

Molecular cloning, expression pattern, and putative cis-acting elements of a 4-coumarate:CoA ligase gene in bamboo (*Neosinocalamus affinis*)

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Abstract

Background: 4-coumarate:CoA ligase (4CL) plays an important role at the divergence point from general phenylpropanoid metabolism to several branch pathways. Although 4CL in higher plants have been extensively studied, little has known about the 4CL gene of bamboo. **Results:** In current study, a *Na4CL* gene putative encoding 4-coumarate:CoA ligase (4CL) and its 5'-flanking region were isolated from bamboo (*Neosinocalamus affinis*) by RACE-PCR and genomic DNA walker, respectively. *Na4CL* encodes a predicted protein of 557 amino acids, with conserved motifs of adenylate-forming enzymes. Phylogenetic analysis showed that *Na4CL* shared 62~85% identity with other known plant 4CLs, and cluster closely with some known 4CLs in monocots. Sequence analysis revealed conserved cis-acting elements (Box A and AC-II element) present in the *Na4CL* promoter. Additionally, a *Na4CL* RNAi construct was transformed into tobacco. Transgenic tobaccos displayed significant down-expression of endogenous 4CL and reduced lignin contents. **Conclusion:** These results contribute to the knowledge of the presence of *Na4CL* gene and its possible role in phenylpropanoid metabolism.

Keywords: expression pattern, *Neosinocalamus affinis*, promoter, RACE, RNAi

INTRODUCTION

Phenylpropanoid pathway is an important secondary metabolism in plants. 4-coumarate:CoA ligase (4CL, EC 6.2.1.12) catalyzes the last reaction of the general phenylpropanoid pathway through converting 4-coumaric acid and other substituted cinnamic acids (such as caffeic acid and ferulic acid) into the corresponding CoA thiol esters (Allina et al. 1998; Hu et al. 1998; Ehltling et al. 1999; Ehltling et al. 2001; Lindermayr et al. 2002; Hamberger and Hahlbrock, 2004; Tsai et al. 2006). Therefore, 4CL is of importance for monolignol biosynthesis and for the production of other secondary metabolites such as flavonoids, isoflavonoids as well as phytoalexins (Allina et al. 1998; Ehltling et al. 1999; Ehltling et al. 2001). Homologous genes encoding 4CLs have been widely reported in numerous plants, such as *Arabidopsis* (Ehltling et al. 1999; Ehltling et al. 2001; Hamberger and Hahlbrock, 2004; Costa et al. 2005), soybean (Lindermayr et al. 2002), rice (Souza et al. 2008; Gui et al. 2011), and woody plants (Zhang and Chiang, 1997; Allina et al. 1998; Hu et al. 1998; Harding et al. 2002). Recently, a 4CL gene family in the moss (*Physcomitrella patens*) has also been identified (Silber et al. 2008). Consisting of a multigene family (Costa et al. 2005), 4CL isoforms display different substrate specificities, suggesting their distinct function in regulation of phenylpropanoid metabolism (Ehltling et al. 1999; Ehltling et al. 2001; Endler et al. 2008; Gui et al. 2011).

Bamboos are ancient grass plants that are widely distributed in tropical and subtropical zones in Asia, Africa and America. Because of morphologically and physiologically unique, bamboos have drawn

much attention in last few years. Peng et al. (2010) reported 10,608 putative full length cDNAs and nearly 38,000 ESTs generated from Moso bamboo (*Phyllostachys heterocycla* cv. *Pubescens*). And there is more than nine thousands ESTs of *Dendrocalamus latiflorus* in GenBank which are available. These sequence data would be useful for studying the structure and function of bamboo genes. However, different from scattered-born bamboo species such as *Dendrocalamus latiflorus* and *Phyllostachys edulis*, *Neosinocalamus affinis* is a typical sympodial bamboo, and it is widely cultivated in south-western of China as a preferable wood substitute for chemical pulping. However there is little genome data available from *Neosinocalamus affinis* in public database.

In this paper, we isolated a cDNA putative encoding 4CL and its upstream region from *Neosinocalamus affinis* by the RACE and genomic DNA walking strategy, respectively. The sequence characteristic, expression pattern, and putative cis-acting elements of the bamboo 4CL gene, designated as *Na4CL* here, were also reported. Additionally, a *Na4CL* RNAi construct was transformed into tobacco. Transgenic tobaccos displayed significant down-expression of endogenous 4CL and reduced lignin content. These results contributed to the knowledge of the presence of *Na4CL* gene and its possible role in phenylpropanoid metabolism.

