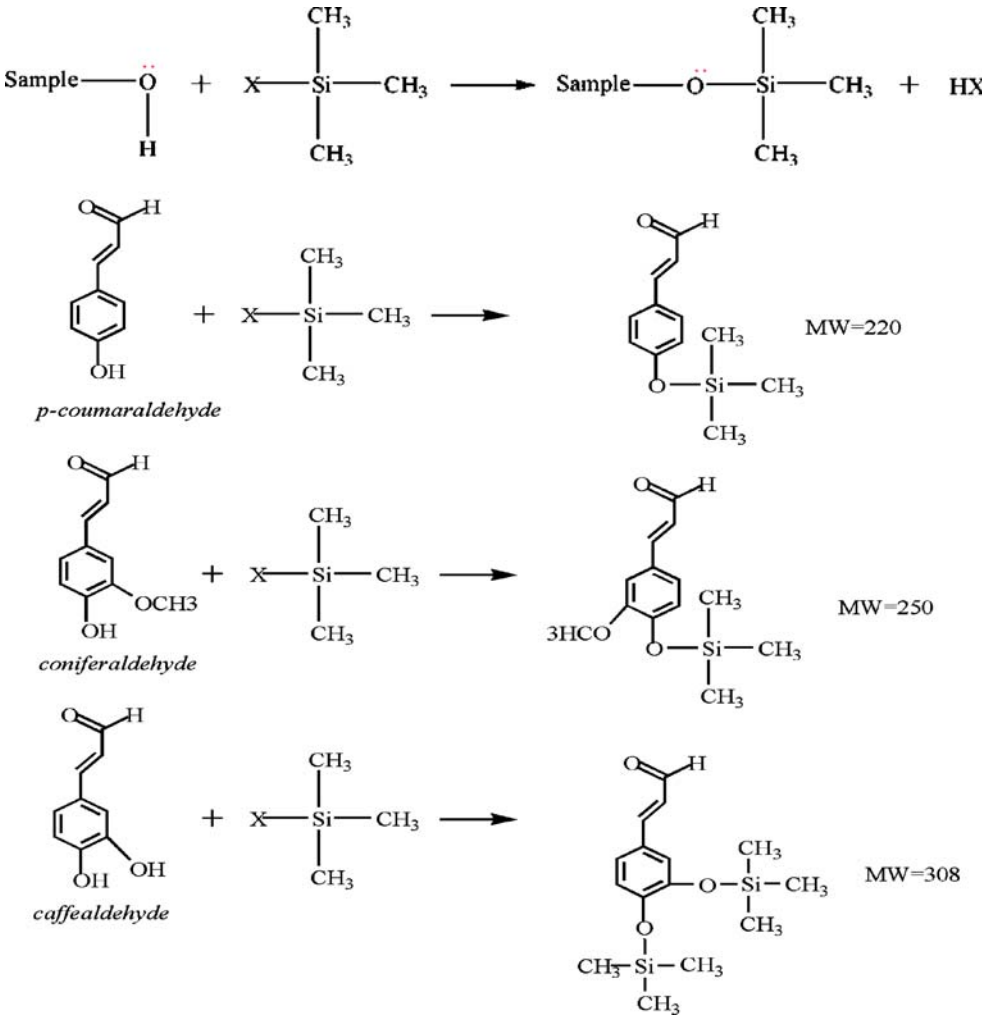


Fig. 5 Trimethylsilylation of CCR product with silylating reagent BSTFA

study, the CCR enzyme had almost the same affinity with feruloyl-CoA, *p*-coumaroyl-CoA, caffeoyl-CoA; it is different from the CCR, which is cloned by Li from aspen, which may be caused by the difference of substrate and experiment method. In Li's experiment, the substrate underwent chemical synthesis. The 4CL enzyme reaction product was extracted and employed in CCR enzyme reaction in this experiment; it was different from the former study. The method that reclaimed the upper reaction as the substrate's next reaction can be expended in many experiments. Through the blast, CCR enzymes existed in the poplar as a family, as the genome DNA sequence of *P. trichocarpa* was sequenced. The lignin biosynthesis should focus on the gene family; more and more enzymes existed as gene family in lignin biosynthesis. In our study, a C4H, PAL, and a CCoAOMT gene in lignin biosynthesis were cloned, too. They all have homoloy genes. Therefore, in the future, the gene family will be the study hot spot of lignin biosynthesis, and the method that reclaimed the 4CL enzyme reaction product was viable and can be expanded in the whole lignin biosynthesis network to detect the activity between two enzymes in the neighborhood.

