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# Molecular cloning of gene fragment encoding 4-coumarate: Coenzyme A ligase of Sengon (*Paraserianthes falcataria*)

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*Abstract*

4-coumarate:Coenzyme A ligase (4CL) plays an important role in lignin biosynthetic pathway that catalyzed the activation of coumaric acid, caffeic acid or ferulic acid to be a syringil monomer. Lignin biosynthesis control through 4CL down regulating would support lower lignin wood production. The objective of this study was to clone conserved region cDNA of gene encoding 4CL. Gene fragment isolation was conducted by means of reverse transcriptase polymerase chain reaction (RT-PCR) using degenerate heterologous primer. The RT-PCR products were purified, sequenced and analyzed to select the highly homologous fragment to 4CL. BLASTanalysis result showed that deduction of amino acid sequences from one of two RT-PCR product nucleotide was highly homologous with the 4CL conserved region from *Rubbus ideaus, Oryza sativa, Populus tomentosa, Populus balsamifera, Betulla platyphilla, Nicotiana tabacum, and Arabidopsis thaliana* with identity ranging from 78-90%.

Key words: 4-coumarate: Coenzyme A ligase, lignin, sengon

## Introduction

Lignin is a major component of wood, the most widely used raw material for the production of pulp and paper. Lignin is a complex phenolic aromatic polymer that constitutes 20–32% of woody plant cell walls, being the second most abundant biopolymer after cellulose. It is deposited in cell walls of certain supportive and water conductive tissues, providing rigidity and structural support to cell wall polysaccharides (cellulose, hemicellulose, pectin) (Harakava, 2005; Bhuiyan *et al.,* 2009; Wagner *et al.,* 2009). During the manufacture of high-quality paper, lignin is chemically separated from the polysaccharide components of wood during pulping and bleaching reactions. Lignin extraction needs large quantities of chemicals and energy leading to a poor environmental image for the industry (Baucher *et al.,* 2003).

Most of the enzymes required for the synthesis of lignin precursor have been characterized, such as p*henylalanine ammonia–lyase* (PAL) (Kao *et al.*, 2002), *o-methyltransferase* (CCoAoMT) (Ibrahim *et al.*, 1998; He *et al.*, 1998), *4-coumarate CoA ligase* (4CL) (Lee *et al.*, 1997; Allina *et al.*, 1998; Ehlting *et al.*, 1999; Cukovic *et al.*, 2000; Ehlting *et al.*, 2001; Rogers *et al.*, 2005; Wagner *et al.*, 2009), *cinnamoyl-CoA reductase* (CCR) and cinnamyl alcohol dehydrogenase (CAD) (Ralph *et al.*, 1998). These enzymes are involved in the initiation of lignin biosynthetic pathway by conversion of these to monolignol formation. 4-coumarate:Coenzyme A ligase (4CL) is an enzyme that functions early in the general phenylpropanoid pathway by producing the

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monolignol precursor p-coumaroyl-CoA. This enzyme catalyzes the activation of conversion of coumaric acid, cafeic and ferulic into syringil monomer (Harding *et al*., 2002). This catalytic process is characterized by the formation of a coumaroyl-adenylate intermediate, which is subsequently converted to the corresponding CoA ester. The activated phenolic acids serve as precursors for the biosynthesis of numerous plant secondary products such as flavonoids, isoflavonoids, coumarins, lignin, suberin, and wall-bound phenolics (Schneider, 2003). These compounds are important for plant growth and development by providing mechanical support and rigidity to cell walls, attracting insects for pollination, or protecting against biotic and abiotic stresses (Boudet *et al*., 2003).

Concerning the important role of 4-CL in many plant species, gene sequence analysis, protein activity and structure would be interesting subject. This enzyme has been isolated and purified ranging from annual plant species such as *Arabidopsis thaliana* (Lee, *et al.*, 1997; Costa, 2005), G*lycine max* (Lindermayr, 2002), *Oryza sativa* (Lee, *et al.*, 2007) to woody plant species such as pine (*Pinus taeda*) (Voo, 1995) and poplar (*Populous tremuloides*) (Hu, *et al*., 1998). Molecular cloning of 4CL of cDNA *Pinus taeda* consisted of 537 amino acid sequence ( Zhang, 1997).

