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Clarification of Cinnamoyl Co-enzyme A Reductase Catalysis in Monolignol Biosynthesis of Aspen

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Cinnamoyl co-enzyme A reductase (CCR), one of the key enzymes involved in the biosynthesis of monolignols, has been thought to catalyze the conversion of several cinnamoyl-CoA esters to their respective cinnamaldehydes. However, it is unclear which cinnamoyl-CoA ester is metabolized for monolignol biosynthesis. A xylem-specific CCR cDNA was cloned from aspen (*Populus tremuloides*) developing xylem tissue. The recombinant CCR protein was produced through an *Escherichia coli* expression system and purified to electrophoretic homogeneity. The biochemical properties of CCR were characterized through direct structural corroboration and quantitative analysis of the reaction products using a liquid chromatography–mass spectrometry system. The enzyme kinetics demonstrated that CCR selectively catalyzed the reduction of feruloyl-CoA from a mixture of five cinnamoyl CoA esters. Furthermore, feruloyl-CoA showed a strong competitive inhibition of the CCR catalysis of other cinnamoyl CoA esters. Importantly, when CCR was coupled with caffeoyl-CoA *O*-methyltransferase (CCoAOMT) to catalyze the substrate caffeoyl-CoA ester, coniferaldehyde was formed, suggesting that CCoAOMT and CCR are neighboring enzymes. However, the in vitro results also revealed that the reactions mediated by these two neighboring enzymes require different pH environments, indicating that compartmentalization is probably needed for CCR and CCoAOMT to function properly in vivo. Eight CCR homologous genes were identified in the *P. trichocarpa* genome and their expression profiling suggests that they may function differentially.

Keywords: Cinnamoyl co-enzyme A reductase (CCR) — Lignin — Monolignol biosynthesis — Xylem.

Abbreviations: AldOMT, 5-hydroxyconiferyl aldehyde *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; CAld5H, coniferyl aldehyde 5-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl co-enzyme A reductase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaroyl-CoA 3-hydroxylase; CYP, cytochrome P450; F5H, ferulic 5-hydroxylase; HPLC-UV/MS, high-pressure liquid chromatography-UV/mass spectrometry; PAL, L-phenylalanine ammonia lyase; PCR, polymerase chain reaction; Rt, retention time.

The nucleotide sequence reported in this paper has been submitted to GenBank under accession number AF217958.

Introduction

Over the past half century, the road map of monolignol biosynthesis in plants has been depicted as a grid of alternative pathways according to the early physiological and biochemical evidence (Whetten and Sederoff 1995, Whetten et al. 1998). In those traditional analyses, the catalysis of many monolignol biosynthesis pathway enzymes was indirectly determined by methods such as UV spectrometry which lack structural and quantitative characterization of the reaction products. Thus the proposed monolignol biosynthesis pathways still remain to be clarified with certainty. Recently, the accuracy of the road map in many steps has been re-examined in studies using more advanced approaches (Osakabe et al. 1999, Li et al. 2000, Li et al. 2001). Using high-pressure liquid chromatography coupled with a UV/mass spectrometry (HPLC–UV/MS) system, the reaction products are structurally corroborated and quantitatively determined; the enzymatic reactions with multiple substrates are kinetically characterized. These new approaches have yielded an array of insightful evidence that suggests a new view of the pathways of monolignol biosynthesis.

Earlier studies suggested that ferulic acid was an intermediate in the monolignol biosynthesis pathways and hydroxylated by ferulic 5-hydroxylase (F5H) that was encoded by a cytochrome P450 gene, *CYP84* (Meyer et al. 1996, Meyer et al. 1998). This scheme was amended recently according to the results of the HPLC-UV/MS-mediated product characterization. From sweetgum (*Liquidambar styraciflua*) (Osakabe et al. 1999), a *CYP84* gene was isolated, and its enzymatic activity demonstrated that coniferaldehyde instead of ferulic acid is the real metabolic substrate of the CYP84 enzyme. Thus, CYP84 is in fact a coniferyl aldehyde 5-hydroxylase (CAld5H). This finding was verified further in the catalysis of *Arabidopsis* CYP84 protein (Humphreys et al. 1999). In connection with the amended pathway mediated by CAld5H, 5-hydroxyconiferaldehyde synthesis is catalyzed by a 5-hydroxyconiferyl alde-

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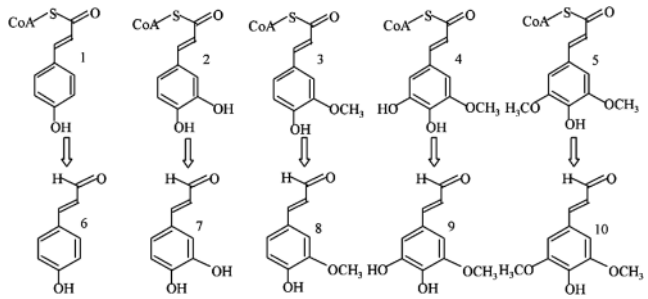

Fig. 1 CCR is believed to catalyze the conversion of various cinnamoyl-CoA esters to their respective cinnamaldehydes. (1) *p*-coumaroyl-CoA, (2) caffeoyl-CoA, (3) feruloyl-CoA, (4) 5-hydroxyferuloyl-CoA, (5) sinapoyl-CoA, (6) *p*-coumaraldehyde, (7) caffealdehyde, (8) coniferaldehyde, (9) 5-hydroxyconiferaldehyde, (10) sinapaldehyde.

hyde *O*-methyltransferase (AldOMT) (Li et al. 2000) that was an enzyme believed for a long time to mediate the methylation of caffeate and 5-hydroxyferulate in monolignol biosynthesis (Kuroda et al. 1975, Kuroda et al. 1981, Bugos et al. 1991, Bugos et al. 1992, Tsai et al. 1998).

Individually, many of the enzymes involved in monolignol biosynthesis are able to use several compounds as substrates. However, the direct product characterization revealed a significant difference in enzyme affinities for those substrates as well as strong substrate competition in the mixed substrate reactions (Osakabe et al. 1999, Li et al. 2000, Li et al. 2001). The results of the competitive interactions among the substrates demonstrated that only one of several possible substrates is predominantly consumed by an enzyme, suggesting that lignin biosynthesis may follow a main metabolic stream, instead of a grid of alternatives in lignifying tissues.