Materials and Methods

Plant materials, *P. tomentosa* (Chinese white poplar), used in the present study were kept in our lab. The whole plant was used, except the root, after 1 month when it was transferred.

Isolation CCR cDNA and DNA Sequence

A mass of 0.1 g fresh plant material was collected, and the total RNA was extracted by Trisol reagent (invitrogen). Based on the mRNA character Poly(A)$^-$, a oligodT primer and a reverse transcription polymerase was employed. One cDNA strand was reverse transcribed. The genome DNA

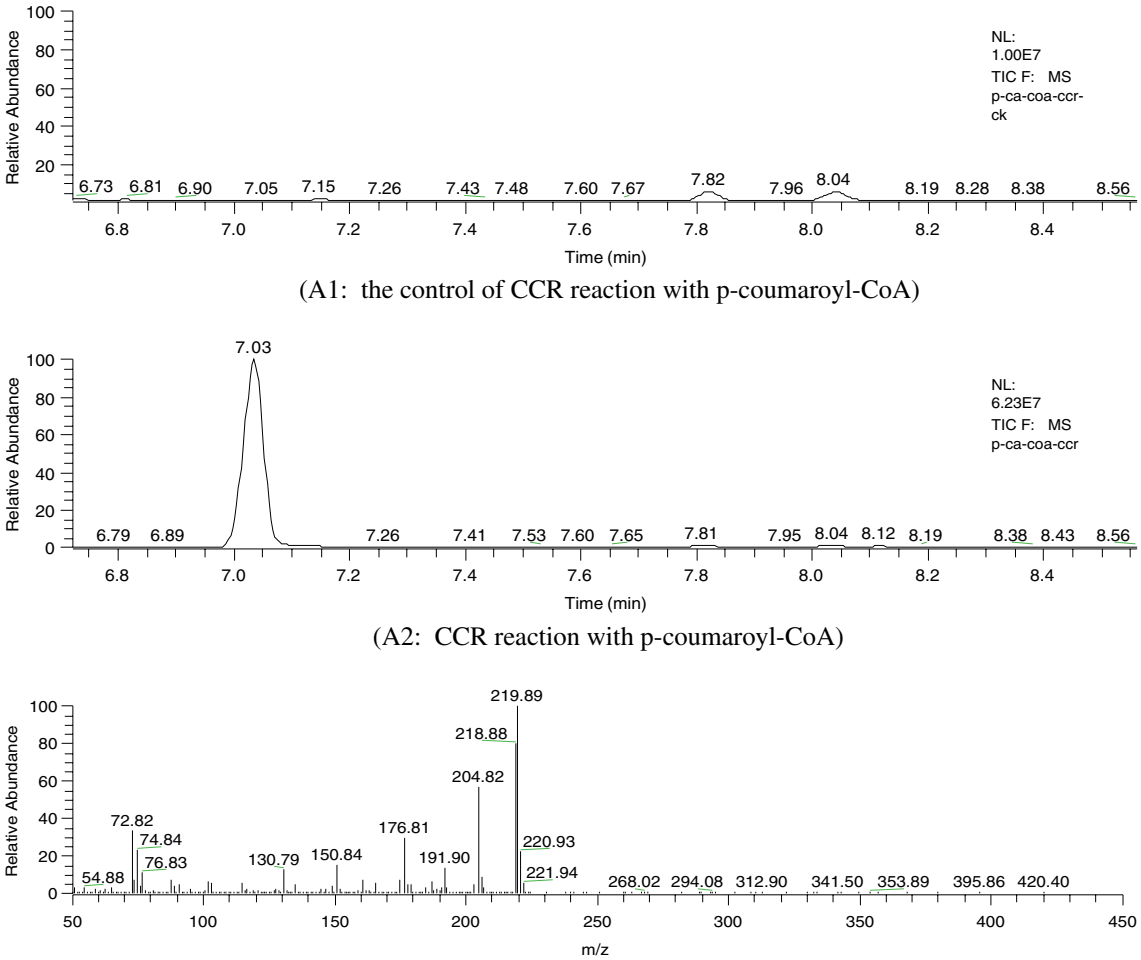
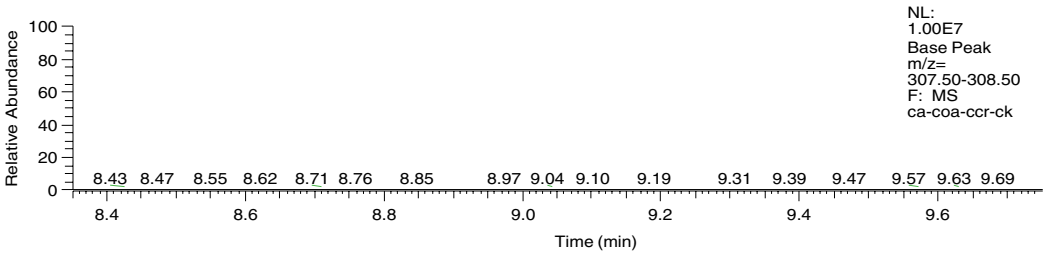


