Isolation and characterization of the 4-coumarate:coenzyme A ligase (4CL1) promoter from *Eucalyptus camaldulensis*

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Abstract The most important enzyme of the phenylpropanoid pathway, 4-coumarate:coenzyme A ligase (4CL), is encoded by several homologous genes including *4CL1*. The 4CL1 promoter is a tissue-specific gene expression element, particularly active in the secondary xylem or older stems. In this study, the 1127 bp 5'-upstream region of the *4CL1* coding sequence from *Eucalyptus camaldulensis*, *Euc4CL1*, was isolated and characterized. Essential putative *cis*-elements in the *Euc4CL1* promoter included: a TATA-box at −22/−28 position, two CCAAT-boxes at −256/−260 and −277/−281 positions, respectively, an AC-element at −328/−336 and A-boxes at −115/−120 and −990/−995 positions. To investigate the effect of the *Euc4CL1* promoter on gene expression, a plant transformation vector, *pEuc4CL1p*, containing the reporter gene for β-glucuronidase (GUS) under the control of *Euc4CL1* promoter was constructed based on the pBI101 backbone and introduced in tobacco plants. Stable expression of the GUS gene in transgenic lines was analysed by a histochemical GUS assay. The results indicated the specific expression of the GUS gene in the stem xylem cells of transgenic tobacco lines was controlled by the *Euc4CL1* promoter. The observations suggest the isolated *Euc4CL1* promoter is a potential candidate for driving the expression of a foreign gene in plant xylem tissues.

Keywords 4-coumarate:coenzyme A ligase · 4CL1 promoter · *Eucalyptus camaldulensis* · GUS gene · Transgenic plant

Introduction

Lignin is one of the most abundant structural components of plants and lignin content is an important trait for forest tree breeding. Wood with low concentrations of lignin is desired for pulp, paper and bioethanol production, and highly lignified wood is required for civil construction, charcoal and furniture production (Shinya et al. 2016).

Lignin biosynthesis involves a number of enzymes, among which phenylpropanoid 4-coumarate:coenzyme A ligase (4CL, EC 6.2.1.12) is considered as the key enzyme (Li and Nair 2015). A number of *4CL* and *4CL*-like genes have been identified from different species such as parsley (Lozoya et al. 1988), potato (Becker-André et al. 1991), pine (Voo et al. 1995), tobacco (Lee and Douglas 1996), *Eucalyptus camaldulensis* (Chen et al. 2006), *Pinus massoniana* (Huan et al. 2012), and mulberry tree, *Morus notabilis* (Wang et al. 2016).

Due to the importance of the 4CL enzyme in plant development, the structures and functions of 4CL have been studied extensively in many species, including in woody plants. Gene sequence analysis demonstrated that 4CL is encoded by either single gene in pine (Voo et al. 1995), duplicated genes in parsley and potato (Lozoya et al. 1988; Becker-André et al. 1991), or multiple genes in soybean, poplar (Lindermayr et al. 2002; Chen et al. 2013; Zhang et al. 2015), sorghum (Saballos et al. 2012) and *Arabidopsis* (Li et al. 2015). Although, the expression of the duplicated *4CL* genes (*4CL1* and *4CL2*) was similar in

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various organs of tobacco, they were divergent in coding sequences (Lee and Douglas 1996). Similarly, *Pinta4CL1* and *Pinta4CL2* genes of pine (*Pinus taeda*) were identical in the coding sequences (Zhang and Chiang 1997). Based on the expression analysis of 4CL-GUS fusions in transgenic plants, Reinold et al. (1993) observed that both tobacco and parsley 4CL genes were expressed specifically during floral organ differentiation. This expression pattern was also observed in raspberry (Kumar and Ellis 2003). Transgenic tobacco plants harboring a sense *Pm4CL1* gene had higher 4CL enzyme activity and lignin content compared with wild-type plants while the lignin content was decreased in the ones expressing antisense *Pm4CL1* (Huan et al. 2012).