Although several steps in monolignol biosynthesis have been elucidated by advanced approaches, the step mediated by cinnamoyl-CoA:NADP oxidoreductase (CCR, EC.1.2.1.44) remains to be clarified. CCR is believed to catalyze the reaction from cinnamoyl-CoA esters to cinnamaldehydes (Fig. 1). In previous studies, a number of cinnamoyl-CoA esters (*p*-coumaroyl-CoA, caffeoyl-CoA, feruloyl-CoA, 5-hydroxyferuloyl-CoA and sinapoyl-CoA) were tested to determine if they were substrates for CCR (Wengenmayer et al. 1976, Luderitz and Grisebach 1981, Sarni et al. 1984, Goffner et al. 1994). The purified CCR enzyme from *Eucalyptus* xylem tissue was shown to be active toward *p*-coumaroyl-CoA, feruloyl-CoA, caffeoyl-CoA and sinapoyl-CoA with approximately equal affinity (Goffner et al. 1994). Similar to the native protein, the recombinant *Eucalyptus* CCR protein was also demonstrated to be active with the substrates *p*-coumaroyl-CoA, feruloyl-CoA and sinapoyl-CoA, individually (Lacombe et al. 1997). Although these results provided a line of biochemical evidence supporting CCR function in connection with monolignol biosynthesis, it still remains to be clarified which of the multiple CoA esters is used as a metabolic intermediate in the monolignol biosynthesis pathways.

To elucidate the metabolic route mediated by CCR in lignin biosynthesis, a xylem-specific CCR cDNA was cloned and the reaction kinetics of recombinant CCR protein were investigated using an HPLC-UV/MS system. The catalytic properties of recombinant PtCCR suggested that CCR primarily converts feruloyl-CoA to coniferaldehyde, which, connecting with its upstream and downstream pathways, constitutes a main metabolic flux towards the biosynthesis of monolignols in aspen xylem. In the *Populus* genome, eight CCR homologous genes could be identified. The expression profiling suggests that the CCR homologous genes, in addition to their function in lignin biosynthesis, may also play roles in other metabolic pathways.

Results

CCR isolation and expression in aspen

To isolate the *CCR* gene from aspen, the *CCR* sequences from *Eucalyptus* (Lacombe et al. 1997), maize (Pichon et al. 1998) and *Arabidopsis* (Lauvergeat et al. 2001) were analyzed for their conserved regions. Based on the conserved sequences, two degenerate primers (5′-TGYTGGAGYGAYCTYGARTW-3′ and 5′-GCYTCTGGTTBGWGAACTTG-3′) were designed for polymerase chain reacion (PCR) amplification of a *CCR* fragment from a developing xylem cDNA library. The amplified PCR fragment was cloned and sequenced. In order to isolate a full-length *CCR* cDNA, the PCR fragment was used as a probe to screen a developing xylem cDNA library. In order to identify all possible homologous *CCR* cDNAs in developing xylem tissue, the library was screened under various low and high stringent conditions. A total of 28 clones were identified and sequenced. The sequence analysis indicated that they all belonged to the same cDNA but varied in length. The longest *P. tremuloides CCR* (*PtCCR*) cDNA clone had 1361 bp and contained a full coding sequence encoding a predicted 37.1 kDa protein. The predicted amino acid sequence shows a range of 72–96% identities with the CCRs from other species (Lacombe et al. 1997, Leple et al. 1998, Pichon et al. 1998, Lauvergeat et al. 2001). The expression pattern of the *PtCCR* gene in aspen was investigated by Northern blotting analysis. Total RNA was isolated from xylem, phloem and leaf tissues of *P. tremuloides* in a growing season and probed with the ^{32}P-labeled 5′ end PtCCR cDNA fragment of about 500 bp. As shown in Fig. 2, Northern blot hybridization revealed that the expression of *PtCCR* was predominant in xylem tissue, weak in phloem and undetectable in leaves. In the stem, *PtCCR* expression was weak in young tips (1st–3rd internode) and strong in mature stems where secondary xylem development becomes apparent (8th internode). This expression pattern agreed with the occurrence of lignin deposition, suggesting a correlation between *CCR* expression and lignin biosynthesis.

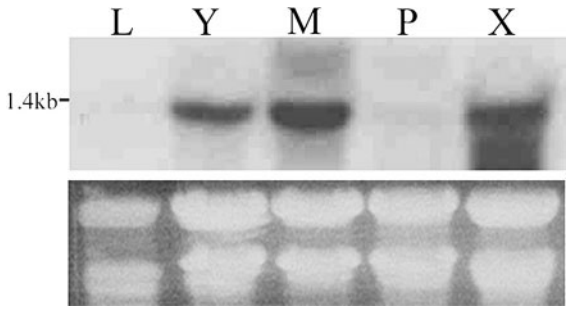

Fig. 2 Northern blotting analysis of CCR expression. A 10 μg aliquot of total RNA from various tissues was loaded into each lane and the gel blot was hybridized with ^{32}P-labeled 5′ end PtCCR cDNA. Ethidium bromide staining of rRNAs is shown below. The RNAs were from leaf (L), young stem (Y), mature stem (M), developing phloem (P) and developing xylem (X).

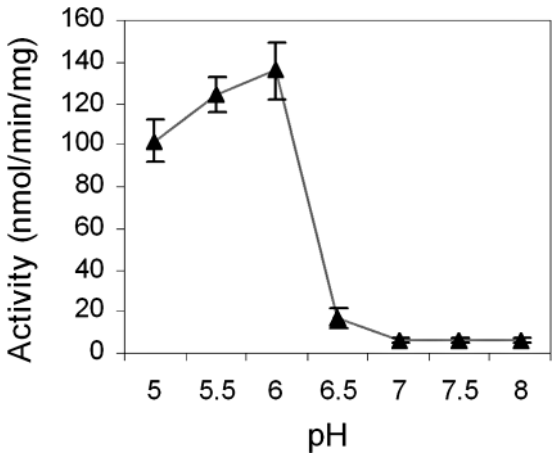

Fig. 3 pH effect on PtCCR catalytic activity. Using feruloyl-CoA as substrate, PtCCR catalysis was conducted from pH 5.0 to 8.0 at intervals of 0.5. Each point of the activity represented the mean of three independent assays.

CCR catalytic properties characterized by direct product corroboration

CCR activity was determined traditionally with spectrophotometric measurement of the absorbance change corresponding to NADPH consumption (Luderitz and Grisebach 1981, Sarni et al. 1984). This method only provided indirect chemical evidence referring to the enzyme activity and overlooked the characterization of the reaction product per se. Although the reaction product could be identified qualitatively (Lacombe et al. 1997), the challenge was how the catalytic properties could be characterized according to the quantitative product measurement and how the enzyme activity could be determined in the mixed substrates reaction. In this study, CCR catalysis was investigated by direct product corroboration using an HPLC-UV/MS system. Therefore, this approach resulted in direct product identification and quantification of the CCR catalysis in the reactions with various compounds.

