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Regulation of *4CL*, encoding 4-coumarate: coenzyme A ligase, expression in kenaf under diverse stress conditions

Md. Emran Khan Chowdhury[1], Bosung Choi[1], Byoung-Kwan Cho[2], Joon Bum Kim[3], Sang Un Park[4], Savithiry Natarajan[5], Hyoun-Sub Lim[6]*, Hanhong Bae[1]*

[1]School of Biotechnology, Yeungnam University, Gyeongsan712-749, Republic of Korea
[2]Department of Biosystems and Machinery Engineering, Chungnam National University, Daejeon 305-764, Republic of Korea
[3]Warm-Temperate Forest Research Center, Korea Forest Research Institute, Seogwipo 697-050, Korea
[4]Department of Crop Science, Chungnam National University, Daejeon 305-754, Korea
[5]US Department of Agriculture, Agricultural Research Service, Soybean Genomics and Improvement Laboratory, 10300, Baltimore Avenue, Beltsville, MD 20705, USA
[6]Department of Applied Biology, Chungnam National University, Daejeon 305-764, Republic of Korea

***Corresponding authors: hyounlim@cnu.ac.kr; hanhongbae@ynu.ac.kr**

Abstract

We cloned the full length *4CL* ortholog encoding 4-coumarate:coenzymeA ligase from kenaf (*Hibiscus cannabiuns*, GenBank Accession No. JX548316) using degenerate primers and RACE (rapid amplification of cDNA ends) method. The 4CL is a key regulatory enzyme of the phenylpropanoid pathway that regulates the activation of cinnamic acid, leading to the synthesis of flavonoids and lignin. The 1,704-bp full length of *4CL* ortholog had a 1,623-bp open reading frame (ORF) encoding a predicted protein of 540 amino acids. The predicted molecular weight and isoelectric point (pI) of the deduced protein was 59.56 kDa and 6.58, respectively. The sequence of the deduced amino acid shared 57-79% identities with other 4CL sequences. 4CL ortholog had two conserved putative AMP (adenosine monophosphate)-binding motifs, the SSGTTGLPKGV and GEICIRG domains. A BlastP analysis showed that kenaf 4CL ortholog showed 79% identity with ri4CL2 of *Rubus idaeus* (AAF91309), which is a class I 4CL involved in lignin synthesis. *4CL* ortholog showed differential expression in all tissues during the developmental stages and was highly expressed in stem and root tissues. However, the lowest expression of *4CL* ortholog was observed in leaf and mature flower tissues. *4CL* ortholog was responsive to various stress conditions in the stem tissues of 3-week-old kenaf plants. Wounding caused biphasic expression at 6 h and 24 h after treatment. Taken together, the results of this study contribute to the knowledge of the presence of *4CL* ortholog and its possible role in lignin biosynthesis, as well as its differential expression during developmental stages.

Keywords: kenaf (*Hibiscus cannabinus*L.), 4-coumarate: coenzymeA ligase (4CL), lignin biosynthesis, abiotic stress, quantitative real-time PCR (QPCR).
Abbreviations: 4CL_4-coumarate:coenzymeA ligase; SA_salicylic acid_ABA, abscisic acid; MeJA_methyl jasmonic acid.