Fig. 6 CCR enzyme reaction detected by GC-MS

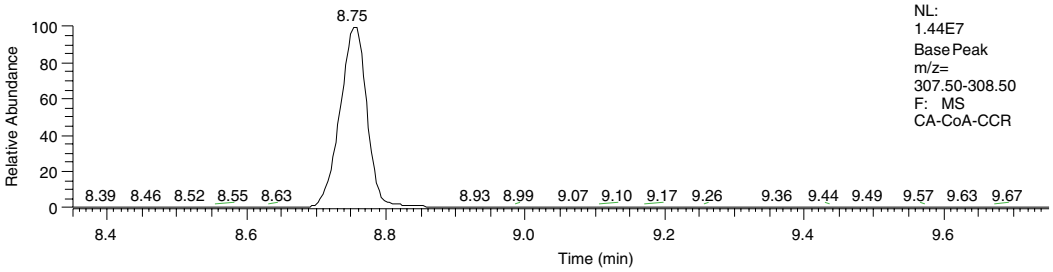
was extracted by 2× CTAB reagent according to the protocol described by Wang et al. (2002). A pair of primers were designed: ATGCCGGTTGATGCTTCATC, GAGGCCTTATTGAATCTTC. Genome DNA and cDNA were amplified as PCR template.

His-tag CCR Recombinant Protein Expression and Purification

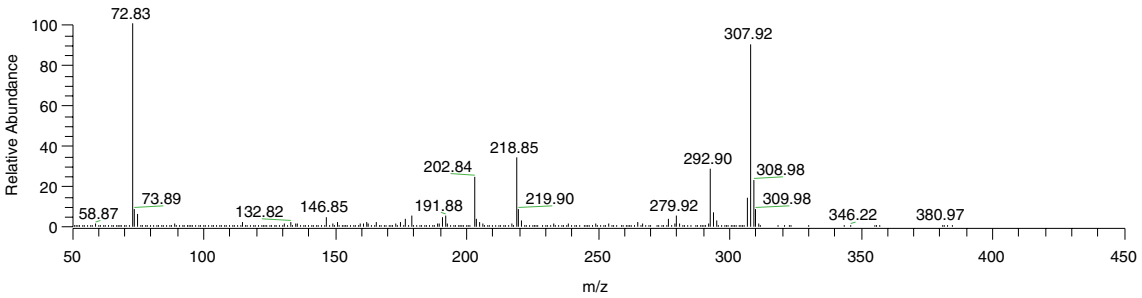
To obtain the CCR protein in *E. coli*, a pair of expression primer with a *sal*I enzyme site respectively was designed, and the CCR cDNA was amplified from the original CCR cDNA sequence. The CCR coding region was sun cloned into expression vector pQE31, in which a six His amino acid tag was fused. The His-tag is very convenient for enzyme purification. The pQE31-CCR vector was expressed in M15 strain, which has a small plasmid in it, which selected for kanamycin and ampicillin resistance. The pQE31-CCR was cultured at 37°C in LB medium containing kanamycin 25 μg/μL and ampicillin 100 μg/μL overnight and then transferred at a ratio of 4% to fresh LB medium. Protein expression was induced by 1 mM IPTG while OD_{600} was about 0.6. After 4 h, the culture was collected and purified by His-tag Ni-NTA protein purification kit from Qiagen. The culture was collected, kept on ice for about 30 min, and then dissolved by buffer A (50 mM NaH_2PO_4, 300mMNaCl, 10 mM imidazole, pH 8.0), sonicated 3× for 40 