Sengon (*P. falcataria* L. Nielsen) which belongs to family leguminoseae and sub family of Mimosoideae is one of the recommended species for industrial timber estates. This species has the ability to grow in poor or marginal land and could make the land fertile as it is categorized as nitrogen-fixing tree species (Binkley et al., 2003; Shively *et al*., 2004; Kurinobu *et al.*, 2007; Siregar *et al*., 2007). This fast-growing native of the Moluccan Islands of Indonesia in the South Pacific has been widely planted throughout many tropical regions of the world and has become naturalized in many of them. *P. falcataria* is known as multipurpose tree with uses including pulp, fiber and particle board, packing cases, boxes, matches, chop sticks and light furniture. Its wood is soft and generally light in color with a reported specific gravity range of 0.20 to 0.49 (NAFTA, 1989).

The benefits of removing as much lignin as possible in order to avoid residual lignin, which causes discoloration and reduces paper brightness, have to be balanced against the loss of pulp quality and strength as the result when cellulose is significantly degraded. An alternative idea has been to modify lignin content or structure in genetically engineered trees to reduce lignin production or to make lignin easier to extract (Baucher, *et al*., 2003). Genetic engineering of low lignin tree would support supply of more suitable raw material for the pulp and paper industry. A wide variety of transgenic plants with altered expression of one or more lignification genes in both models and economically important species have become available, with the principal knowledge of the lignin biosynthetic pathways and modifying lignin content (Church and Galston, 1988; Kajita,*et al.*, 2002; Wagner, *et al*., 2009).

Although the role of 4CL has been studied in a large number of plants species, there has been little reported about molecular study of 4CL in tropical woody plants. The aim of the study was to isolate and characterize the 4CL gene of sengon using RT-PCR technique. Cloning and sequencing of 4CL could be used to construct 4CL antisense to repress lignin content in sengon. Low lignin wood would have important economic and environmental benefits.

## Materials and methods

### *RNA extraction and RT-PCR*

Total RNA was isolated from the xylem of 2 months old sengon seedling using Trizol reagent (Invitrogen). RT-PCR reactions were carried out in a one step reaction in which

cDNA synthesis and Reverse Transcription reactions were performed directly in the same tube using a kit of ready to go RT-PCR (Amersham). The bead of ready to go RT-PCR was diluted with 43.9 ml RNase free water (Gibco BRL) and then 1.1 ml of the first strand of pd(T) 12-18 concentrations (0.5 µg /ml), reverse and forward degenerate primers (10 pmole), and 5 mg RNA template were added into the bead solution. The solution was then incubated at 42°C for 30 min for cDNA synthesis. Reaction was stoped at 95°C for 5 min for PCR reaction. Degenerate primer pairs used were DP-F (5'-CCTCATCTTCCGGTCCAAGYTNCMNGAYAT-3') and DP-R (5'-CGCAGGTCCTTCCGCARDATYYYNCC-3'). Primers were designed according to CODEHOP techniques (Consensus-degenerate hybrid oligonucleotide primers) based on alligment of 4CL gene sequences of some plants *Arabidopsis thaliana*, parsley (*Petrocelinum crispum*), vanilla (*Vanilla planifolia*), *Solanum tuberosum*, tobacco (*Nicotiana tabacum*), *Pinus taeda* and *Oryza sativa*. PCR condition were : 95°C, 5 min (pre-PCR); 95°C,45 seconds; 50°C - 56.8°C (annealing temperature), 45 seconds and 72°C, 60 seconds.

*Sequence analysis and cloning of RT-PCR product*

RT-PCR products were purified according to the Sephaglas Brandprep kit protocol (Ammersham). The purified fragments were then subjected to sequence analysis by using fluorescent automated sequencing with ABI Prism ™. Competent cell preparation of *Escherichia coli* DH5 a and bacterial transformation were carried out according to Tomley (1996). The purified RT-PCR product was cloned into PGEM ®-T Easy cloning vector.

Results and Discussion

*Isolation of total RNA*

Electrophoresis of total RNA of sengon seedling xylem was carried out to determine the RNA integrity. The isolated RNA showed the high quality RNA indicated by the non degraded ribosomal RNA 28S and 18S bands (Figure 1). The result showed that RNA of sengon could be used for RT-PCR analysis. RT-PCR technique was carried out through *one step* RT-PCR in which synthesized cDNA as DNA template to further PCR reaction using specific designed primer of 4CL directly in the same tube.