Introduction

Kenaf (*Hibiscus cannabinus* L.), which is the third largest fiber crop, is native to central Africa (Dempsey, 1975). Kenaf is a short-day, rapid growing, annual crop grown in temperate and tropical areas that has been used as a cordage crop to produce twine, rope and sackcloth for over six millennia (Dempsey, 1975; Rymsza, 1999). As an alternative to woody plants, kenaf has great potential for making diverse agricultural and industrial products such as paper pulp, thermoplastic, composite, geotextile, fabrics, and industrial absorbents (Pande and Roy., 1996; Ahmed et al., 1998). Kenaf stem mainly consists of bark (35-40% of the total stem weight) and an inner core (60-65% of the total stem weight), which has become a great potential source for alternate raw materials for pulp and fiber (Kuroda et al., 2002; Lin et al., 2004). Per unit production of kenaf is higher (3-5 times) than that of pulp wood trees, and the quality of pulp is equal or superior to that of many woody plants. Additionally, making pulp using kenaf requires less energy and chemical inputs than standard wood sources (Nelson et al., 1962). The content and quality of pulp are greatly affected by plant cell wall compositions. The cell walls of higher plants are mainly composed of cellulose, hemicellulose, pectin, proteins and lignin (Popper et al., 2011). Lignin provides mechanical support to the entire plant and its content varies according to cell type. Lignin protects cell wall polysaccharides from microbial degradation; however, it is one of the major obstacles for conversion of plant biomass to pulp or biofuels (Vanholme et al., 2010; Neutelings et al., 2011). The by-products that are generated during the lignin removal step also hinder the saccharification and fermentation processes (Vanholme et al., 2008; Parawira and Tekere, 2011; Paliwal et al., 2012). The lignin removal step is also expensive and generates environmentally hazardous by-products. Therefore, wood that accumulates either less lignin and/or produces lignin that is more amendable to chemical degradation can reduce obstacles for pulp or biofuel production (Sticklen,

2008; Weng et al., 2008; Mansfield, 2009; Jung et al., 2012). Lignin is synthesized by all vascular plants and is the second most abundant aromatic cell wall polymer that results from the oxidative combinatorial coupling of three hydroxycinnamyl alcohol monomers (reviewed by Vogt, 2010). Lignin biosynthesis begins with the synthesis of cinnamic acid from phenylalanine by phenylalanine ammonia lyase (PAL). The three hydroxycinnamyl alcohols (monolignols), ρ-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), are the precursors of lignin biosynthesis. The amount of different monolignols in lignin varies according to plant species and cell type. Lignin in gymnosperms is mainly composed of G units with few H units, while lignin in angiosperms is composed of G and S units (Uzal et al., 2009). Kenaf has a high ratio of S/G (5.4), with few H units (S 83.3, G 15.4 and H 1.3%) (Gutiérrez et al., 2004). Plants require three enzymes, phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate:CoAligase (4CL), for the sequential reaction of the general phenylpropanoid pathway. As the last enzyme of the general phenylpropanoid pathway, 4CL regulates the activation of cinnamic acid and its derivatives to their corresponding thioesters. These thioesters are central intermediates for specific branched pathways that lead to the synthesis of flavonoids and lignin (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995; Douglas, 1996), which control the various physiological functions in plants and facilitate adaptation to environmental perturbations (Dixon and Paiva, 1995). Down-regulation of the *HCT* (encoding hydroxycinnamoyl transferase), *C3H* (encodingρ-coumarate 3-hydroxylase*)* or *4CL* was shown to reduce the lignin content and composition in plants (Li et al., 2008; Xu et al., 2008). Additionally, down-regulation of *4CL* in aspen (*Populus tremuloides*) was found to decrease lignin by up to 45%, increase cellulose, and increase plant growth (Hu et al., 1999). It was also reported that the amount of lignin decreased significantly in response to down-regulation of the *4CL* gene in tobacco (Kajita et al., 1996, 1997) and Arabidopsis (Lee et al., 1995; Lee et al., 1997). In this study, we investigated the expression pattern of *4CL* ortholog in kenaf during the developmental stages of various tissues and in response to diverse abiotic stresses as well as elicitors such as SA (salicylic acid), MeJA (methyl jasmonic acid), ABA (abscisic acid), NaCl, wound, cold, H_2O_2 and drought in 3-w-old stem tissues.