s with 6-s pauses on ice, and then centrifuged at 12,000 rpm for 25 min. The supernatant was removed from the column with Ni-NTA, shacked slowly for 1 h, the mixture washed with buffer B (50 mM NaH_2PO_4, 300mMNaCl, 20 mM imidazole, pH 8.0), making sure that no extra protein is present except target protein, which is detected by Bradford working solution. Then, the target protein was eluted by buffer C (50 mM NaH_2PO_4, 300 mMNaCl, 250 mM imidazole, pH 8.0). The concentration of the protein was qualified by the Bradford assay with bovine serum albumin as standard.



(B1: the control of CCR reaction with caffeoyl-CoA)



(B2: CCR reaction with caffeoyl-CoA)



(B3: the spectrum picture for B2)

– B –

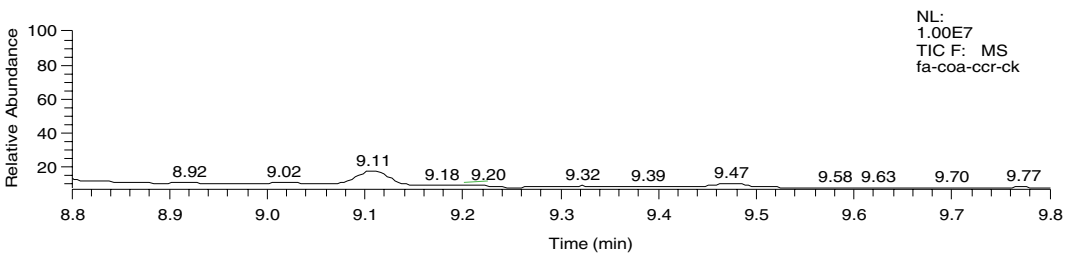
Fig. 6 (continued)

The CCR Protein Analysis by HPLC

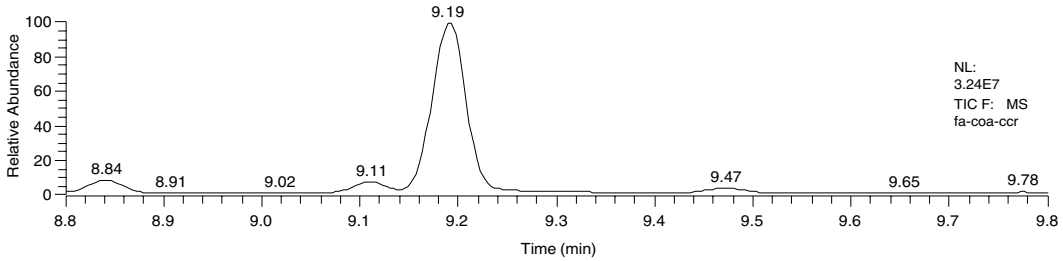
To make sure of the CCR protein sequence, a HPLC-MS was used here. The purified CCR protein (1 μg) was digested by trypsin (1 μg) for 4 h under 37°C, and then 10% formic acid was added to stop the reaction. The mixture was centrifuged at 10,000 g/min for 5 min and then analyzed by HPLC-MS. A gradient program with A (98% ACN, 2% H_2O, and 0.1% HAC) and B (98% H_2O, 2% ACN, and 0.1% HAC) was carried out: for the first 10 min, 100% B; from 10 to 50 min, 100% to 60% B; from 50 to 60 min, 60% to 20% B; then 100% B for 15 min. The result was analyzed by Biowork software.

