Plant Physiology and Biochemistry 49 (2011) 138–145

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Expression analysis of cinnamoyl-CoA reductase (*CCR*) gene in developing seedlings of *Leucaena leucocephala*: A pulp yielding tree species

Sameer Srivastava [a,1], Ranadheer K. Gupta [b], Manish Arha [a], Rishi K. Vishwakarma [a], Shuban K. Rawal [c], P.B. Kavi Kishor [b], Bashir M. Khan [a,*]

[a] *Plant Tissue Culture Division, National Chemical Laboratory, Homi Bhabha Road, Pune-411 008, Maharashtra, India*
[b] *Department of Genetics, Osmania University, Hyderabad 500 007, India*
[c] *Molecular Biology Division, Ajeet Seeds, Aurangabad, Maharashtra, India*

ARTICLE INFO

Article history:
Received 7 January 2010
Accepted 1 November 2010
Available online 11 November 2010

Keywords:
Cinnamoyl-CoA reductase
Developing seedling
Leucaena leucocephala
Lignin biosynthesis

ABSTRACT

Removal of lignin is a major hurdle for obtaining good quality pulp. *Leucaena leucocephala* (subabul) is extensively used in paper industry in India; therefore, as a first step to generate transgenic plants with low lignin content, cDNA and genomic clones of *CCR* gene were isolated and characterized. The cDNA encoding CCR (EC 1.2.1.44) was designated as *Ll-CCR*; the sequence analysis revealed an Open Reading Frame (ORF) of 1005 bp. Phylogenetic analysis showed that *Ll-CCR* sequence is highly homologous to *CCR*s from other dicot plants. The 2992 bp genomic clone of *Leucaena CCR* consists of 5 exons and 4 introns. The haploid genome of *L. leucocephala* contains two copies as revealed by DNA blot hybridization. *Ll-CCR* gene was over-expressed in *Escherichia coli*, which showed a molecular mass of approximately 38 kDa. Protein blot analysis revealed that Ll-CCR protein is expressed at higher levels in root and in stem, but undetectable in leaf tissues. Expression of *CCR* gene in *Leucaena* increased up to 15 d in case of roots and stem as revealed by QRT-PCR studies in 0–15 d old seedlings. ELISA based studies of extractable CCR protein corroborated with QRT-PCR data. CCR protein was immuno-cytolocalized around xylem tissue. Lignin estimation and expression studies of 5, 10 and 15 d old stem and root suggest that *CCR* expression correlates with quantity of lignin produced, which makes it a good target for antisense down regulation for producing designer species for paper industry.

1. Introduction

Worldwide annual production of paper has increased more than three fold in the past forty years, amounting to a total production of 330 million tonnes (FAO, Forest report 2003). The growth of paper and paper product consumption in world is expected to be the highest during the coming decade. Paper industry mainly uses poplar, bamboos, *Eucalyptus* sp. and *Casuarina* sp. as a source of raw material for paper and pulp production. However *Leucaena leucocephala* is widely used in India because of its high rate of biomass production and easy acclimatization. In India about 25% of raw material for paper industry is contributed by this plant.

The biosynthesis of lignin begins with the common phenylpropanoid pathway starting with the deamination of phenylalanine and leading to the cinnamoyl-CoA ester [1,2] that plays key role during plant development and defense. Cinnamoyl-CoA esters are then channeled into the lignin branch pathway to produce monolignols via two reductive steps using *CCR* and cinnamyl alcohol dehydrogenase (CAD). Lignins, second to cellulose in abundance, are traditionally considered to be polymers of three monolignols: *p*-coumaryl alcohol (H unit), coniferyl alcohol (G unit), and sinapyl alcohol (S unit) monomers differing in their degree of methoxylation [3–5]. Each type of precursor may form several types of bonds with other monolignols to produce a lignin polymer [6]. Out of three monomers (H, G and S units), S and G lignins are found predominantly in angiosperms. A higher S/G ratio is desirable for paper industry as S lignin is less compact (higher degree of methoxylation) than G lignin and easier to remove from pulp.