To investigate PtCCR catalytic properties, the reaction parameters were optimized. The pH effect on PtCCR activity was tested from pH 5.0 to 8.0 in intervals of 0.5. When feruloyl-CoA ester was used as a substrate, the peak activity occurred at pH 6.0 (Fig. 3). When the pH was >6.5, the activity became negligible. This pH optimum was consistent with that of eucalyptus CCR (Goffner et al. 1994, Lacombe et al. 1997). It was also found that PtCCR activity was affected by reductants. 2-Mercaptoethanol sustained higher PtCCR activity than dithiothreitol (DTT). The optimal concentration of 2-mercaptoethanol was 10 mM. Optimization of PtCCR reaction parameters revealed that the highest CCR activity occurred in a 0.5 ml reaction mixture containing 100 mM sodium phosphate buffer (pH 6.0), 10 mM 2-mercaptoethenol, 0.5 mM NADPH and 100 ng of purified CCR protein. With a saturated concentration of substrates (1 mM), the product accumulation was linearly correlated with time within the first 4 min of the reaction.

In order to test whether the catalytic function of PtCCR is affected by a tag sequence in the recombinant protein, we chose two protein expression systems, pET-41a(+) and pET-23b(+) (Novagen Inc., Madison, WI, USA). With the pET-41a(+) expression system, the purified recombinant protein was subsequently subjected to the removal of the fusion tag and, therefore, the final recombinant CCR protein was in its native sequence without any additional tag peptide. Meanwhile, the pET-23b(+) vector system synthesized a larger scale (milligram level) of PtCCR protein fused with a histine tag at the C-terminus. The PtCCR proteins from both expression systems were analyzed for catalytic properties.

Five cinnamoyl CoA esters (*p*-coumaroyl-CoA, caffeoyl-CoA, feruloyl-CoA, 5-hydroxyferuloyl-CoA and sinapoyl-CoA) were synthesized via acyl *N*-hydroxysuccinimide esters as described (Stöckigt and Zenk 1975, Osakabe et al. 1999). The authenticity of the intermediate esters was confirmed by ^{1}H nuclear magnetic resonance (NMR) and MS and that of the final CoA esters was confirmed by UV spectrometry and MS (Osakabe et al. 1999, Li et al. 2000). CCR protein was incubated with these substrates and the reaction products were separated by HPLC and characterized by MS. *o*-Coumaric acid was used as an internal standard for quantitative measurement. As shown in Fig. 4, four products were able to be detected from five CoA ester substrates individually. They were caffealdehyde (Fig. 4A, retention time 13.282 min) from caffeoyl-CoA, coniferaldehyde (Fig. 4B, retention time 19.784 min) from feruloyl-CoA, 5-hydroxyconiferaldehyde (Fig. 4C, retention time 12.557 min) from 5-hydroxyferuloyl-CoA, and sinapaldehyde (Fig. 4D, retention time 16.381 min) from sinapoyl-CoA. However, no respective product was detected from *p*-coumaroyl-CoA. The detailed kinetics of PtCCR catalysis were characterized further in terms of the K_m, V_{max} and K_{cat} with various substrates. As shown in Table 1, PtCCR had a K_m of 13.7 μM with feruloyl-CoA, while the K_m value was 76.3 μM with caffeoyl-CoA, 99.0 μM with 5-hydroxyferuloyl-CoA and 104.5 μM with sinapoyl-CoA, indicating that PtCCR affinity

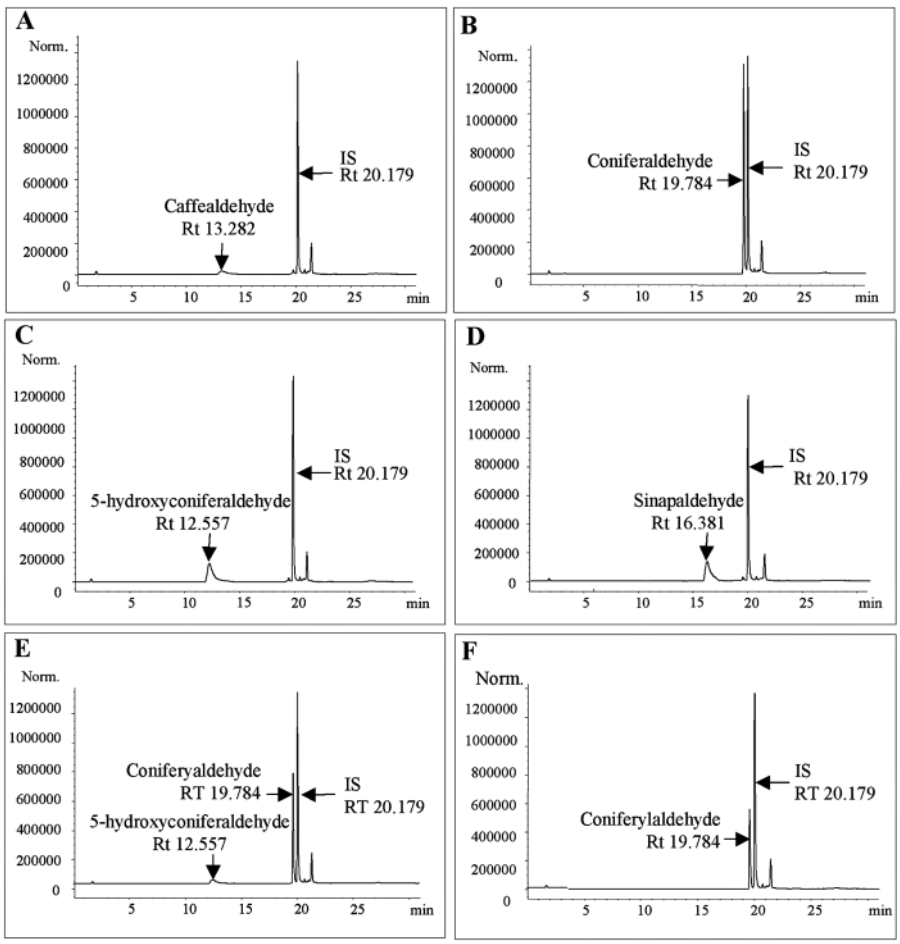

Fig. 4 HPLC-MS determination of the products from PtCCR catalysis with various substrates. PtCCR recombinant protein (100 ng) or xylem crude protein (10 μg) was incubated with various substrates. The reaction products were separated by HPLC and detected by a UV/MS system. The authenticity of the products was confirmed with the UV/MS signatures of the standard compounds. *o*-Coumarate was used as an internal standard (retention time = 20.178 min by MS). (A) Caffealdehyde (retention time = 13.282 min by MS) was produced from caffeoyl-CoA substrate. (B) Coniferaldehyde (retention time = 19.784 min by MS) was produced from feruloyl-CoA substrate. (C) 5-Hydroxyconiferaldehyde (retention time = 12.557 min by MS) was produced from 5-hydroxyferuloyl-CoA substrate. (D) Sinapaldehyde (retention time 16.381 min by MS) was produced from sinapoyl-CoA substrate. (E) Coniferaldehyde (retention time = 19.784 min by MS) was a primary product from the five mixed substrates reaction mediated by PtCCR recombinant protein. (F) Only coniferaldehyde was detected in the five mixed substrates reaction mediated by the developing xylem crude protein extract.