The 4CLs and lignin-related genes of the *Eucalyptus* genus, the main hardwood trees for plantations worldwide, have been investigated in different species. According to Haracava (2005), 4CL proteins of this genus were divided into three classes, in which the 4CL class I protein was the main iso-enzyme involved in lignin biosynthesis and predicted to be targeted to the endoplasmic reticulum. Northern and Western hybridization analyses verified that the *Euc4CL1* gene expressed in secondary developing xylem tissues is associated with lignin biosynthesis (Chen et al. 2006). Negishi et al. (2011) found that the transcript abundances of 4CL were highly correlated with lignin content in the basal part of stems of *E. globulus*. In *E. grandis*, there is a single member *Egr4CL1* in class I, which is strongly and specifically expressed in developing xylem (Carocha et al. 2015). The control mechanisms of lignifications in *Eucalyptus* have been studied at the level of gene expression regulatory elements. For example, the SND2 promoter (Botha et al. 2011) and the cellulose synthase (*CesA*) promoter (Creux et al. 2011, 2013) from *Eucalyptus* have been functionally characterized. In this study, the promoter region of the 4CL1 gene from *E. camaldulensis* was isolated and characterized by expression analysis of promoter-GUS fusion construct in transgenic tobacco plants.

Materials and methods

Materials

Plants of *Nicotiana tabacum* variety K326 were grown in Murashige and Skoog (MS) medium under 16 h light and 8 h dark at 25 °C, and then potted into soil. Cells of *Escherichia coli* strain DH5 α were used for the cloning and propagation of all recombinant vectors. *Agrobacterium tumefaciens* strain C58 (pGV2260) was used for tobacco leaf disc transformations.

Extraction of genomic DNA from *E. camaldulensis* leaves

Genomic DNA was extracted from young leaves of *E. camaldulensis* following the method of Angeles et al. (2005) with some modifications. Leaves (5 g) were homogenized in liquid nitrogen and then added 250 mg PVPP. The ground tissues were then transferred to a 50 ml Falcon tube which contained 25 ml of the 65 °C DNA extraction buffer: 2 M NaCl, 0.2 M TrisHCl pH8, 0.07 M β -mecaptoethanol, to which was then added 0.5 ml 20 % SDS and incubated at 65 °C for 1 h. The solution was treated with phenol, chloroform, isoamylalcohol and centrifuged at highest speed. Genomic DNA was precipitated in absolute ethanol and its concentration was determined by spectrophotometry and checked by electrophoresis on a 0.8 % agarose gel (Sigma, USA).

Isolation of 4CL1 putative promoter by genome walker DNA walking

The upstream sequence region of the 4CL1 gene was isolated using Universal Genome Walker™ Kit (Clontech Laboratories, Palo Alto, CA). In order to generate two genome walker libraries, isolated genomic DNA was digested with two restriction enzymes *DraI* and *PvuII*, respectively and ligated to a genome walker adaptor. The first round of DNA amplification was performed using the GenAmp® PCR System 9700 (Applied Biosystems) in 25 μ l reactions containing 1.0 unit of DreamTaq DNA polymerase (Fermentas, EU), 14.6 μ l 10 $\times$ buffer, 2 μ l 10 mM dNTPs, 1.0 μ l of each 0.1 μ M primer (Pro4CL1.5R, a gene specific primer; and AP1, the outer adaptor primer; Table 1), and 50 ng DNA template. The two-step PCR cycle was as follows: 25 s at 94 °C, 3 min 72 °C, (7 cycles); 25 s at 94 °C, 60 s at 65 °C, (32 cycles) and 5 min at 72 °C.

The second round of PCR (the nested PCR) was performed using the diluted PCR products generated in the first round of PCR, the nested gene specific primer (Pro4CL1.4R) and the nested adaptor primer (AP2) (Table 1). The major bands of secondary PCR products were purified from agarose gels using GeneJET™ Kit (Fermentas, EU), cloned into pJET 1.2/blunt vector (CloneJET™ PCR Kit – Fermentas, EU) and sequenced (Sambrook and Russell 2001; Rishi et al. 2004).