Abbreviations: AMV-RT, Avian Myeloblastosis Virus Reverse Transcriptase; BCIP, 5-Bromo-4-Chloro-3′-Indolyphosphate p-Toluidine Salt; CCR, Cinnamoyl-CoA Reductase; cDNA, complementary DNA; DFR, Dihydroflavonol-Reductase; DKFR, Dihydro Kempferol Reductase; ELISA, Enzyme-Linked Immuno-Sorbent Assay; G unit, Guaiacyl unit; kDa, Kilo Daltons; *Ll-CCR*, *Leucaena leucocephala CCR*; NBT, Nitro-Blue Tetrazolium Chloride; PAGE, Poly Acrylamide Gel Electrophoresis; RACE, Rapid Amplification of cDNA Ends; QRT-PCR, Quantitative Real-Time PCR; S, Syringyl unit.

* Corresponding author.
E-mail address: bm.khan@ncl.res.in (B.M. Khan).
[1] Present address: Prince of Wales Clinical School, Lowy Cancer Research Center, Adult Cancer Program, UNSW, Sydney, Australia.

0981-9428/$ – see front matter © 2010 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.plaphy.2010.11.001

Among tree species very few plant systems have been studied for down regulation of *CCR* gene. In case of Norway spruce down-regulated for *CCR* gene, transcript abundance of the sense *CCR* gene was reduced up to 35% relative to the transformed control and the corresponding reduction in lignin content was up to 8% [7]. Recent studies have also shown that down regulation of *CCR* gene in transgenic poplar (*Populus tremula* × *Populus alba*) was associated with up to 50% reduced lignin content and the reduced levels of lignin and hemicellulose were associated with an increased proportion of cellulose [8]. All these studies provide a good platform for generating designer *L. leucocephala* species to meet the increasing demand of high quality wood for paper industry.

This report illustrates isolation, cloning and molecular characterization of *CCR* gene from *L. leucocephala*. In addition expression analysis of *CCR* gene in developing seedling has been studied, which suggests that composition of lignin in developing seedling might be dependent on expression of *CCR* gene. No study has been carried out on lignin biosynthetic pathway gene(s) in *L. leucocephala* and the study of cinnamoyl-CoA reductase will help in better understanding the lignin biosynthesis pathway and its manipulation in a tree species.

2. Materials and methods

2.1. Plant material

Xylem tissue of mature and healthy *L. leucocephala* cv. K636 plants (7–8 years) growing in NCL campus was harvested according to the experimental need. Outer bark was scraped to expose the xylem tissue. Xylem tissue was scraped and ground using liquid nitrogen and RNA was isolated for first-strand synthesis and RACE PCR. Seeds of *L. leucocephala* were treated according to the protocol described by Shaik et al. (2009) [9]. The day of inoculation was considered as zero day. Root, shoot and leaves were harvested from 5, 10, 15 d and 8 week and 12 week old plants. Experiments were conducted in a growth chamber with temperatures of 25 ± 2 °C under a 16 h photoperiod, 70% relative humidity, with a light intensity of 24.4 μmol m^{-2} s^{-1}.

2.2. Isolation of full-length Ll-CCR gene

The CCR protein sequences available from various species in the NCBI database were aligned by Clustal X program to obtain the conserved amino acid sequences [10,11]. These conserved domains (amino acid sequences) were used for designing five sets of primers: *CCR*-F2 5′-CCGTGAGGGCAAAGY NMGNAAYCC-3′, *CCR-R*1 5′-GTCTTGGCGGAGCCGKYNARRTAYTT-3′, *CCR*-R2 5′-CACCGTCTTG CCGTAGCARTACCARTT-3′, SCH-F2 5′-GATG AGGTGGTTGACGAGTC-3′, Sam-r1 5′-TTAGCTGAGCCAGTGAGG TACTT-3′. Total RNA was isolated from xylem (lignified tissue) using TRI reagent (Sigma, GmbH). First-strand cDNA synthesis was performed with AMV-RT according to the manufacturer's protocol (Promega Corp., Madison, USA), which was followed by PCR for partial amplification of *CCR* cDNA. SMART-RACE cDNA amplification kit (Clontech Laboratories Inc., Takara bio, Japan) was used to amplify the 5′ and 3′ ends of *L. leucocephala CCR* cDNA. The 5′ and 3′ RACE was done according to manufacturer's instruction in two rounds of PCR amplification with the *L. leucocephala CCR* gene-specific primers: 5′RACE primer: Sam-r1 5′-TTAGCTGAGCCAGTGAGGTACTT-3′, 3′RACE primer: SCH-F2 5′-GATGAGGTGGTTGACGAGTC-3′. Sequence information of 5′ and 3′ RACE PCR products were used to design primers for the amplification of *CCR* coding sequence. Following primers: *CCR*-F2 5′-ATGCCTGCTGCCGCCCCCG-3′, *CCR*-R 5′-TTACTTGGTGGGAAGAGGAA GA-3′ were used for full-length amplification. PCR was performed using Stratagene HiFi Taq DNA polymerase. The same primers were used to amplify *CCR* genomic fragment using genomic DNA as a template.