MATERIALS AND METHODS

Amplification of 4CL gene from *Neosinocalamus affinis*

Total RNAs from young shoots of *Neosinocalamus affinis* were extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instruction, and treated with DNase (TaKaRa, Dalian, China). The AccuScript High Fidelity First Strand cDNA Synthesis Kit (Stratagene) was used to synthesize first strand cDNA. A partial cDNA fragment was isolated firstly by RT-PCR with a pair of degenerate primer (DP1 and DP2: 5'CAGGCACTACAGGTTTGGCNAARGGNGT 3' and 5'ATCCAATGTCTCCAGTRTGNAG CCANCC3'; R means A or G; N means A, C, G, T) and LA-Tag polymerase adding high GC buffer (TaKaRa).

The extension of the 3'-cDNA end was performed with a 3' anchor primer (5'GACCACGGC TATCGATGGCTCA3') and a sense primer (5'GGCTATGGGATGACCGAGGCT3'). 5'-RACE was carried out according to the protocol of the 5'-full RACE core set (TaKaRa). Gene-specific primer GSP1 (5' GCAGCACCGAGTTGA 3') was used for reverse transcription, and then the first and second round PCR was performed with a set of nested primers L1/R1 (5'TTCGGGTTCTCCCCATCCAC3'/5' CTGTGTGTGCTGCCGCTGTT3) and L2/R2 (5'TGACCAGGCTGCGGTGCGT3'/5' TGCCGCTGTTCCACATCTACTCG 3') respectively. The full length of the open reading frame (ORF) of *Na4CL* was further confirmed with gene-specific primers 4CLF/4CLR(5'ATACATATGGGCTCCATCGCGCA3' and 5'ACCCTCGAGTTAGCTTTGGACTGTG3'). PCR products were subsequently cloned into the pMD19 vector (TaKaRa) and then sequenced.

Isolation of putative promoter by GenomeWalker

The extension of 5'-flanking region was carried out according to the protocol of GeneWalker Universal Kit (Clontech). Isolated genomic DNA from young shoots of *Neosinocalamus affinis* was digested with four restriction enzymes (DraI, EcoRV, PvuII, and StuI, respectively), and then ligated to a GenomeWalker adaptor. The primary PCR was performed with a gene-specific primer (P1: 5'TAGGTGTACACCGCCCCGCTCT3') and the outer adaptor primer (AP1: 5'GTAATACGACTCACTATAGGGC3'), and then the secondary PCR was performed with nested gene-specific primer (P2: 5'CTCCTCTGCCGCGATGGAGCCC3') and the nested adaptor primer (AP2: 5'ACTATAGGGCACGCGTGGT3'). The secondary PCR products were cloned into pMD19 Vector (TaKaRa) and then sequenced.

Sequence alignment and protein structure prediction

Multiple alignments were performed by ClustalW. A phylogenetic tree was generated with MEGA 4.0 based on deduced protein sequences of plant 4CL homologs. The protein three-dimensional structure

was simulated using SWISS MODEL and RasMol software. Putative cis-acting elements were identified by Plantcare database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>).

Expression analysis of the *Na4CL* gene

Total RNAs were extracted from various tissues using Trizol reagent following the protocol provided by the manufacturer (Invitrogen, Carlsbad, CA) and treated with DNase (TaKaRa, Dalian, China). About 1 µg of total RNA was used for first-strand cDNA synthesis. RT-PCR was performed using a pair of specific oligonucleotide primer (P11 and P12: 5'GAGACATCGGTTATGTTGACGA3' and 5'GGAATCCGTGAAGAAAACCTTTG3') derived from 3' -end of the *Na4CL* cDNA. A partial housekeeping *Actin* cDNA (JN869246) was employed as internal control using gene-specific primers (P21 and P22: 5' gtattgtcagcaactggatga 3' and 5' gcgtacaaggaaagaacagctt 3'). Real-time quantitative PCR reaction was performed using SYBR Green PCR Master Mix (Applied Biosystem) and each sample was amplified in triplicate. REST software was used to quantify mRNA level of *Na4CL* with actin normalization by the 2-Ct method.

RNAi construct and tobacco transformation

For a *Na4CL* RNAi construct, an inverted repeat construct with ~600-bp sequence (spanning from 600~1264 bp downstream of the initial codon of *Na4CL* ORF) was generated via pSK-int vector and inserted into pBI121 vector. Tobacco transformation was performed as described by Horsch et al. (1985). Kanamycin-resistant transformants were identified by PCR using primers for NPTII (5'-TGAATGAACTGCAGGACGAG-3' and 5'-AGCCAACGCTATGTCCTGAT-3'). The expression of endogenous tobacco *Nt4CL 1* (U50845) was detected using primers 5'-ACCATAGCCACAATGCCAAT-3' and 5'-CGTCAACATCACACCTTTTCG-3'. The tobacco *Actin* (JQ435884) was amplified as a control using primers 5'-TACAACGAGCTTCGT GTTGC-3' and 5'-TTGATCTTCATGCTGCTTGG-3'.