4CL Enzyme Reaction and Extract of the Products

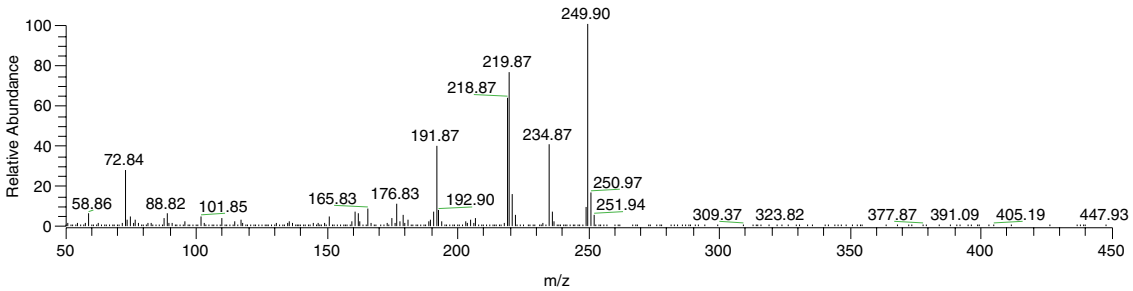
The cpt4CL1 was cloned and kept in our lab, and the activity was identified by Fan. The 4CL gene was subcloned into pQE31; a single clone was inoculated in LB medium containing 100 μg/ml Amp and 25 μg/ml Kan, overnight and then transferred 4% with final concentration of 0.4 mM IPTG. The culture was incubated at 28°C for 12 to 14 h. The 4CL was purified by Ni-NTA. The 4CL reaction system included 0.2 M Tris buffer pH 7.8, 5 mM Mg2+, 5 mM ATP, 0.3 mM CoA, 0.4 mM *p*-coumarate, caffeate, and ferulate (Fan et al.). To get enough substrates for CCR reaction, 10 ml reaction system was made. The reaction mixture was kept at 30°C for 16 min. Then, the reaction was boiled for 10 min for termination. To get rid of the protein and buffer and gain products at the same time, a C-18 column was used. The column was saturated with 5 ml methanol and 0.1 M HAC, then the mixture was loaded on the column. Then, the column was washed with 5 ml 0.1 M HAC. Forty percent HCN in 0.1 M HAC was used to elute the products. At that step, the elution was collected and dried by vacuum in the dark; after this, methanol was added to dissolve the product as the substrates for CCR. The 4CL reaction products were monitored by HPLC-MS; this process is shown in Table 1.



(C1: the control of CCR reaction with feruloyl-CoA)



(C2: CCR reaction with feruloyl-CoA)



(C3: the spectrum picture for C2)

- C -

Fig. 6 (continued)

The Activity Analysis of the CCR by GC-MS

A GC-MS was employed to identify the activity of CCR. The product of the reaction was measured by the GC-MS. The CCR reaction system included 50 mM sodium buffer, pH 6.25, 0.5 mM NADPH, 0.1-0.3 mM substrate, and 30 ng CCR protein; the final volume was 200 µl. The reaction was initiated by adding NADPH, 30°C, 30 min (Li et al. 2005; Baltas et al. 2005). To stop the reaction and get the products, 200 µl/per ethyl acetate was added and extracted twice (Kawasaki et al. 2006). The organic phase was collected and dried under vacuum condition, and then 10 µl BSTFA was added and kept at 80°C for 2 h, then subjected to GC-MS to identify the products. CCR enzyme reaction were analyzed with a GC-MS system equipped with a DB-5MS (30 m×0.25 mm).