Result and Discussion

Cloning and sequence characterization of kenaf 4CLortholog

We cloned the full-length kenaf *4CL* ortholog (JX548316) using degenerate primers and the RACE (rapid amplification of cDNA ends) system. The cloned full-length kenaf *4CL* ortholog is 1,704-bp long with a 1,623-bp open reading frame (ORF) that encodes 540 amino acids (Fig.1). The predicted molecular weight and isoelectric point (pI) of the deduced amino acid sequence is 59.56kDa and 6.58, respectively, as calculated by the ExPASy Proteomic Server. Analysis by SingalP 4.0 revealed that the deduced amino acid sequence has no cleavage site, indicating that it is probably a non-secretory protein. According to TargetP V1.1 analysis, there is no signal peptide for sub-cellular localization in the cloned kenaf 4CL ortholog, indicating that it might be a cytoplasmic protein. A BlastP search showed that the deduced kenaf *4CL* ortholog shared 57-79% identities with other 4CL proteins from *Populus trichocarpa* (79%), *Ricinus communious* (79%), *Rubus idaeus* (78%), *Paulownia fortune* (75%),

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1      ggcagcaaaagcaATGgaggccaaccaagatgggcatgaattcatc  46
                    M  E  A  N  Q  D  G  H  E  F  I
47   tttcggtccagtctcccagacataaacatcccaaaccacctccctttg  94
     F  R  S  S  L  P  D  I  N  I  P  N  H  L  P  L
97   Catacttactgttttgagaacctttccaactttaaagatggtccttgc 142
     H  T  Y  C  F  E  N  L  S  N  F  K  D  G  P  C
143  Ttaatcaacgctcctaccgggcgggtatatacttatgcccaagtccat 190
     L  I  N  A  P  T  G  R  V  Y  T  Y  A  Q  V  H
191  Ctcacttgtcgtaaagtggccgccggtctcaacaaactgggcatccaa 238
     L  T  C  R  K  V  A  A  G  L  N  K  L  G  I  Q
239  Cagggagatgtcatcatgcttttgcttcacaactctcccgaattcgtc 286
     Q  G  D  V  I  M  L  L  L  H  N  S  P  E  F  V
287  Tttgctttccttggtgcatcgttccgtggagccatcaccactaccgcc 334
     F  A  F  L  G  A  S  F  R  G  A  I  T  T  T  A
335  Aatccctttttttacccccgccgagattgcgaaacaggcctcggcctcc 382
     N  P  F  F  T  P  A  E  I  A  K  Q  A  S  A  S
383  Aaaactaggctgtttataactcaagcagtttatgcagagaaagtgaag 430
     K  T  R  L  F  I  T  Q  A  V  Y  A  E  K  V  K
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     N  F  A  L  D  K  D  I  K  I  I  T  I  D  T  T
479  Cccgagggttgtttgcatttctcggagttgactcgggtacacgaggat 526
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     E  I  P  A  V  K  I  N  P  D  D  V  V  A  L  P
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623  Aaaagtctcgtaaccagcgtcgctcaacatgttggtggagacaatccc 670
     K  S  L  V  T  S  V  A  Q  H  V  G  G  D  N  P
671  Aacatttatttccacgagagagatgtgattctctgcttgctccctta 718
     N  I  Y  F  H  E  R  D  V  I  L  C  L  L  P  L
719  Ttccacatatactcactcaattgcatcttgctttgctctttgagagca 766
     F  H  I  Y  S  L  N  C  I  L  L  C  S  L  R  A
767  Ggggctgcaatcttgattatgcaaaagttcgagatcctccattaatg 814
     G  A  A  I  L  I  M  Q  K  F  E  I  L  P  L  M
815  Gagcttgtggagaaatacagtgtaaccatcgccccttcgtaccgcca 862
     E  L  V  E  K  Y  S  V  T  I  A  P  F  V  P  P
863  Attattttggccatcgccaagactccagacatccaaaaatacgacctt 910
     I  I  L  A  I  A  K  T  P  D  I  Q  K  Y  D  L
911  Tcgtcatccgaatggtgatttctggcgccgctccgatgggaagaag 958
     S  S  I  R  M  V  I  S  G  A  A  P  M  G  K  K
959  Ctagaggatgctgttagagacaggctcccaaacgcaaaactgggacag 1006
     L  E  D  A  V  R  D  R  L  P  N  A  K  L  G  Q
1007 ggctatgggatgaccgagacagtgctagcattgaacttagcttttgcg 1054
     G  Y  G  M  T  E  T  V  L  A  L  N  L  A  F  A
1055 aaagagccgtgggagacaaaatctggtgcatgtggcacggtcgtaagg 1102
     K  E  P  W  E  T  K  S  G  A  C  G  T  V  V  R
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     N  A  E  M  K  I  V  D  P  E  T  G  T  S  L  P
1151 cgaaatcagtcgggcgaaatttgcattcggggtagtcagatcatgaaa 1198
     R  N  Q  S  G  E  I  C  I  R  G  S  Q  I  M  K
1199 ggttatcttaatgatccggaagctacaaagagaacaatagacaaagaa 1246
     G  Y  L  N  D  P  E  A  T  K  R  T  I  D  K  E
1247 ggatggttgcacacgggcgacattgggtacattgatgaagataatgag 1294
     G  W  L  H  T  G  D  I  G  Y  I  D  E  D  N  E
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1342 caagtggcacccgcagagctggaagcaatgttgatttcccaccccaac 1390
     Q  V  A  P  A  E  L  E  A  M  L  I  S  H  P  N
1391 atctctgatgctgctgttgtacctatgaaagatgaagcagctggagag 1438
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     V  P  V  A  F  I  V  R  S  N  H  S  N  I  T  E
1487 gatgagatcaagcaattcatctcaaagcaggttgtgttttacaagagg 1534
     D  E  I  K  Q  F  I  S  K  Q  V  V  F  Y  K  R
1535 ctggcccgggttttctttgtggatacgatccctaaagctccctctggc 1582
     L  A  R  V  F  F  V  D  T  I  P  K  A  P  S  G
1583 aagattttgcggaaagatgtacgagcaaagcttgctgcacatgtaccc 1630
     K  I  L  R  K  D  V  R  A  K  L  A  A  H  V  P
1631 aacTAGcatttgtttattgtctgcggtccacagatgtgtaatagcttc 1678
     N  *
1679 tttttacgaaagatacaaatgatggg                        1704
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Fig 1. Full length coding sequence and amino acid sequence of kenaf 4CL ortholog. The start codon (ATG) and stop codon (TAG) are indicated by uppercase. The asterisk indicates the stop codon.