Sequencing and sequence analysis

Sequencing reactions were performed with M13 forward and M13 reverse primers using BigDye^R Terminator v3.1 Cycle Sequencing Kit. The sequencing PCR products were applied to the ABI PRISM™ 3100 Genetic Analyzer

Table 1 Primers used to amplify and subclone 5'flanking region of *4CL1* gene

Primers' name	Sequence (5'- 3')
Pro4CL1.4R (GSP2)	5' GGT GGC CCC GTT GAT GAC GC 3'
Pro4CL1.5R (GSP1)	5' ACC GGA AGA TGA ACT CGC GG 3'
AP1	5' GTAATACGACTCACTATAGGGC 3'
AP2	5' ACTATAGGGCACGCGTGGT 3'
Pro4CL1-Bam-R	5' GCG <u>GGA TCC</u> GAG AAT CGA GAG ACA 3'
Pro4CL1-Hind-1.1F	5' CGT <u>AAG CTT</u> TTA TTC GGT TCC GAG 3'

Restriction enzyme recognition sites are underlined

(Applied Biosystems, USA) after purification through precipitation with 95 % ethanol and 3 M NaOAc (pH 5.2). Nucleotide data was analysed with Sequencing Analysis 5.2, SeqScape 2.5 Software and the BLAST Tool on the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST/>). Putative *cis*-acting element searches were performed with PLACE database (<http://www.dna.affrc.go.jp/htdocs/PLACE>) (Higo et al. 1999).

Construction of the plant transformation vector containing *Euc4CL1*-GUS gene cassette

The 1.1 kb putative promoter fragment of the *Euc4CL1* gene (−1004/+109 bp) was produced by PCR with DreamTaq DNA polymerase (Fermentas, EU) using forward primer Pro4CL1-Hind-1.1F (with *Hind*III site) at 5' end, and reverse primer Pro4CL1-Bam-R (Table 1). The PCR products and the binary *A. tumefaciens* transformation vector pBI101 (Clontech Laboratories, Palo Alto, CA) were both digested with *Hind*III and *Bam*HI, then ligated to create the vector p*Euc4CL1*p containing the *Euc4CL1* promoter upstream of the GUS gene. The chimeric GUS gene fusion was verified by DNA sequencing to eliminate possible PCR—introduced mutations.

Plant transformation

A. tumefaciens strain C58 (pGV2260) containing the promoter-GUS fusion construct was used to transform to *N. tabacum* by the leaf-disc method according to Draper et al. (1988). As a control, a vector harboring a promoterless GUS gene (pBI101) was used. To confirm the integration of GUS gene, genomic DNA was isolated from the putative transgenic and control plants and used as templates in PCR that was conducted using the primers as shown in Table 1. The PCR products were then checked by restriction enzyme *Spe*I.

Analysis of GUS activity

Expression analysis of reporter genes in transgenic plants is a common approach to study the activities of promoters. Histochemical GUS assay was performed following the

method of Jefferson et al. (1987). Tissues were incubated in staining solution (0.05 % X-gluc, 50 mM NaH₂PO₄, 50 mM Na₂HPO₄ and 0.1 % Triton X100) and kept in the dark at 37 °C for 24–48 h. After staining, tissues were rinsed in 70 % ethanol, three times for 5 min each. The tissue localization of GUS activity was observed by light microscopy. Photographs were taken with a photomicroscope and 8.0 pixel Canon camera.

Results and discussion

Molecular cloning and nucleotide sequence analysis of the *Euc4CL1* promoter

The proximal sequence upstream of the *Euc4CL1* gene was isolated and sequenced by the Genome Walker DNA Walking method using gene specific primers which were designed based on the known *4CL1* coding sequence of *E. camaldulensis*. Exact 14 nucleotides in the coding region and 104 nucleotides in the non translated region were identical with the cDNA of *4CL1* gene. Therefore, the identified sequence was confirmed to be upstream genomic DNA region of *Euc4CL1* gene. This 1127 bp sequence was submitted to GenBank (Accession No. HE 576688).