Histochemical analysis and lignin contents

Fresh freehand sections from the bottom of stems in tobaccos (12-week old) were subjected to lignin staining using the 0.1% phloroglucinol-HCl (Li et al. 2003). For analysis of lignin content, leaves and stems of transgenic tobaccos were ground with liquid nitrogen, and non-covalently-bound compounds were sequentially removed by successive extractions with 80% ethanol at room temperature. The obtained material was called Cell-Wall Residue (CWR). Lignin content in CWR was estimated by the AcBr method as modified by Jourdes et al. (2007). Measurements were performed in triplicate.

RESULTS AND DISCUSSION

Cloning of a bamboo 4CL gene by RACE procedure

To isolate a conserved 4CL sequence from *Neosinocalamus affinis*, a RT-PCR was performed using a pair of degenerate oligonucleotide primers (DP1 and DP2) designed corresponding to highly conserved regions of other plant 4CL sequences. The resulting cDNA sequence of ~700 bp showed significant similarity with other 4CL sequences. To obtain the complete 4CL gene from *Neosinocalamus affinis*, a series of gene-specific primers and nested primers (see Materials and Methods) were designed targeting the conserved sequence. Subsequently, 3' and 5'-rapid amplification of cDNA ends (RACE) were performed, separately. In 3' RACE, five 750~860 bp fragments were cloned, which have similar sequences (87% identity) but with different 3'-UTR length (data not shown), while only a 702 bp cDNA fragment was isolated by 5' RACE (data not shown).

A non-overlapping cDNA sequence with the length of 1852 bp was obtained through assembling the conserved region and the cDNA fragments from 5' and 3' RACE, and then was confirmed by RT-PCR using gene-specific primers (4CLF and 4CLR). The isolated cDNA, designated as *Na4CL* here (GenBank accession EU327341), contains a complete open reading frame (ORF) of 1674 bp, which encodes a predicted protein of 557 amino acids with a calculated molecular mass of 59.6 kDa and a pI of 5.1.

Sequence analysis and multiple alignments

BLASTp searches showed the Na4CL from *Neosinocalamus affinis* shares 62~85% amino acid identity with other known plant 4CLs in the GenBank database. Multiple alignments of Na4CL and other characterized 4CL highlighted two conserved motifs that are common to a group of adenylate-forming enzymes (Figure 1; Schmelz and Naismith, 2009). Among them, Box I (SSGTTGLPKGV) is conserved in all known 4CLs, and is considered as the putative AMP binding domain (Uhlmann and Ebel, 1993; Stuible and Kombrink, 2001). Another highly conserved motif, Box II (GEICIRG), has been proposed to be associated with stability and catalytic activity of 4CL and related enzymes (Schmelz and Naismith, 2009). As is clear from the presence of conserved sequences, Na4CL might be a member of the ANL superfamily of adenylating enzymes. However, L (Leu) in the common SSGTTGLPKGV motif of 4CLs is substituted by M (Met) in Na4CL protein, and it is not known whether this amino acid-substituent has any impact on the function of Na4CL.

Phylogenetic analysis previously had shown that most 4CL homologs in dicot could be divided into two distinct group, 4CL class I and class II (Ehrling et al. 1999; Ehrling et al. 2001; Lindermayr et al. 2002). In general, class I 4CLs (such as *Arabidopsis* At4CL1 and 2) are probably associated with lignin accumulation, while class II 4CLs (such as At4CL3, soybean Gm4CL3 and 4) are assigned to flavonoid branches of phenylpropanoid metabolism (Lindermayr et al. 2002). However, because monolignol compositions and phenylpropanoid profile in monocotyledons are different from those of dicots, the differentiation between evolutionary distance and metabolic function is not certain for isoenzymes of 4CLs in monocots (Hatfield et al. 2008; Hatfield et al. 2009). In phylogenetic tree, bamboo Na4CL clustered closely with *Lolium perenne* Lp4CL2, *Zea mays* Zm4CL, *Oryza sativa* Os4CL1 and Os4CL4, suggesting that 4CLs in monocots likely had more functional conservation (Figure 2).

Structural comparisons revealed that the overall fold is highly conserved among ANL enzymes (Hu et al. 2010). The predicted structure overall structure of Na4CL is similar to that of *Populus tomentosa* 4CL1 with resolved crystal structure by X-ray diffraction (Pt4CL1; PDB id: 3A9U; Hu et al. 2010). Na4CL contains two distinct N- and C-domains, and the N- domain is composed of N1, N2, and N3 subdomains, which is much larger than that of the C-domain (Figure 3). The major conserved SSGTTGLPKGV motif as a putative AMP-binding domain is located at the position of N3 domain (Figure 3).

Isolating and sequence analysis of 5'-upstream region of *Na4CL* gene

To isolate the 5'-flanking region of *Na4CL* gene, a genomic DNA walking strategy was performed by using gene-specific primers (P1 and P2) and adapter primers (APs) derived from the *Na4CL* cDNA sequence and its promoter region, respectively (see Materials and Methods), and yielded a DNA clone that contains a 420-bp 5'-upstream sequence and a 176-bp coding region of *Na4CL* gene. A putative TATA box (TATATTAA), a core element in eukaryotic promoter, was located at -137 bp relative to the ATG translation initiation site (Figure 4, boxed).