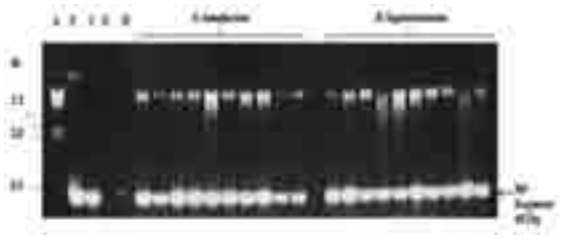

Figure 1. Total RNA of 2 months old sengon seedling xylem

*RT-PCR analysis*

CODEHOP technique was applied to design *degenerate* primer of 4CL (Rose *et al.*, 1998). Each primer consist of a short 3' degenerate core region and a longer 5' consensus clamp region. Only 3 - 4 higly conserved amino acid residues are necessary for design of the core, which is stabilized by the clamp during annealing to the template molecules. During later rounds of amplification, the non-degenerate clamp permits stable annealing to the product molecules. Figure 2 showed the design of *degenerate* 3' amino acid core and 5' region consensus clamp as basic of designing hybrid *degenerate-consensus* primer to amplify 4CL gene fragment of sengon.

```
|4CL1_ARATH        (  33) DVIFRSKLPDIYIPNHLSLHDYIFQNIS  64
4CL2_ARATH         (  33) DVIFRSRLPDIYIPNHLPLHDYIFENIS  61
4CL4_ARATH         (  33) DFIFRSKLPDIFIPNHLPLTDYVFQRFS  84
4CL1_PETC.         (  33) DLIFRSKLPDIYIPKHLPLHTYCFENIS  40
4CL2_PETCR         (  33) DLIFRSKLPDIYIPKHLPLHTYCFENIS  40
4CL_VANPL          (  33) DIIFRSKLPDIYIPKNLPLHSYCFENIS  50
4CL1_SOLTU         (  33) DLIFRSKLPDIYIPKHLPLHSYCFENLS  39
4CL2_SOLTU         (  33) DLIFRSKLPDIYIPKHLPLHSYCFENLS  39
|4CL1_TOBAC        (  33) DLIFRSKLPDIYIPKHLPLHSYCFENIS  39
4CL2_TOBAC         (  33) DIIFRSKLPDIYIPNHLPLHSYCFENIS  39
|4CL_PINTA         (  33) EHLYRSKLPDIEISDHLPLHSYCFERVA  96
4CL2_SOYBN         (  33) SHVFKSKLPDIPISNHLPLHSYCFQNLS  78
|4CL3_ARATH        (  33) PRIFRSKLPDIDIPNHLPLHTYCFEKLS  62
|4CL2_ORYSA        (  33) VTVFRSKLPDIDIPSHLPLHEYCFARAA  90
4CL1_ORYSA         (  33) EIIFRSKLQDIAITNTLPLHRYCFERLP 100

Block primer forward

          H  I  F  R  S  K  L  P  D  I
oligo:5'-CCTCATCTTCCGGTCCAAGytncmngayat-3' degen=128 temp=60.6

|4CL1_ARATH        ( 545) SELSEDDVKQFVSKQVVFYKRINKVFFTESIPKAPSGKILRKDLRAKLA  54
4CL2_ARATH         ( 545) SNISEDEIKQFVSKQVVFYKRINKVFFTDSIPKAPSGKILRKDLRARLA  53
4CL4_ARATH         ( 545) SQLTEDDVKSYVNKQVVHYKRIKWVFFIEVIPKAVSGKILRKDLRAKLE  88
4CL1_PETC.         ( 545) FTTTEEEIKQFVSKQVVFYKRIFRVFFVDAIPKSPSGKILRKDLRARIA  55
4CL2_PETCR         ( 545) FTTTEEEIKQFVSKQVVFYKRIFRVFFVDAIPKSPSGKILRKDLRAKIA  55
4CL_VANPL          ( 545) HNITEDEIKQFISKQVIFYKRINRVFFVEAIPKAPSGKILRKDLRARLA  53
4CL1_SOLTU         ( 545) STITEDEVKDFISKQVIFYKRIKRVFFVETVPKSPSGKILRKDLRARLA  49
4CL2_SOLTU         ( 545) STITEDEVKDFISKQVIFYKRIKRVFFVETVPKSPSGKILRKDLRARLA  49
|4CL1_TOBAC        ( 545) SAITEDEVKDFISKQVIFYKRVKRVFFVETVPKSPSGKILRKDLRARLA  57
4CL2_TOBAC         ( 545) STITEDEVKDFISKQVIFYKRIKRVFFVDAIPKSPSGKILRKDLRAKLA  46
|4CL_PINTA         ( 545) SEISEQEIKEFVAKQVIFYKKIHRVYFVDAIPKSPSGKILRKDLRSRLA  68
4CL2_SOYBN         ( 545) FDLTEEAVKEFIAKQVVFYKRLHKVYFVHAIPKSPSGKILRKDLRAKLE  62
|4CL3_ARATH        ( 545) NDITEEDVKEVVAKQVVFYKRLHKVFFVASIPKSPSGKILRKDLKAKLC  77
|4CL2_ORYSA        ( 545) SDITEESIKEFISKQVVFYKRLHKVHFIHAIPKSASGKILRRELRAKLA  82
4CL1_ORYSA         ( 545) SELSEDDVKQFVAKEVIYYKKIREVFFVDKIPKAPSGKILRKELRKQLQ 100

Primer REVERSE

G  K  I  L  R  K  D  L  R
ccnttytadraCGCCTTCCTGGACGC oligo:5'-CGCAGGTCCTTCCGCardatyttncc-3'
degen=48 temp=63.3
```