towards feruloyl-CoA was much higher than towards other CoA ester substrates. Moreover, the PtCCR catalysis on feruloyl-CoA had a V_{max} of 158.6 μM min^{-1} and a K_{cat} of 7.8 min^{-1}. This was much higher than those on the other substrates. Taken together, these results indicated that feruloyl-CoA was the most favorable substrate for the PtCCR enzyme. Meanwhile, the catalytic activities of two PtCCR recombinant proteins, with and without the histidine tag, were examined in the reactions with various substrates. The results did not show any statistical difference between the two proteins (data not shown).

Table 1 Kinetic properties of CCR catalysis with various cinnamoyl-CoA esters

Substrate	K_m (μM)	V_{max} (μM min^{-1})	K_{cat} (min^{-1})
Caffeoyl-CoA	76.3 ± 5.1	13.4 ± 0.9	0.7 ± 0.05
Feruloyl-CoA	13.7 ± 2.1	158.6 ± 9.1	7.8 ± 0.5
5-Hydroxyferuloyl-CoA	99.0 ± 1.0	97.0 ± 3.0	4.9 ± 0.2
Sinapoyl-CoA	104.5 ± 5.5	105.0 ± 5.0	5.3 ± 0.3

Values were the mean ± SE, n = 3 independent assays.

Kinetic interaction of CCR substrates

Individually, feruloyl-CoA was catalyzed by PtCCR as the most favorable substrate. To understand further how the substrates interacted in the mixed substrates reaction, a substrate mixture containing an equal concentration of *p*-coumaroyl-CoA, caffeoyl-CoA, feruloyl-CoA, 5-hydroxyferuloyl-CoA and sinapoyl-CoA was catalyzed by PtCCR. The reaction products were analyzed qualitatively and quantitatively using the HPLC-UV/MS system. The results showed that the major product from the five mixed substrates was coniferaldehyde, along with a trivial amount of 5-hydroxyconiferaldehyde, but no other products were detected (Fig. 4E). This indicated that the mixed substrates were catalyzed by PtCCR in a manner different from how they were individually. Meanwhile, to understand how the mixed substrates were used by native proteins, the crude protein was extracted from developing xylem tissue. When mixed substrates were incubated with 10 μg of the developing xylem protein extract, the only product was coniferaldehyde (Fig. 4F), similar to the case with of PtCCR recombinant protein, indicating that the biochemical properties of the recombinant PtCCR were the same as those of the native protein and that PtCCR may be a major CCR protein in developing xylem tissue.

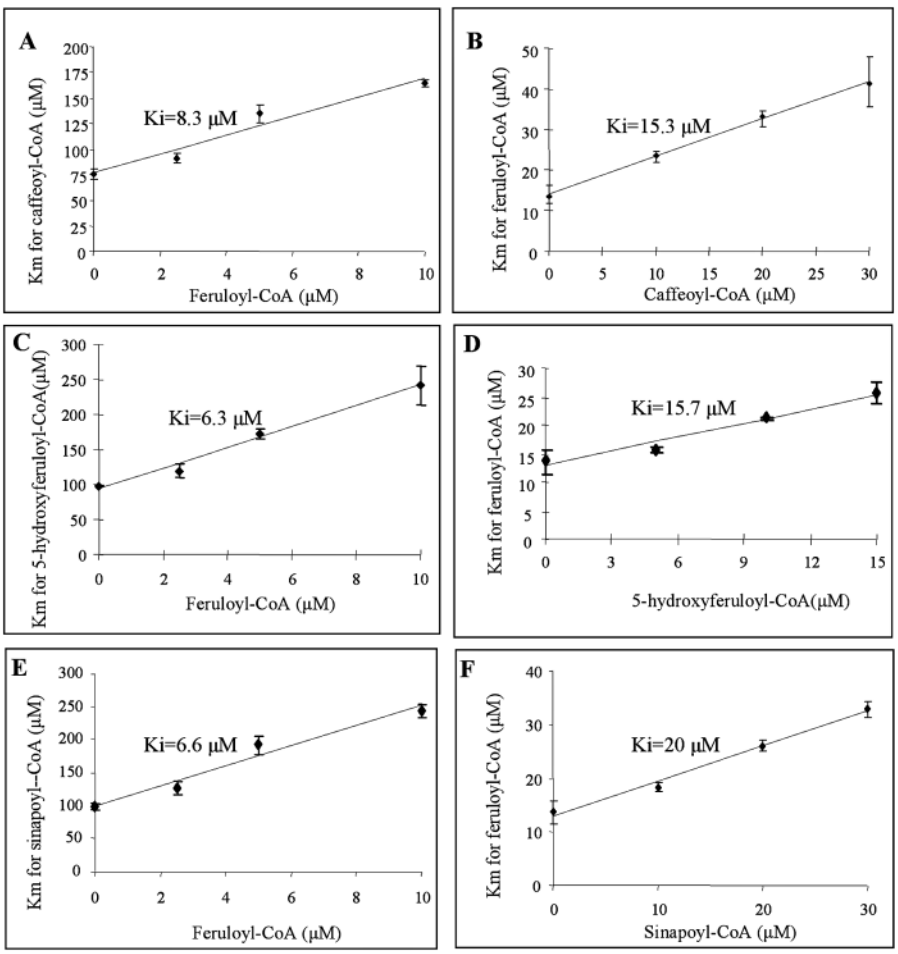

Fig. 5 Kinetic analysis of the substrate interactions. The mixed substrates at a series of concentrations were co-catalyzed by PtCCR recombinant protein. The reaction products were separated and quantitatively characterized using the Agilent 1100 HPLC-MS system. (A) Competitive inhibition of feruloyl-CoA on the caffeoyl-CoA reaction, K_i = 8.3 μM. (B) Competitive inhibition of caffeoyl-CoA on the feruloyl-CoA reaction, K_i = 15.3 μM. (C) Competitive inhibition of feruloyl-CoA on the 5-hydroxyferuloyl-CoA reaction, K_i = 6.3 μM. (D) Competitive inhibition of 5-hydroxyferuloyl-CoA on the feruloyl-CoA reaction, K_i =15.7 μM. (E) Competitive inhibition of feruloyl-CoA on the sinapoyl-CoA reaction, K_i = 6.6 μM. (F) Competitive inhibition of sinapoyl-CoA on the feruloyl-CoA reaction, K_i = 20 μM. Each point represented the mean of at least three independent assays.