AC-rich cis-acting elements, such as 'P' and 'L' boxes, have been known to be highly conserved in the promoter regions of a number of known 4CL genes (Hauffe et al. 1991; Hamberger and Hahlbrock, 2004). Furthermore, these AC elements, which have been shown to match the binding sites of MYB-related transcription factors, also conserved in the promoters of other phenylpropanoid pathway genes, including phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and caffeoyl-CoA 3-O-methyltransferase (COMT) (Hatton et al. 1995; Raes et al. 2003; Deluc et al. 2006; Legay et al. 2007; Fornalé et al. 2010). Accumulated researches demonstrated that cis-elements P (C/TTT/CC/TA/CA/CCA/CAA/CCC/AC/AC) and L (T/CCT/CC/TACCTACC) boxes, together with another common box A (C/ACGTCT/C), were required for UV- and stress-elicitor responses, and this combination of cis-elements were assumed to be related with coordinate expression of lignin-biosynthetic genes in various stimulus conditions (Logemann et al. 1995; Koopmann et al. 1999). In this study, Plantcare program analysis revealed two box A-like sequences (CCGTCC, underline in Figure 4) and an AC-II element (ACCAACC, underline in Figure 4) present in *Na4CL* promoter, suggesting its roles in lignin biosynthesis.

Expression analysis of the *Na4CL* gene

Numerous studies have been shown that, the expressions of *4CL*, and other lignin-involved genes, such as *PAL*, *C4H*, and *CAD*, are directed to lignifying tissues during plant development (Kao et al. 2002; Eudes et al. 2006; Tsai et al. 2006). To investigate the expression pattern of *Na4CL* gene in *Neosinocalamus affinis*, a pair of specific oligonucleotide primer (P11 and P12) was designed from the particular 3' end region of the *Na4CL* cDNA. Total RNAs were isolated from young leaves (YL), mature leaves (ML), 15-day old seeding (Se), young stems (YS) in branch, and mature stems (MS) of 3-year old *Neosinocalamus affinis* plants. RT-PCR analyses showed the *Na4CL* is expressed both in seeding, stem and leaf, without apparent tissue specificity, and increased levels of *Na4CL* mRNA during the later development stage of leaf and stem indicated that *Na4CL* expression is developmentally regulated (Figure 5).

A *Na4CL*-RNAi construct resulted in reduced lignin in tobacco transformants

Furthermore, a *Na4CL* RNAi construct was generated and then was transformed into tobacco. Eight out of 14 kanamycin-resistant transformants exhibited apparent down-regulation in expression of tobacco endogenous *4CL* (*Nt4CL1*) as compared with that of wild type (Figure 6), hinting the *Na4CL* RNAi construct effectively resulted in gene silence of tobacco endogenous *4CL*. Furthermore, analysis of histochemically stained with phloroglucinol-HCl, which specifically reacts with lignin in plant tissues, showed transgenic plants had less red coloration in xylem of stem compared with those of the wild-type (Figure 6). These results were supported further by reduced lignin content in these transgenic tobacco lines. As showed in Figure 7, lignin contents both in stems and leaves of *4CL*-downregulation transgenic lines (T5, T7, T9~T14) decreased significantly, only accounting for 72.7%~88.1% of the wild-type. These results, together with *Na4CL* expression pattern and AC elements present in its promoter, indicated that *Na4CL* might be involved in lignin biosynthesis and be a potential alternative gene for modifying the content of lignin to improve bamboo paper-making efficiency.

Nucleotide and amino acid sequences

Accession Number for the *Na4CL* sequence reported in this article is EU327341.