Figure 2. Primer design strategy of hybrid *core degenerate*-consensus clamp to ampify 4CL gene fragment of sengon based on 4CL of *A. thaliana*, peterseli (*Petrocelinum crispum*), vanilla (*Vanilla planifolia*), potato (*Solanum tuberosum*), tobacco (N. tabacum), *Pinus taeda and* rice (*O. sativa*) sequences.

Annealing temperatures gradients ranging from 50°C to 56.8°C were optimized to obtain optimum PCR condition. RT-PCR products of 300 to 500 bp were revealed at annealing temperature of 52.2 °C and 54,4 °C. The two bands of PCR product obtained at annealing temperature of 54,4 °C (Figure 3) were then isolated and separated prior to sequence analysis (Figure 4). RT-PCR technique also using degenerate and non degenerated primers has been widely used to isolate another gene such as carotenoid biosynthetic pathway gene in wild and cultivated *Solanum lycopersicon* (Araújo *et al*., 2007), lettuce (*Lactuca sativa*) aminocyclopropane-1-carboxylic acid (ACC) synthase (Takahashi *et al.*, 2003), ferns (*Adiantum capillus-veneris*) phosphoglucose isomerase (*PgiC*) (Ishikawa *et al.*, 2002), and oil palm (*Elaeis guineensis*) ACCase subunit biotin carboxilase (Budiani, *et al*., 2006).

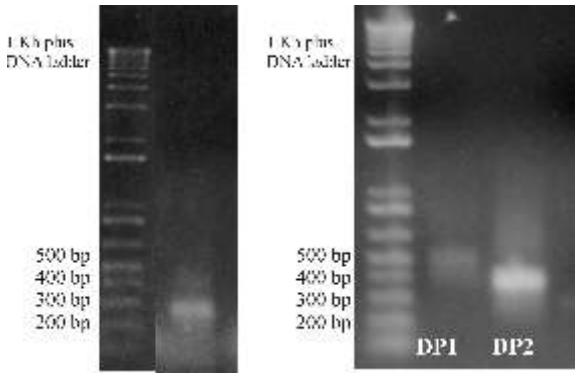


Figure 3. The RT-PCR amplification product of 300 and 500 bp generated by degenerate primer at annealing temperature of 54,4·C for 45 seconds and 60 seconds of extension (a) and separated PCR product by using sephaglass band prep kit (b).

*Sequence analysis*

The sequencing result obtained of RT-PCR product using degenerate primer (342 bp) and its translated amino acid are shown in Figure 5. Analysis of that sequence using BLAST N and Blast P (Table 1) for the amino acid sequence showed that fragment of one of the RT-PCR product namely DP2 had a high homology with the 4 CL of many plant species such as *A. thaliana*, *Medicago truncatula*, *Populus tomentosa*, *Populus balsamivera*, *Betulla platyphylla* with 78% - 90% identity. The 4CL full length sequence size of *Glycine max* (Uhlman and Ebel, 1993), *P. crispum (*Lozoya *et al*.,1988*)*, *A. thaliana* (Shockey *et al*., 2003), *Pinus radiata (*Wagner, 2009*)* and *Solanum tuberosum* (Becker *et al.*, 1991) ranged from 1021 bp to 4021 bp.