The results of the mixed substrates reaction indicated a selective reaction and possible interactions among substrates. In order to understand the selective reactivity, in which feruloyl-CoA was mostly preferred, we analyzed the interaction of feruloyl-CoA with other cinnamoyl-CoA esters. Fig. 5 shows an interaction profile of feruloyl-CoA with caffeoyl-CoA, 5-hydroxyferuloyl-CoA and sinapoyl-CoA. When feruloyl-CoA and caffeoyl-CoA were co-catalyzed by PtCCR, feruloyl-CoA exerted a strong competitive inhibition (K_i, 8.3 μM, Fig. 5A) on caffeoyl-CoA reactivity, while caffeoyl-CoA showed a much lower inhibition (K_i, 15.2 μM, Fig. 5B) on feruloyl-CoA. Likewise, when feruloyl-CoA was co-catalyzed with 5-hydroxyferuloyl-CoA and sinapoyl-CoA, respectively, feruloyl-CoA always displayed a strongly competitive inhibition over the reactivity of the rival substrate, showing a K_i of 6.3 μM to 5-hydroxyferuloyl-CoA (Fig. 5C) and 6.6 μM to sinapoyl-CoA (Fig. 5E), whereas the inhibition of 5-hydroxyferuloyl-CoA (Fig. 5D) and sinapoyl-CoA (Fig. 5F) on feruloyl-CoA was much weaker. Taken all together, the interactions of various CoA ester substrates in the CCR reaction revealed that feruloyl-CoA was the most competitive substrate as well as exhibiting the reactivity of other CoA esters.

CCR pathway coupled to CCoAOMT catalysis

Previously, CCoAOMT was demonstrated to convert caffeoyl-CoA to feruloyl-CoA (Ye et al. 1994, Meng and Campbell 1998, Li et al. 1999). The present results indicate that feruloyl-CoA serves as a CCR substrate in the immediately subsequent step of the monolignol biosynthesis pathway. Thus, when CCoAOMT and CCR are coupled together, they should be able to convert caffeoyl-CoA to coniferaldehyde. We cloned *PtCCoAOMT* cDNA from aspen developing xylem and PtCCoAOMT recombinant protein was expressed in *Escherichia coli* and purified. Caffeoyl-CoA as a sole substrate was catalyzed by mixed enzymes, PtCCoAOMT and PtCCR. The catalysis was carried out in three pH conditions, which were constant pH 7.5, constant pH 6.0 and pH change from 7.5 to 6.0. At pH 7.5, which is the optimal pH for CCoAOMT activity (Ye et al. 1994, Meng and Campbell 1998, Li et al. 1999), coniferaldehyde could not be detected as a product. Similarly, the two enzymes did not produce coniferaldehyde at pH 6.0, which is the optimum for PtCCR activity. When the catalysis was carried out at pH 7.5 with PtCoAOMT protein for 10 min first, then the catalysis system was adjusted to pH 6.0 and PtCCR protein was added, coniferaldehyde was

Table 2 CCR homologous genes in the genome of *P. trichocarpa*

CCR homolog	Sequence location in *P. trichocarpa* genome
CCR-H1	LG_III:16014069–16020928
CCR-H2	LG_I:3141926–3234493
CCR-H3	Scaffold_208:244587–356480
CCR-H4	Scaffold_208:244587–356480
CCR-H5	Scaffold_2524:530–1345
CCR-H6	LG_XIII:3208597–3209347
CCR-H7	Scaffold_2804:588–1329
CCR-H8	Scaffold_5382:88–865

detected after another 10 min catalysis. To examine further if the pH adjustment was required with plant native proteins, the developing xylem crude proteins were used to conduct the coupling reactions. Similarly, the coupling reactions catalyzed by the xylem crude proteins were set up under three pH conditions. Coniferaldehyde was produced only in the reaction when the pH was adjusted from 7.5 to 6.0. The different pH requirement of the two neighboring enzymes strongly suggested that the in vivo location of CCoAOMT and CCR might be spatially separated and compartmentalized in a way that renders the microenvironments needed for these two enzymes to function properly.

The expression profiling of CCR homologs in the poplar genome

In this study, we only cloned one *PtCCR* cDNA from developing xylem tissue, while Southern blotting analysis indicated that a small family of *CCR* gene was present in the aspen genome (data not shown). In order to investigate whether there are other CCR homologous genes in a tree species, we searched the *Populus* genome sequence database (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) and identified eight loci in the genome containing CCR homologous sequences. They were designated CCR homolog-1 (*CCR-H1*), CCR homolog-2 (*CCR-H2*), CCR homolog-3 (*CCR-H3*), CCR homolog-4 (*CCR-H4*), CCR homolog-5 (*CCR-H5*), CCR homolog-6 (*CCR-H6*), CCR homolog-7 (*CCR-H7*) and CCR homolog-8 (*CCR-H8*) (Table 2). The eight genomic homologs were scanned to predict their transcripts by GENESCAN (http://genes.mit.edu/GENSCANinfo.html). The results indicated that three predicted transcript sequences (*CCR-H1*, *CCR-H2* and *CCR-H3*) had full coding sequences, and the others were partial due to gaps and the incompleteness of the genome assembly. The sequence comparison further revealed that the transcripts of *CCR-H1*, *CCR-H2* and *CCR-H3* shared 68–95% homology with the *PtCCR* coding region, and *CCR-H1* was 98% identical to the cDNA isolated from *P. trichocarpa* previously (Leple et al. 1998).