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Figures

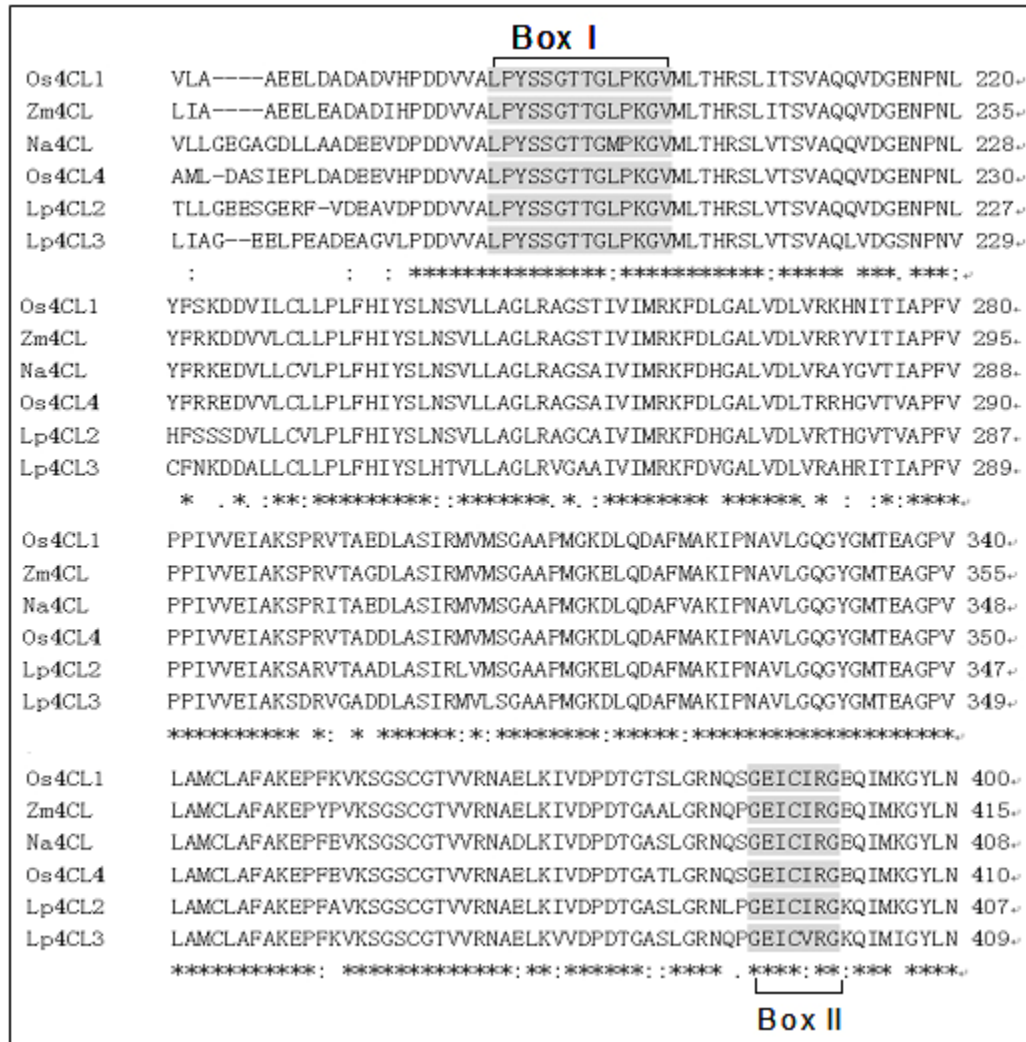


Fig. 1 Amino acid alignment of *Neosinocalamus affinis* Na4CL (ACA09448) with other monocot 4CLs using ClustalW. Identical amino acids are marked (*), strongly similar residues (:), and weakly similar residues (.). Box I and Box II represent the putative AMP binding domain and GEICIGR motif, respectively. Abbreviations and accession numbers, Os: *Oryza sativa*, Os4CL1 (NP001046069), Os4CL4 (NP001058252); Zm: *Zea mays*, AAS67644; Lp: *Lolium perenne*, Lp4CL2 (AAF37733), Lp4CL3 (AAF37734).