The identified *Euc4CL1* promoter sequence was referred to the PLACE database. Figure 1 demonstrates the nucleotide sequence of the isolated promoter region (−1004/+123), in which a number of potential *cis*-acting elements and putative transcription start site (+1) are noted. The length from TSS to the initiation codon (Met) of the *Euc4CL1* gene is 108 nucleotides (Fig. 1). The sequence contains a putative TATA-box (TATATTA) at position −22/−28 relative to the TSS and putative CAAT-boxes at −256/−260, and −277/−281 which are generally consistent with the characteristics of eukaryotic promoters. An AC-element (TCACCAACC) identified at position −328/−336 is required for lignin biosynthetic gene expression in lignifying tissue (Zhong and Ye 2009) and was found in all lignin biosynthetic gene promoters of *Arabidopsis* (Raes et al. 2003). G-boxes, also considered to be consensus elements involved in xylem localized expression (Hatton et al. 1995) were located at two positions −174/−183 and −238/−247

Fig. 1 The nucleotide sequence of *Euc4CL1* putative promoter region. Potential transcription factor binding sites are underlined; the transcription start site (TSS) is denoted +1; the start codon is *boxed*; *SpeI* site is the binding site of restriction enzyme *SpeI*

TAAATTATT <u>CCGTTC</u> CGAGAGCCAAACATATGAAGGAAATTTCAAACCGCCCCCTCTCTTCCA	941
A-box	I-box
AACATGGGAGGACGGGATGAAAAGTGTGGTAATGTTGGGAATTTGACCATTAAAGGTAGAGAG	877
W-box	
GCCATGTACGAAAAAGGGGAAAAAATCTGGCCTCCCGATGCCCATGTACAAATGTTCTCCCTC	813
AAGCCCATGTGAGATGAATGGGCTGGGCCGATTCAATTTTCTTTACATTGATTGATGGGGTCTTG	749
TCT-element	
ACAATATTTAGGTGCTCAAAAGTGGAACCGTGTAGATAAGGGATTGGACTTGTATTATCTTTA	685
TTATTATTTTTGGGAAATGAAAAATATTAGATTTGTCGAGCCCTAAATGACATGGGTATTTTCA	621
SpeI site	
GCGCTGCTTTTTACATATATCAGAATTA ACTAGT GGAATCTGACTAATAGAGAGCATGAATAAT	557
TTCGATGCCACGTTCAATGTCCAGCTCGGGCGATGTGGGGGTGCCGTTTAATCGGGAAGGAGG	493
GCCCGCTTTGGTAGGTGAAATCCT ACGTG GCACAGCATTGCTGGCCAAGGTCAAA ACGTG GCAT	429
ABRE-element	ABRE-element
GCACTAAAATAAT CCCGAAA GGGCACCCAAAAAAGGGGAAGACGAAAAGAAAACGAGG	365
LTR-element	
GACTTTTGTATGACCAAAATGTCCCT TCACCAACC TCCGATGGCTCCTTGCCCGGCGTGGTA	301
AC-element	
GGTGACGCCCCGAGACGAC CCAAT CTCCCTCCCTCCCT CCAAT CGCAGCGCG GCCACGTGCGC	237
CCAAT-box	CCAAT-box
AACAAGACAAGCGAAGACGCCCCGGGAAGTCCCGTCAAGGCTAAGCCTAGAGCG GGCACGTGGCG	173
G-box	G-box
GGTCGCGATTGGCCCCGCCAAAGGGACAGCCCGGGCGGACTTCTCCACCG CCGTCC CTGGCC	109
A-box	
AGGGCGGTACGAAAGGACGGAAATGCCCCCATCGAACCCGACTCCACAGCTCCTCTAC	45
CCGCCACAGCTCGTG TATATTA CCCGCAACCAAGCTCAAAC A AGCCTCAGGGAAGGGACGA	20
TATA-box	+1 TSS
AGGCGCCACCACCAACGCTCACCTTCTCATCATCAGCCTTCTGTCTGTCTGTCTGTCTGTC	84
TCTCGATTCTCCGCCCCCAGAC ATG AGGGCGAAGCC	123