To understand how these CCR homologous genes are expressed in association with tree development, total RNAs were isolated from eight different tissues of *P. trichocarpa*

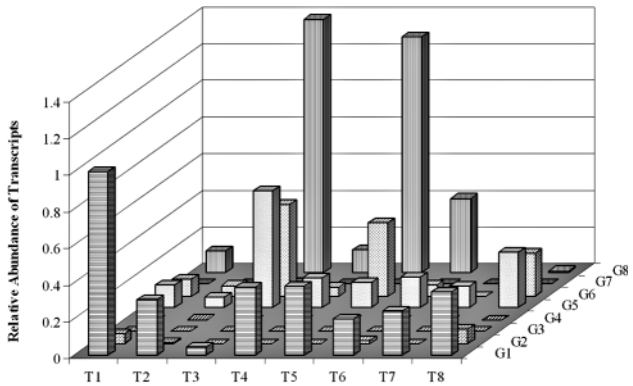

Fig. 6 The expression profile of CCR homologous genes in *P. trichocarpa*. The transcripts of the CCR homologous genes were quantitatively determined by real-time PCR. The determined value was normalized by 18S RNA transcripts which were assumed to be constantly expressed in all tissues, and the relative abundance was calculated for each gene in various tissues. T1, xylem from the 8th to the 15th internode; T2, xylem from the 3rd to the 6th internode; T3, young leaves (1st–3rd from the top); T4, mature leaves; T5, stem tip; T6, young roots (1–3 cm); T7, phloem; T8, callus tissue; G1–G8, predicted CCR homologous gene CCR-H1 to CCR-H8, respectively. Each value represents the mean of three experimental repeats.

(Nisqually-1), the same clone used for the genome sequencing. The tissues included xylem from the 8th to the 15th internodes, xylem from the 3rd to the 5th internodes, young leaves (1st–3rd from the top), mature leaves (7th–8th), stem tip, young roots (1–3 cm in length), phleom and calli. Then we measured the transcripts of the eight *CCR-H* genes in those total RNAs using real-time PCR qualification. The results (Fig. 6) revealed that the *CCR-H* genes exhibited different expression patterns in the studied tissues. The expression of *CCR-H1* displayed a xylem-specific pattern, similar to that of *PtCCR* expression illustrated above by Northern blotting analysis. This gene also had the highest sequence homology (95%) with *PtCCR*. In contrast to *CCR-H1*, *CCR-H8* was strongly expressed in young leaf, young stem and young root. Similar to *CCR-8*, *CCR-H6* was mainly expressed in young leaf, young stem and calli. *CCR-H5* was expressed in all the tissues, and strongly in young leaf. On the other hand, the expression of *CCR-H2*, *CCR-H3*, *CCRH-4* and *CCR-H7* was barely detected in all the studied tissues. Therefore, the different manners of expression may indicate that these CCR homologous genes play disparate roles in tree growth and development. *CCR-H1* was probably a *PtCCR* ortholog in *P. trichocarpa* and a major one expressed in developing xylem, while in leaf and shoot tip *CCR-H8* and others were strongly expressed.

Discussion

For many years, monolignol biosynthesis pathways have been one of the major topics in studying plant secondary metabolism and cell wall biosynthesis (Whetten and Sederoff

1995, Boudet 1998, Whetten et al. 1998, Humphreys and Chapple 2002, Raes et al. 2003). Apparently, during wood formation, the biosynthesis of lignin serves as the main metabolic stream sinking photosynthesis-fixed carbon and energy into cell walls. To meet the requirement for amassing a substantial cell wall, highly efficient biosynthesis pathways need accordingly to be evolved in wood formation. In aspen lignifying xylem tissue, recent studies of monolignol biosynthesis have demonstrated that many of the steps of the pathway follow a principal route rather than wander via a grid of alternative routes (Osakabe et al. 1999, Li et al. 2000, Li et al. 2001). The pathway of the reduction from cinnamoyl-CoA to cinnamyl aldehyde is a crucial step committed to monolignol biosynthesis. However, whether this step metabolizes through a main route or goes by multiple alternative pathways has not been demonstrated unequivocally. The *CCR* gene has been cloned and investigated in several species, including *Arabidopsis* (Lauvergeat et al. 2001), maize (Pichon et al. 1998) and *Eucalyptus* (Lacombe et al. 1997). According to those studies, five cinnamoyl-CoA esters can be used in CCR-mediated reduction. In the present study, the biochemical data suggest that PtCCR selectively catalyzes cinnamoyl-CoA esters, and the CCR catalytic property may contribute a biochemical mechanism in vivo mainstreaming the metabolic flux mediated by CCR from feruloyl-CoA to coniferaldehyde, in aspen lignifying xylem tissues. This biochemical insight into CCR catalysis provides a line of new knowledge to understand lignin biosynthesis pathways, although the pathways may be regulated by other environmental and cellular signals in plants. Consistent with the view of the CCR main pathway, down-regulation of *CCR* expression in other plant species such as tobacco (Piquemal et al. 1998, Chabannes et al. 2001) and *Arabidopsis* (Goujon et al. 2003) results in a drastic decrease of lignin content in stem tissue.

The reaction conditions for aspen CCR in vitro are optimal at pH 6, consistent with what was reported previously for eucalyptus CCR (Lacombe et al. 1997). Interestingly, the enzyme CCoAOMT, which catalyzes the immediately upstream reaction in monolignol biosynthesis, had a remarkable difference in pH dependence. As reported in several species, including aspen, the pH optimum for CCoAOMT catalysis is 7.5 (Ye et al. 1994, Meng and Campbell 1998, Li et al. 1999), while the activity of CCR under that condition is undetectable. Our results also revealed that the metabolic flow through the two neighboring reactions mediated by CCR and CCoAOMT requires a pH change in vitro, indicating that a probable compartmentalization is required to accommodate the respective pH environments in order for CCR and CCoAOMT to function properly.

The biosynthesis of monolignols involves multiple enzymes. However, how those enzymes are organized to facilitate the flow of the metabolism pathways is unknown. Currently, we generally assume that monolignols are synthesized in the cytosol and exported out of the plasma membrane for lignin polymerization in cell walls. It remains to be investigated how the entire pathway is organized in the cytosolic micro-compartmental complex. In order to synthesize monolignols, three hydroxylation steps are involved to modify the phenolic ring of cinnamyl alcohols. These reactions are catalyzed through three CYP proteins. They are CYP73 for cinnamic acid 4-hydroxylase (C4H) (Teutsch et al. 1993, Urban et al. 1994), CAld5H (Humphreys et al. 1999, Osakabe et al. 1999) and CYP98 (Schoch et al. 2001, Franke et al. 2002) for an enzyme hydroxylating the 3′ position of the phenolic ring. These P450 enzymes are localized on the microsomal membrane, while the others are thought to be soluble in cytosol. It has been suggested that L-phenylalanine ammonia lyase (PAL) and C4H form a reaction complex chaining two reactions together (Czichi and Kindl 1975, Achnine et al. 2004). Thus, the remaining question is whether the entire set of monolignol biosynthesis enzymes are spatially organized into a reaction chain, channeling metabolism from phenylalanine to cinnamyl alcohols in a way that efficiently streamlines the metabolic flow.