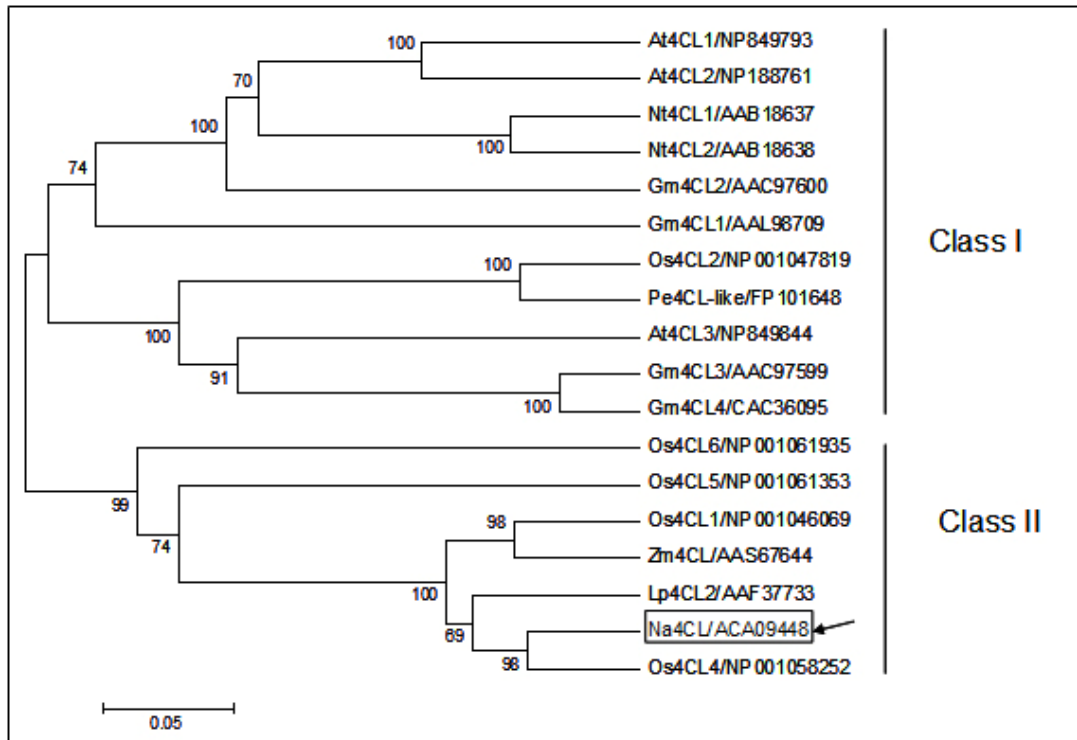


Fig. 2 Phylogenetic analysis of 4CLs from different plant species. Phylogenetic tree was generated with MEGA 4. Scale bar represents genetic distance and node numbers indicate bootstrap support values. Plant species are as follows: Na: *Neosinocalamus affinis*; Os: *Oryza sativa*; At: *Arabidopsis thaliana*; Gm: *Glycine max*; Nt: *Nicotiana tabacum*; Lp: *Lolium perenne*; Pe: *Phyllostachys edulis*.

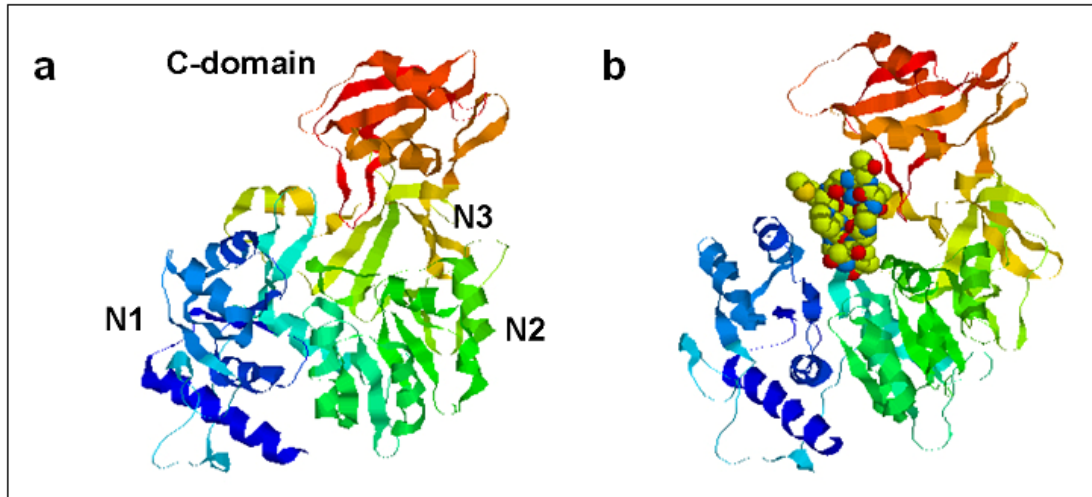


Fig. 3 The predicted three-dimensional structure of Na4CL protein by SWISS-MODEL. (a) The structure of Na4CL based on the known structure of Pt4CL1 from *Populus tomentosa* (Hu et al. 2010); (b) The AMP-binding domain of bamboo 4CL is shown as balls, with C, O, and N atoms coloured in yellow, red, and blue, respectively. These pictures were created with RasMol software.