Although 4CL putative fragments of sengon showing only a short sequence, this result would be useful for further experiments to create antisense construct to suppress expression of 4CL gene. Unlike gene overexpression that requires full length sequence, mRNA gene fragment would be effective to repress the amino acid translation process through interfering with mRNA product by doubled stranded hybrid mRNA complement of sense and antisense mRNA at certain locations.

```
5' CCC CGG GGC TTT CCC CAG GAT TTT TTC CCA TCA CGA GGA AAT TTT AAA GAT GGA
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Pro Arg Gly Phe Pro Gln Asp Phe Phe Pro Ser Arg Gly Asn Phe Lys Asp Gly

            63          72          81          90          99          108
   CCG GGC GAG TCG GCC TTC TTG ATT TTA AGG GTG GTG GTG CAT GGC CGT TTT CAG
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Pro Gly Glu Ser Ala Phe Leu Ile Leu Arg Val Val Val His Gly Arg Phe Gln


            117         126         135         144         153         162
   TTG GTG GAG AGA ACA GTT AAG GGC ATT ACA GTC CCG ATA TTG CCT CAA ACT TCC
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Leu Val Glu Arg Thr Val Lys Gly Ile Thr Val Pro Ile Leu Pro Gln Thr Ser


            171         180         189         198         207         216
   TTG GCC CGA ATG CGG AGA GTC CCT TTA AGA AGC TGG CCT TTG GAG GGT ACC TCC
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Leu Ala Arg Met Arg Arg Val Pro Leu Arg Ser Trp Pro Leu Glu Gly Thr Ser

            225         234         243         252         261         270
   GCA TAC CTA GTT AGC AAG GGA GGT CTA GTT CGT AAA CGG AAT AAA CCA ACA AGT
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Ala Tyr Leu Val Ser Lys Gly Gly Leu Val Arg Lys Arg Asn lys Pro Thr Ser


            279         288         297         306         315         324
   CGG GGG ACC AAC GAG AAA CGC CTA CCT CCT CCA AGA AAG ATA AAA ACG AAA ACA
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
   Arg Gly Thr Asn Glu Lys Arg Leu Pro Pro Pro Arg Lys Ile Lys Thr Lys Thr


            333         342
   CCT AGT TTA CCT TTT GTT CC 3'
   --- --- --- --- --- --- --
   Pro Ser Leu Pro Phe Val
```

Figure 4. Putative sengon cDNA 4CL partial sequences obtained from RT-PCR product using degenerate primer

Table 1. BLAST P results of sengon (*P. falcataria*) translated amino acid sequence obtained from RT-PCR.

| Gene bank number | Enzyme | Organism | Score (bits) | E value |
|---|---|---|---|---|
| AAG43823.1 | 4-Coumarate:CoA ligase | Capsicum annuum | 39.7 | 0.099 |
| AAD40665.1 | 4-Coumarate:CoA ligase | Solanum tuberosum | 39.7 | 0.099 |
| P31685 | 4-Coumarate:CoA ligase 2 | Solanum tuberosum | 39.7 | 0.099 |
| O24145 | 4-Coumarate:CoA ligase 1 | Nicotiana tabacum | 39.7 | 0.099 |
| P14912 | 4-Coumarate:CoA ligase 1 | Petroselinum crispum | 39.7 | 0.099 |
| P31684 | 4-Coumarate:CoA ligase 1 | Solanum tuberosum | 39.7 | 0.099 |
| BAA08365.1 | 4-Coumarate:CoA ligase | Lithospermum erythrohizon | 39.2 | 0.13 |
| AAL02145.1 | 4-Coumarate:CoA ligase | Populus tomentosa | 37.5 | 0.43 |
| AAC97600.1 | 4-Coumarate:CoA ligase isoenzyme 2 | Glycine max | 37.5 | 0.43 |
| AAK58908 | 4-Coumarate:CoA ligase 3 | Populus balsamifera subsp trichocarpa | 37.5 | 0.43 |
| AAV65114.1 | 4-Coumarate:CoA ligase | Betulla platyphylla | 36.7 | 0.78 |
| AAC24503.1 | 4-Coumarate:CoA ligase | Populus tremuloides | 36.7 | 0.78 |
| ABD59789.1 | 4-Coumarate:CoA ligase | Arnebia euchroma | 36.3 | 1.0 |
| AAP03020.1 | 4-Coumarate:CoA ligase – like protein | Arabidopsis thaliana | 35.8 | 1.4 |
| BAA07828.1 | 4-Coumarate:CoA ligase | Nicotiana tabacum | 35.8 | 1.4 |
| AAQ86591.1 | 4-Coumarate:CoA ligase isoform 5 | Arabidopsis thaliana | 35.8 | 1.4 |
| AAP68991.1 | 4-Coumarate:CoA ligase 2 | Salvia miltiorrhiza | 34.6 | 3.4 |
| AAS48417.1 | 4-Coumarate:CoA ligase 1 | Allium cepa | 33.7 | 6.1 |

*Cluster analysis of 4CL putative gene fragment*

Cluster analysis of sengon 4CL putative fragments (Figure 5) by using CLUSTAL W software showed that 4CL sengon sequence was a unique cluster. This is probably due to only short sequence being compared to complete fragment of others.