Table 1 HPLC-MS condition for detecting the 4CL enzyme reaction

	Time (min)	Flow rate (mlmin)	Acetonitrile (%)	Methanol (%)	0.2%NH_4AC and 0.1%HAC (%)
1	0.00	0.15	0.00	0.00	100.00
2	24.00	0.15	35.00	0.00	65.00
3	27.00	0.15	100.00	0.00	0.00
4	32.00	0.15	100.00	0.00	0.00
5	35.00	0.15	0.00	0.00	100.00
6	55.00	0.15	0.00	0.00	100.00

Helium (1 ml/min) was used as a carrier gas. The injector was set for splitless injection at 250°C, and the detector was set at 280°C. The oven was set to120°C for 1 min after injection, and then the temperature was increased: increased to 200°C at a rate of 20°C/min for 2.5 min, increased to 240°C at a rate of 10°C/min for 8 min, and increased to 260°C at a rate of 20°C/min for 10 min. The mass/charge ratio was 45:450, with an energy of 70 electron volts. Eluting compounds were identified using a GC-MS library and by comparison of mass spectra and retention times with authentic samples.

Acknowledgement The work is jointly supported by the 973 Project (G1999016005) and Natural National Science Foundation of China (NSF 30630053) granted to Professor Dr. Xiangning JIANG.

References

Baltas M, Lapeyre C et al (2005) Kinetic and inhibition studies of cinnamoyl-CoA reductase 1 from *Arabidopsis thaliana*. Plant Physiol Biochem 43(8):746–753

Barriere Y, Ralph J et al (2004) Genetic and molecular basis of grass cell wall biosynthesis and degradability. II. Lessons from brown-midrib mutants. C R Biol 327(9–10):847–860

Besseau S, Hoffmann L et al (2007) Flavonoid accumulation in *Arabidopsis* repressed in lignin synthesis affects auxin transport and plant growth. Plant Cell 19(1):148–162

Freudenberg K (1959) Biosynthesis and constitution of lignin. Nature 183(4669):1152–1155

Goffner D, Van Doorsselaere J et al (1998) A novel aromatic alcohol dehydrogenase in higher plants: molecular cloning and expression. Plant Mol Biol 36(5):755–765

Gross GG, Kreiten W (1975) Reduction of coenzyme A thioesters of cinnamic acids with an enzyme preparation from lignifying tissue of Forsythia. FEBS Lett 54(2):259–262

Fan B-Y, Hu S-Y, Lu H, Jiang X-N (2006) Soluble prokaryotic expression of 4-coumarate:coenzyme A ligase from *Populus tomentosa* and enzyme activity of recombinant 4CL1. Journal of Beijing Forestry University 28(2):1–8

Hu WJ, Kawaoka A et al (1998) Compartmentalized expression of two structurally and functionally distinct 4-coumarate: CoA ligase genes in aspen (*Populus tremuloides*). Proc Natl Acad Sci U S A 95(9):5407–5412

Huntley SK, Ellis D et al (2003) Significant increases in pulping efficiency in C4H–F5H-transformed poplars: improved chemical savings and reduced environmental toxins. J Agric Food Chem 51(21):6178–6183

Inoue K, Sewalt VJ et al (1998) Developmental expression and substrate specificities of alfalfa caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase in relation to lignification. Plant Physiol 117(3):761–770

Jones L, Ennos AR et al (2001) Cloning and characterization of irregular xylem4 (irx4): a severely lignin-deficient mutant of *Arabidopsis*. Plant J 26(2):205–216

Kawasaki T, Koita H et al (2006) Cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis, is an effector of small GTPase Rac in defense signaling in rice. Proc Natl Acad Sci U S A 103(1):230–235

Lee D, Meyer K et al (1997) Antisense suppression of 4-coumarate: coenzyme A ligase activity in *Arabidopsis* leads to altered lignin subunit composition. Plant Cell 9(11):1985–1998

Li L, Cheng X et al (2005) Clarification of cinnamoyl co-enzyme A reductase catalysis in monolignol biosynthesis of Aspen. Plant Cell Physiol 46(7):1073–1082

Ma QH (2007) Characterization of a cinnamoyl-CoA reductase that is associated with stem development in wheat. J Exp Bot 58 (8):2011–2021

Ruegger M, Chapple C (2001) Mutations that reduce sinapoylmalate accumulation in Arabidopsis thaliana define loci with diverse roles in phenylpropanoid metabolism. Genetics 159(4):1741–1749

Wang G et al (2002) Principle and technology of plant gene engineering. (M)