In most higher plant cells, the cytoplasm has a slightly basic pH (7.4–7.5) and the vacuole is acidic with a pH in the range of 4.5–6 (Kurkdjian and Guern 1989, Guern et al. 1991). In monolignol biosynthesis, pH 7.4–8.5 is required for most of the pathway enzymes, such as C4H (Teutsch et al. 1993, Strack 2001), PAL (Strack 2001), CCoAOMT (Ye et al. 1994, Meng and Campbell 1998, Li et al. 1999), 4-coumaroyl-CoA 3-hydroxylase (4CL; Strack 2001), AldOMT (Li et al. 2000) and CAld5H (Osakabe et al. 1999). Cinnamyl alcohol dehydrogenase (CAD) activities that catalyze alcohol oxidation to aldehyde and the reverse reaction are regulated by pH. At pH 8.8, the catalysis is favored towards oxidation. When the pH is <8, the reaction is favored for reduction (Wyrambik and Grisebach 1975, Li et al. 2001). The present results indicate that CCR is not active at pH 7.5. This raises an interesting question of why the pH environment required by CCR is so different from that of its neighboring enzymes. The reaction mediated by CCR is considered as the first step committed to monolignol biosynthesis (Lacombe et al. 1997) and its upstream reactions are networked to many other secondary metabolisms (Croteau et al. 2000). Thus the distinct pH for CCR catalysis may play a role in regulating lignin biosynthesis. Nevertheless, how CCR acquires the specific pH micro-environment in the cytoplasm remains a question in the full understanding of the process of monolignol biosynthesis.

A small family of *CCR* genes is found in various species. For example, *CCR* members can be identified in six loci in the *Arabidopsis* genome (http://www.tigr.org/tdb/e2k1/ath1/). To investigate how many CCR homologous genes there are in the genome of a tree species and how those genes are expressed in a way associated with tree growth and development, we searched the *Populus* genome sequence database and found that there are eight loci in the genome containing the CCR homologous sequences. Although the full transcripts from the eight genes are unable to be entirely predicted due to the

incompleteness of the genome assembly, real-time PCR can be employed to determine whether the genes are expressed according to the predicted exon sequences. According to the expression results, it appears that four of the eight predicted genes were expressed in the tested tissues while the transcripts of the other four were hardly detected. The four expressed genes displayed a variety of expression patterns in eight different tissues. Among them, *CCR-H1*, which shares 95% homology with *PtCCR* and was expressed in a xylem-specific manner similar to *PtCCR*, is probably a *PtCCR* ortholog in *P. trichocarpa*. Thus *CCR-H1* can be a CCR gene for lignin biosynthesis. However, *CCR-H5*, *CCR-H6* and *CCR-H8* genes displayed different expression patterns that appeared irrelevant to lignin biosynthesis. On the other hand, for the four genes whose expression was undetected, it is unclear at this time whether they are expressed in other tissues or developmental stages that were not included in the current study, or whether the prediction of the gene is correct.

In the *P. trichocarpa* genome, *CCR-H1* was indicated in association with lignin biosynthesis while the others were not, possibly being involved in the metabolism of other phenolics. More work is needed to dissect fully the functions of all CCR homologous genes in the tree genome. The characterization of two *Arabidopsis CCR* genes suggests that distinct physiological functions can be assigned to different members (Lauvergeat et al. 2001). It is also known that two *4CL* genes in aspen function distinctively, one being expressed specifically in xylem and involved in lignin biosynthesis while the other functions in epidermic tissue not relevant to lignin biosynthesis (Hu et al. 1998). Thus, that the genes of homologous sequences may function differentially suggests caution when the gene function is discussed based simply on sequence homology.

Materials and Methods

Plant materials used in the present study were collected from greenhouse-grown quaking aspen (*P. tremuloides*). In a vibrant growing season, the young leaf, phloem and xylem tissues were directly harvested into liquid nitrogen and stored until later use.

Isolation of CCR cDNA

A pair of degenerate primers (5′-TGYTGGAGYGAYCTYGARTW-3′ and 5′-GCYTCTGGTTBGWGAACTTG-3′) was synthesized on the basis of a *CCR* gene conserved region. Using the phage DNAs isolated from an aspen developing xylem cDNA library as templates, PCR was applied to amplify the corresponding fragment flanked by the two degenerate primers. The PCR fragment with a size of about 0.45 kb was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) and characterized. The results suggested that the PCR clone was a candidate fragment of the CCR sequence according to sequence homology analysis. Then this fragment was used as a probe to screen an aspen developing xylem cDNA library according to Li et al. (1997). The screening of 3.0×10^4 plaque-forming units resulted in 28 positive hybridization signals, and the subsequent purification of the positives led to the isolation of a full-length aspen CCR cDNA, designated PtCCR. The cDNA was sequenced through primer walking from both ends.

RNA blotting analysis

Total RNA was isolated from various aspen tissues according to Bugos et al. (1995). RNA gel blotting and hybridization were performed under high stringent condition using ^{32}P-labeled 5′ end PtCCR cDNA as a hybridization probe according to Li et al. (1999).

PtCCR and PtCoAOMT recombinant protein expression and purification

In order to synthesize PtCCR recombinant protein using an *E. coli* expression system, the coding region of PtCCR cDNA was amplified from the original cDNA clone. Two oligos, one primed at the 5′ end with an additional *Nco*I site (5′-CCC ATGGCTGTTGATGCTTCA-3′) and the other primed at the 3′ end of the antisense strand with an additional *Xho*I site (5′-CCTCGAGTTTTTTCACAGA-3′), were synthesized and used for PCR amplification. The PCR-amplified CCR coding region was cloned and confirmed by sequencing, and then subcloned into an expression vector, pET-41a(+) (Novagen Inc., Madison, WI, USA), which allowed the fusion tag in the recombinant protein to be cleaved by enzymatic digestion. pET-41a(+) PtCCR plasmid DNA was transferred into the BL21(DE3) strain and selected for kanamycin resistance. The selected clone was cultured at 37°C in LB medium containing 50 mg l^{-1} of kanamycin. Protein expression was induced at 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after the cell density was cultured to OD_{600} 0.6. Following the manufacturer's instructionsa, recombinant CCR protein was purified using a BugBuster GSTclosed circleBind purification kit, and the fused tag of PtCCR recombinant protein was cleaved and removed using the Enterokinase Cleavage capture kit (both from Novagen Inc.). Furthermore, PtCCR was also expressed in pET-23b(+) (Novagen Inc., Madison, WI, USA) in order to produce a larger amount of recombinant protein, according to Li et al. (2000).