2.3. Heterologous expression, purification, production of antiserum, protein blot analysis

The *CCR* gene coding sequence was amplified with primers: *CCR*pet-F 5′-CATATGCCTGCTGCCGCCCCAGCC-3′, *CCR*pet-R 5′-AAGC TTCTTGGTG GGAAGAGGAAGA-3′, containing restriction sites (Forward primer: *Nde* I and Reverse primer: *Hind* III) and was directionally cloned in pET30b + vector. The recombinant plasmid was introduced into *E. coli* BL21 (DE3) cells for over-expression. Purification of recombinant CCR protein was performed using Ni-NTA Agarose beads (Qiagen) and analyzed through SDS-PAGE. All the purification procedures were performed at 4 °C [12]. Purified protein from inclusion bodies was used for raising antibodies in New Zealand white rabbit. Protein blot analysis was performed for analyzing the plant native CCR protein from the crude protein extracts of stem, root and leaf. Prior to this, antibodies were tested for its specificity against purified soluble recombinant CCR protein (Titre; 1:100,000). *L. leucocephala* plant, approximately 7–8 year old, was used for extraction of protein in appropriate buffer (100 mM Tris–HCl, pH.7.5, 2% PVPP, 2% PEG 4000, DTT 5 mM and PMSF 1 mM). A total of 100 μg protein was separated on a 10% SDS-PAGE and electro-transferred onto nitrocellulose membrane (Schleicher and Schuell, Barcelona, Spain). Signals were detected with NBT/BCIP in alkaline phosphatase buffer according to the standard protocol [13].

2.4. Quantitative real-time PCR

Total RNA was extracted individually from root and shoot at different time intervals from developing seedling of *L. leucocephala*. Total RNA (1.0 μg) was used for making cDNA using ImProm cDNA synthesis kit (Promega, Madison, USA). Brilliant SYBRGreen QPCR kit (Stratagene, USA) and Stratagene Mx3000P real-time PCR machine was used for all quantitative real-time PCR reactions. The primer sequences that were designed for specific amplification of *L. leucocephala CCR* gene and 5.8S rRNA are: Sam-F1 5′-CTAAC ACCACCTCATCAGGTT-3′, *CCR*intrev 5′-GTTCTTAGAATCATCTGGAT TTCTG-3′and CT-F 5′-CTAAACGACTCTCGGCAAC-3′, CT-R 5′-TTCA AAGACTCGATGGTTCAC-3′, respectively. Optimal number of PCR cycles within the linear range of amplification for each gene was determined in preliminary experiments. QRT-PCR reactions were performed under the following conditions: 2 min at 50 °C, followed by 10 min at 94 °C, 42 cycles (for *Ll-CCR*) or 30 cycles (for 5.8S rRNA) of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C. The reaction was run in duplicates and repeated twice. It was ensured that equal quantity of RNA template was used for each reaction by normalizing against 5.8S rRNA expression [14,15].

2.5. Enzyme extraction from developing seedlings and ELISA analysis

Stem and root samples were harvested from 5, 10, 15 d and 8 week and 12 week after germination and homogenized in buffer (Section 2.3). Leaf tissue was not considered for this experiment, as expression of *CCR* gene was very low. All samples (total protein 25 μg) were coated in triplicates on 96 well microtitre plate. PNPP (p-nitro phenyl phosphate) was used as a substrate for all reactions at a concentration of 1 mg mL^{-1}. Prior to this a standard graph was made using 1 ng–32 ng of purified recombinant soluble Ll-CCR protein. Experiment was repeated twice.