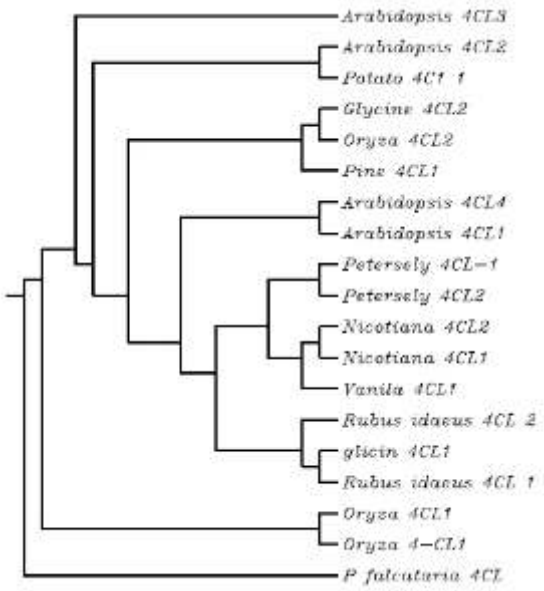


Figure 5. Dendogram of 4CL constructed based on some plants 4CL nucleotide sequences .

*Restriction site analysis*

Restriction site analysis of 4CL gene fragment showed many restriction sites such as XmaI, SmaI, DraI, NlaIII, KpnI, AvaII, Hinc II indicating that there were different restriction sites of pGEMT easy multiple cloning sites so that 4CL fragments could be easily separated from the vector (Figure 6). Information of enzyme restriction site of sequence would assist further gene construct design.

Display: - All commercial single cutter restriction enzymes
- Main non-overlapping, min. 100 aa ORFs
GC=48%, AT=52%

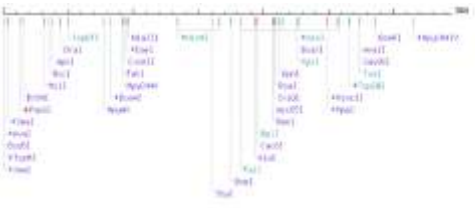

Figure 6. Result of restriction site analysis of 4CL putative gene fragment of sengon by using NEB cutter software (New England Biolabs).

*Transformation of putative 4CL putative gene fragment of sengon into TA cloning vector*

High homology 4CL RT-PCR product was ligated with PGEM®-T Easy cloning vector and transformed into *Escerichia coli* DH5á. Ligation of linear vector of PGEM®-T Easy without inserting activated *lacZ* encoding b-galaktosidase expressed with IPTG induced agent and converted X-gal into blue indicating product reaction. Fragment of putative 4CL inserted between *Eco*R1 and *Not*1 sites of *lacZ* sequence resulted in inactivated b-galaktosidase gen indicated by white *E. coli* (Figure 7)

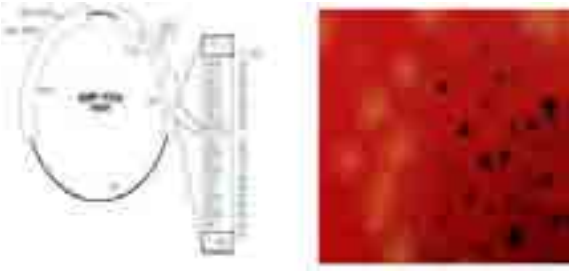

Figure 7. PGEM®-T Easy vector map restriction site (a) and colony of transformed *E. coli* DH5a harboring recombinant plasmid containing putative fragment *4CL* of sengon (b).

Obtained 4CL putative fragment clone could be used to amplify gene fragment in *E.coli* cells in further work such as fragment characterization, as a probe for full length sequence isolation and gene construct.

## Conclusion

The cDNA fragment of 342 bp in size were obtained using degenerate primer designed by CODEHOP technique. This fragment was confirmed as putative 4 CL gene fragment that has high homology with other plants 4CL sequences. This putative 4CL is a fragment which can be potentially be used in genetic engineering study of repression of 4CL expression in order to reduce lignin content of sengon.

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