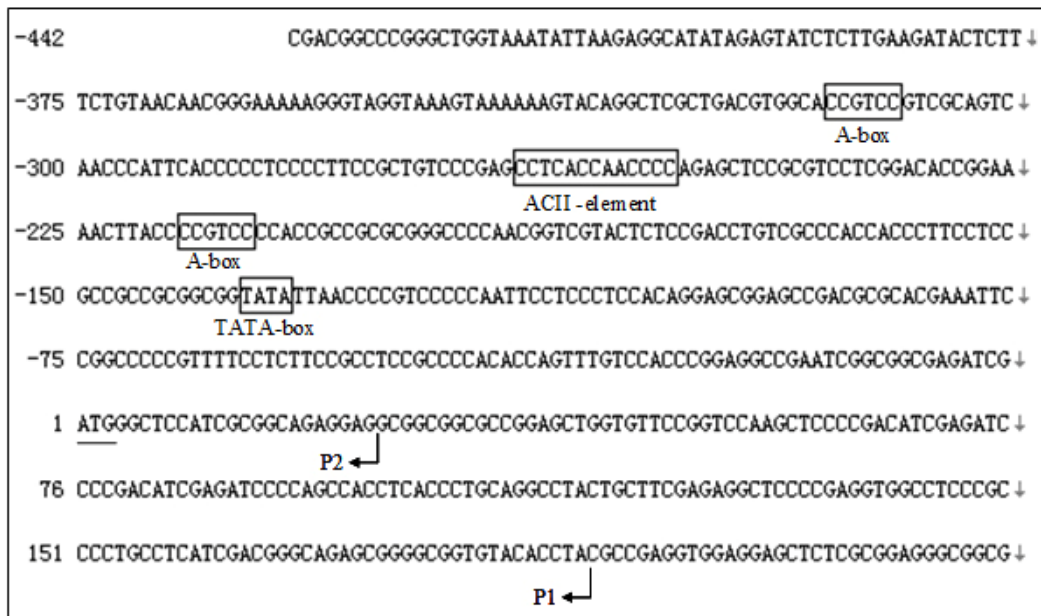


Fig. 4 5'-flanking region of the *Na4CL* gene. The translation initiation codon ATG is located at +1. Putative TATA box and cis-acting elements are boxed. Arrows indicated the position and direction of the nested primers P1 and P2.

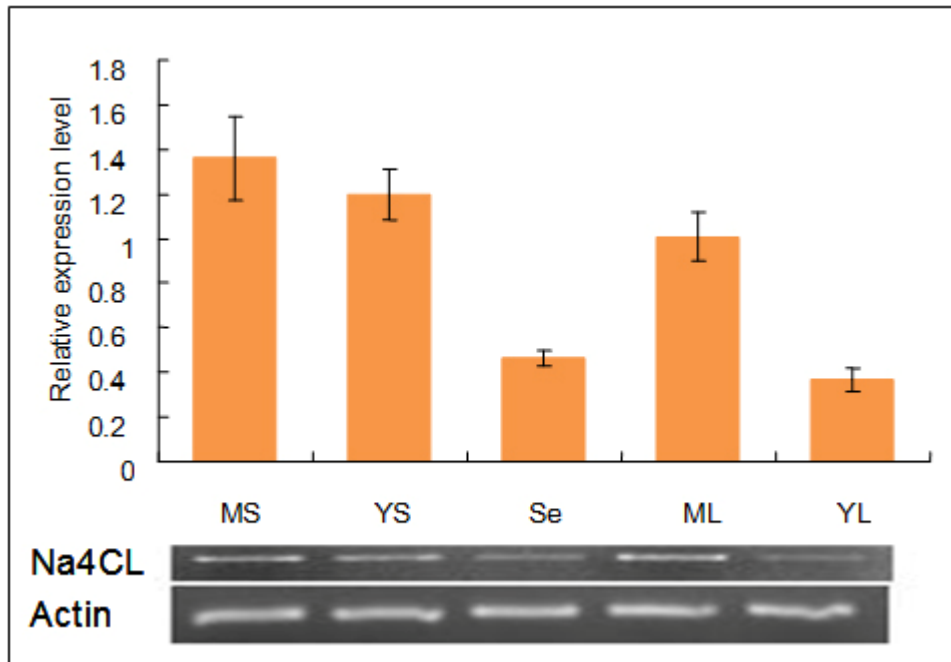


Fig. 5 Expression analysis of the *Na4CL* gene. cDNAs from young leaves (YL), mature leaves (ML), 15-day old seeding (Se), young stems (YS) in branch, and mature stems (MS) of 3-year old *Neosinocalamus affinis* plants were used as templates for semi-quantitative and real-time quantitative RT-PCR analysis. Each bar represents three replications from each RNA sample. Actin from *Neosinocalamus affinis* was employed as positive internal control. Each bar represents three replications from each RNA sample. Error bars represent standard errors shown in each case.