Melissa officinalis (73%), *Nicotiana tabacum* (73%), *Solanum tuberosum* (73%), *Coffea arabica* (72%), and *Salvia miltiorrhiza* (72%). Multiple alignment indicated that kenaf *4CL* ortholog has two conserved putative AMP (adenosine monophosphate)-binding motifs that were common to a group of adenylate-forming enzymes (Fig. 2) (Schmelz and Naismith, 2009). Among them, the first motif (the first box in Fig. 2) is SSGTTGLPKGV, which is conserved in all 4CL sequences and considered the putative AMP-binding domain (Uhlmann and Ebel, 1993; Stuible and Kombrink, 2001). The second conserved motif (the second box in Fig. 2) is GEICIRG, which has been proposed to be associated with the

stability and catalytic activity of 4CL and related enzymes (Schmelz and Naismith, 2009). The existence of the conserved motifs suggest that kenaf 4CL ortholog might be a member of the ANL superfamily of adenylating enzymes (Cao et al., 2012). Six conserved cysteine (C) residues commonly found in the 4CL sequences of all plants (Weitzel and Petersen, 2010) were also found in kenaf 4CL ortholog; however, the fourth cysteine (C, 343 amino acid residue) was substituted by asparagine (N). It is not known whether this amino acid (N) substituent in kenaf 4CL ortholog has any impact on function. A phylogenetic tree was constructed using 4CL amino acid sequences by the neighbor joining method using MEGA5 to investigate the relationship between kenaf 4CL ortholog and other plant 4CL proteins (Fig. 3). 4CL sequences of all dicot plants can be divided into two distinct phylogenetic classes, 4CL class I and class II (Cao et al., 2012). The class I 4CL is associated with lignin accumulation (Hamberger and Hahlbrock, 2004; Cao et al., 2012). In the phylogenetic tree, kenaf 4CL ortholog was clustered closely (79% identity) with ri4CL2 of *Rubus idaeus* (AF239686), which is associated with class I 4CL (Kumar and Ellis, 2003). These results indicate that kenaf 4CL ortholog might be class I 4CL that is involved in lignin biosynthesis. Therefore, kenaf *4CL* ortholog was designated as *Hc4CL*.