of the *Euc4CL1* promoter. In addition, those G-boxes, I-box (TTTCAA) at −959/−965 position, and TCT-element (TCTTAC) at −768/−773 position are considered to be flanking regulatory elements responded to the light. Two consensus PAL and 4CL *cis*-acting elements, the A-boxes (CCGTCC/CCGTTC), related to meristem specific expression were also identified at positions −115/−120 and −990/−995. Another element (W-box/TTGACC) related to fungal elicitor response was also observed in the *Euc4CL1* promoter at −892/−897. Finally, an LTR-element (CCCGAAA), a *cis*-acting regulatory related to low temperature responsiveness, and two ABRE-elements (ACGTG) responsive to abscisic acid are located at −409/−415, −433/−437 and −464/−468, respectively (Table 2). Thus, the *Euc4CL1* promoter had typical characteristics and contributed to the expression of phenylpropanoid genes.

***Euc4CL1* putative promoter directed GUS expression in the xylem of tobacco transgenic plants**

In our experiments, the specific expression in xylem tissues of the *Euc4CL1* promoter was clearly indicated by the

expression pattern of the GUS reporter gene. The full length *Euc4CL1* promoter fragment (1.1 kb) with all of the predicted elements was inserted upstream of the GUS reporter gene to generate the expression vector *pEuc4CL1p*, in which GUS was expressed under the control of *Euc4CL1* promoter (Fig. 2). The construct was then transferred into tobacco plants using *A. tumefaciens* C58 (pGV2260). The GUS expression level in transgenic plants was compared with the control plants transformed with the pBI101 vector containing a promoterless GUS gene.

Before testing GUS activity by histochemical assays, all transgenic lines were checked for the presence of the *Euc4CL1* promoter in the genome by PCR using specific primers and genomic DNA. Most of the ninety checked lines showed the PCR products expected for *Euc4CL1* promoter (data not shown). Eight transgenic lines, which gave positive PCR results (Fig. 3a) were selected for *SpeI* restriction digest analysis. The *Euc4CL1* promoter contained one *SpeI* site at −591. Two expected fragments with the sizes of approximate 700 and 400 bp were appeared by digestion with *SpeI* (Fig. 3b). These transgenic lines were histochemically examined for GUS activity in stems. The

Table 2 Essential *cis*-regulatory elements in the *Euc4CL1* promoter

Elements	Sequences	Position	Functions
TATA-box	TATATTA	−22/−28	<i>cis</i> -regulatory element
G-box	CACGTG	−174/−183 and −238/−247	<i>cis</i> -acting regulatory element involved in light responsiveness
CCAAT-box	CCAAT	−256/−260 and −277/−281	<i>cis</i> -regulatory element
AC-element	TCACCAACC	−328/−336	Element responsible for restricting vascular expression to the xylem
LTR-element	CCGAAA	−409/−415	<i>cis</i> -acting regulatory related to low-temperature responsiveness
ABRE-element	ACGTG	−433/437 and −464/−468	<i>cis</i> -acting element involved in the abscisic acid responsiveness
TCT-element	TCTTAC	−768/773	Element involved in light responsiveness
W-box	TTGACC	−892/−897	Element involved in fungal elicitor responsiveness
I-box	TTTCAA	−959/−965	Element involved in a light responsiveness
A-box	CCGTTC/CCGTCC	−115/−120 and −990/−995	<i>cis</i> -acting regulatory element related to meristem specific activation

Fig. 2 Chimeric promoter–reporter gene construct. The *Euc4CL1* promoter was inserted upstream of the GUS reporter gene of the binary *A. tumefaciens* transformation vector pBI1101 to generate the expression vector p*Euc4CL1p*

bud of full grown transgenic lines were transferred into new medium, and partial stems were used in histochemical assays. The results showed that all of the tested transformants exhibited GUS expression in stem pieces (data not shown).