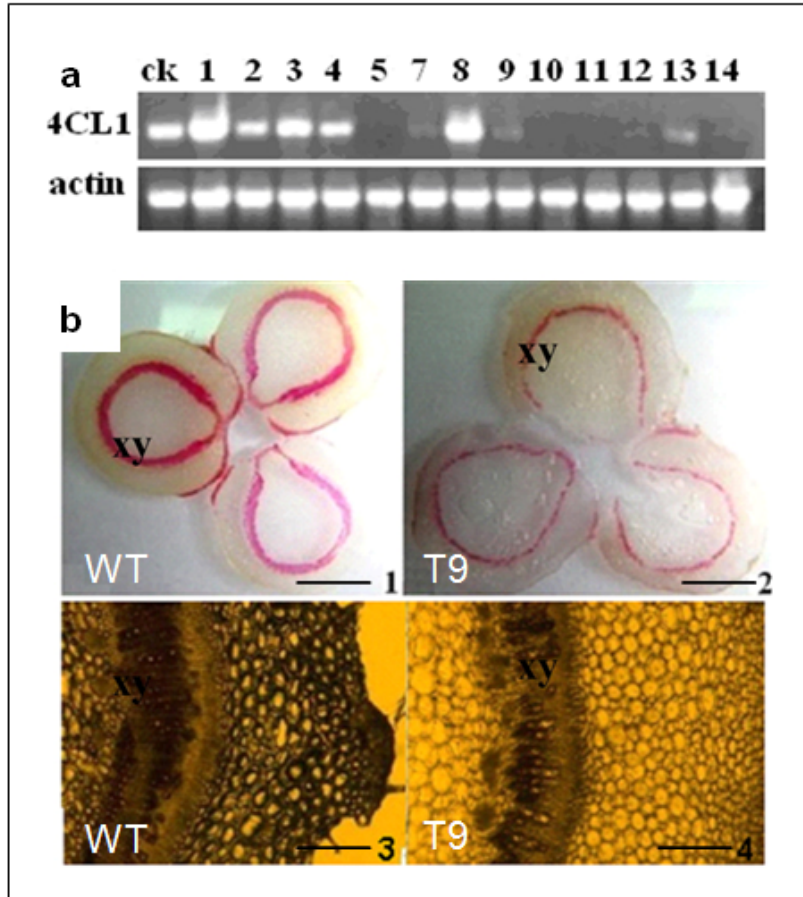


Fig. 6 Transgenic tobacco lines with *Na4CL* RNAi construct. (a) Expressions of endogenesis *4CL* in transgenic tobaccos. RT-PCR was performed on tops of stems from tobacco plants. Amplification was carried out for 32 and 30 cycles for *Nt4CL1* and *actin*, respectively. ck: the wild-type; 1~14: tobacco transgenic line 1~14; (b) Phloroglucinol-stained stem transversal sections photographed under digital camera (1~2) and under microscope (LEICA CIR6000; 3~4); Xy, xylem; rules in figure 1~2 and figure 3~4 represent 200 mm and 500 μ m, respectively.

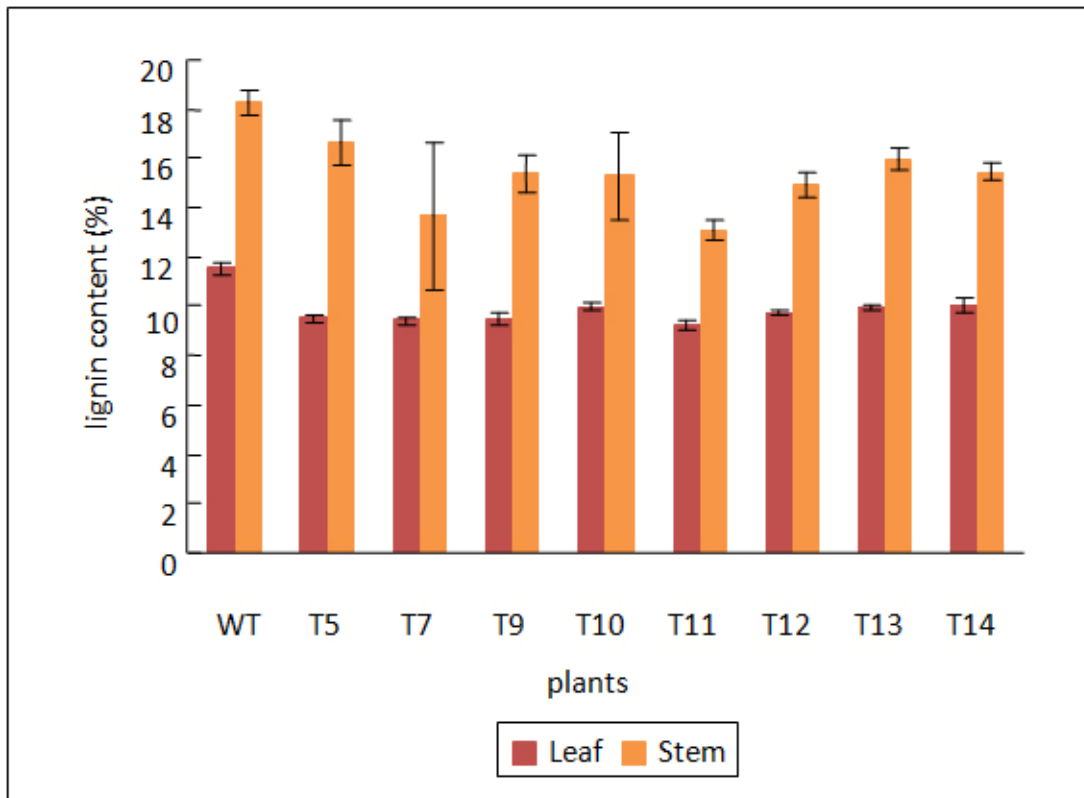


Fig. 7 Lignin contents in transgenic tobaccos. Each bar represented the mean of three determinations per line including standard deviation. WT, the wild- type; T5~T14, transformants with *Na4CL* RNAi construct.