From the developing xylem cDNA library, on the other hand, an aspen CoAOMT cDNA was isolated and shown to be identical to the sequence that Meng and Campbell (1998) cloned previously. The recombinant protein was expressed in the pET-23b(+) system and purified to electrophoretic homogeneity.

Plant protein extract

Developing xylem tissue (10 g) was ground in liquid nitrogen and homogenized at 4°C for 2 min in 25 ml of extraction buffer (0.1 M Tris–HCl, pH 7.0) containing 1 mM EDTA, 40 mM ascorbic acid, 15% glycerol, 1% polyvinylpyrrolidone-40, 1 mM phenylmethylsulfonoyl fluoride, 1 μg/ml leupeptin and 5 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000×*g* for 10 min at 4°C, and the supernatant was used for enzyme assay.

Chemicals

The Co-A ester substrates (*p*-coumaroyl-CoA, caffeoyl-CoA, feruloyl-CoA, 5-hydroxyferuloyl-CoA and sinapoyl-CoA) were synthesized via acyl *N*-hydroxysuccinimide esters as described (Stöckigt and Zenk 1975, Osakabe et al. 1999). The authenticity of the intermediate esters was confirmed by ^{1}H NMR and MS and that of the final CoA esters was confirmed by UV spectrometry and MS (Osakabe et al. 1999, Li et al. 2000). The standard chemicals used for quantitative calculation of PtCCR reaction products were from the following sources: *p*-coumaryl aldehyde, caffealdehyde, sinapaldehyde and coniferaldehyde were from Sigma/Aldrich; 5-hydroxylconiferaldehyde was synthesized by ourselves and confirmed as described by Osakabe et al. (1999).

HPLC-UV/MS analysis of enzyme function and reaction kinetics

An HPLC-UV/MS system (Agilent 1100, Agilent, Palo Alto, CA, USA) was employed for the measurement of the products of the

enzymatic reaction. The accuracy and reproducibility of the determination were carefully validated under the experimental conditions. The enzymatic reaction system, including reaction time, pH, amount of protein and buffer components, was optimized for PtCCR. The optimized conditions were as follows: reaction at 30°C for 4 min in 500 µl of reaction mixture containing 50 mM sodium phosphate buffer (pH 6.0), 10 mM β-mercaptoethanol, 500 µM NADPH and 100 ng of recombinant protein. The reaction was stopped by addition of 1.5 ml of ice-cold ethyl acetate. *o*-Coumaric acid (100 ng) was integrated into the terminated reaction mixture as internal standard for quantitative measurement. After the reaction products were extracted twice by 1.5 ml of ethyl acetate, the upper solvent extract phase was combined and evaporated under vacuum conditions. The dried extracts were then dissolved in 30 µl of HPLC mobile phase buffer and an aliquot of 15 µl was immediately injected into the HPLC-UV/MS system. The conditions for HPLC separation and UV/MS detection were as below. A Hewlett-Packard (HP) 1100 liquid chromatography system was equipped with a Supelcosil LC-ABZ column(15 cm×4.6 mm×5 µm; Supelco, Bellefonte, PA, USA) and automatic sample injection. The column temperature was set at 40°C and the flow rate was at 0.25 ml min^{-1}. The gradient program was 20% acetonitrile in 10 mM formic acid (pH 2.5) for 12 min and then 20–100% acetonitrile from 12 to 16 min, and held at 100% acetonitrile for 5–10 min, and detected with an HP 1100 diode array detector and an HP 1100 liquid chromatography–mass spectrometer detector system with an atmospheric pressure ionization electrospray source in negative ion mode. The reaction products were identified and confirmed by comparing the ion fragmentation patterns of the product and the authentic standard in MS scanning mode at 70 V. The product quantity and K_m, V_{max}, k_{cat} and K_i values (means ± SE) were determined as described (Osakabe et al. 1999, Li et al. 2000, Li et al. 2001).

Transcript quantification of CCR homologous genes in P. trichocarpa by real-time PCR

Eight CCR homologous genes in the *P. trichocarpa* genome were identified by blasting the *Populus* genome database (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). The PCR primers were selected in the sequence region specific to each CCR homolog and the PCR product was designed at a length of 90–120 bp flanked in one exon to ensure the PCR amplifications were accurate and distinct from each other. The primers to amplify the transcripts of the eight genes were as follows (sequence direction 5′ to 3′): *CCR-H1* (forward, CTTTGCTCTGAGAGCGTTCTC; reverse, AACCCAGATCCCTTAGCTTCT), *CCR-H2* (forward, ATTTGCGCTGAGAGAATGCTC; reverse, TGCCCAGATCCTTGATCTTCT), *CCR-3H* (forward, ATTTGCTTTGAGAAAATGCTT; reverse, AAGCACTGTTTTACTGGGGTG), *CCR-4H* (forward, CGGCAGATAGGACAAACTGTA; reverse, ATATACCGACGGCCTAGCTCC), *CCR-5H* (forward, TTGCCATGCCGTCCAATATGT; reverse, ACACATTGCAGCCATCTTCTT), *CCR-6H* (forward, AGAGCAAGGACATTATTTTCC; reverse, CACAGTTTCATAGAGACATTG), *CCR-7H* (forward, GGTGATGTAATTTCATTATTC; reverse, CACAGTCTTTCCATAGCAATA) and *CCR-8H* (forward, GGAGTTTTCCATACTGCTTCT; reverse, GTAACACATAGCTCAACTGAA).

In addition, two primers (forward, TTCTCATAAGGTGCTGGCGGA; reverse, AAGACGATCAGATACCGTCCT) to amplify the 18S transcript were also designed as an internal standard for quantification.

Total RNA (200 ng) was reverse transcribed into cDNA using SYBR Green PCR Master Mix and an RT–PCR kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's manual. Real-time PCR was conducted using Applied Biosystems' 7900HT Sequence Detection System. For each reaction, the 25 µl mixture contained the synthesized cDNA (equivalent to 100 pg of total RNA), 5 pmol each of the forward and reverse primer, and 12.5 µl of 2× SYBR Green PCR Master Mix. The program for amplification was as follows: 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. After amplification, a thermal denaturing cycle was added to determine the dissociation curve of PCR products for verifying amplification specificity. Each reaction was repeated three times. The data were normalized by 18S transcripts that were assumed to be constantly expressed in all tissues, and the relative abundance of the transcript in various tissues was calculated.

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