GUS expression was also observed in other organs as indicated in transgenic line No 5. Although the expression levels at different plant parts were variable, the GUS expression driven by the *Euc4CL1* promoter consistently exhibited the highest level in the stem and petiole core; and no expression was found in epithelial leaves and roots (Fig. 4d–f). Furthermore, the blue color on the horizontal and vertical stained slices of stem sections only appeared in xylem cells (Fig. 4a–c). The AC-elements and G-box of the PAL2 promoter have been studied intensively. These *cis*-acting elements were responsible for xylem specific

expression of the PAL2 promoter in transgenic tobacco plants (Hatton et al. 1995), suggesting that the presence of both AC-element and two G-boxes in the *Euc4CL1* promoter contribute to xylem-specific expression. More over, previous studies demonstrated that the AC-element was a responsive element to nonpathogenic attack (Imura et al. 2000), and that the G-box was likely involved in the wound- and light responsiveness (Jin et al. 2012). Thus, *cis*-acting elements in 4CL1 promoter are not only important for lignin biosynthesis but are also involved in the defense responses of plant cells. Others 4CL1 promoters from plants have been characterized for xylem-specific expression. Lu et al. (2004) reported that 4CL1 enzyme activity in transgenic tobacco plants containing the 4CL1 gene driven by the *Pto4CL1* promoter of *Populus tomentosa* was doubled in stems but there was no increase in the leaves. Using a 4CL-GUS construct, Li et al. (2015) found that 4CL1 and 4CL2 of *Arabidopsis* were strongly expressed in lignified tissue. The histochemical localization of GUS expression in this study suggested that *Euc4CL1* promoter has similar function with other 4CL1 promoters.

The results in this study clearly indicate that the *Euc4CL1* promoter can specifically regulate gene expression in the xylem. Together with a large number of

Fig. 3 Presence of the *Euc4CL1* promoter in transgenic lines confirmed by PCR (**a**) and restriction digest (**b**) analysis. **a** Molecular marker (M); PCR amplification of *Euc4CL1* promoter using DNA of eight transgenic lines (lanes 1–8), positive control vector (lane +),

and wild type plant (lane WT); **b** Molecular marker (M); *SpeI* restriction digests of PCR products of eight transgenic lines (lanes 1–8), positive control vector (lane +), and wild type plant (lane WT)

Fig. 4 Histochemical analysis of GUS expression driven by *Euc4CL1* promoter in the transgenic tobacco line No 5. **a** Horizontal slice stem ($\times 40$); **b** horizontal slice stem ($\times 10$); **c** vertical slice stem ($\times 10$); **d** whole transgenic plantlet; **e** spatial stem; **f** petiole and leaf

available promoters (Dutt et al. 2014), it could provide an additional tool for genetic engineering especially of wood properties. In the past, the application of a xylem specific promoter of cinnamate 4-hydroxylase (C4H) promoter to control the expression of an *Arabidopsis* ferulate 5-hydroxylase (F5H) gene led to significantly increase lignin content in transgenic hybrid poplar trees (*Populus tremula* $\times$ *P. alba*) (Stewart et al. 2009). Therefore, our isolated *Euc4CL1* promoter could be used in future transgenic work to modify lignin content in transgenic plants or drive other xylem-specific traits.

Conclusions

In summary, the *Euc4CL1* promoter from *E. camaldulensis* plant has been cloned and characterized. This novel sequence was submitted to GenBank with Accession No. HE576688. The isolated promoter contains all of the important regulatory regions essential for gene expression in the phenylpropanoid pathway such as an AC-element, A-box, G-boxes, and drives GUS expression in the xylem of transgenic tobacco lines. This xylem-specific expression

pattern of the *Euc4CL1* promoter may be useful for genetic transformation of plants.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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