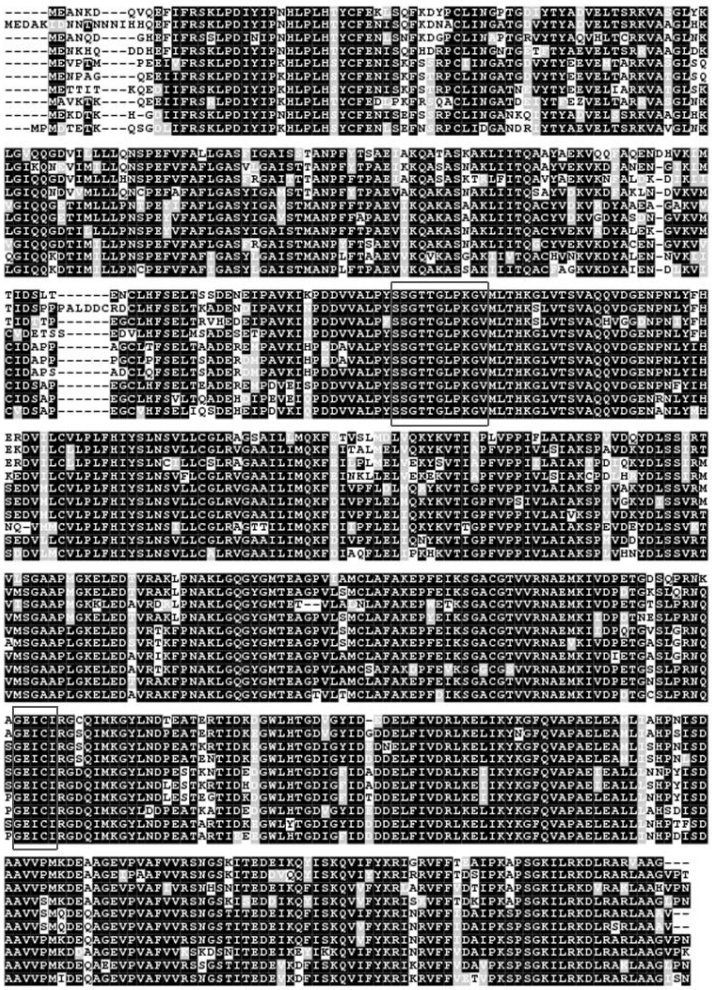

Fig 2. Multiple alignment of deduced amino acid sequence of kenaf 4CL ortholog with other plant 4CL sequences. Boxes are the two putative AMP-binding structural motifs. Sequence alignment was performed using ClustalW and the BOXHADE sequence alignment program in Biology WorkBench. The following 4CL sequences were used for alignment: 1) *Populus trichocarpa* (XP002324477), 2) *Ricinus communious* (XP002533186), 3) *Hibiscus cannabinus* (JX548316), 4) *Rubus idaeus* (AF239686), 5) *Salvia miltiorrhiza* (AAP68991), 6) *Melissa officinalis* (CBJ23825), 7) *Paulownia fortunei* (ACL31667), 8) *Coffea Arabica* (AFP49811), 9) *Nicotiana tabacum* (BAA07828), and 10) *Solanum tuberosum* (AAD40664).

Expression of Hc4CL ortholog in different tissues and organs

The expression pattern of the *Hc4CL* transcript was analyzed in different tissues and organs (root, stem, leaf, petiole and flower) by quantitative real-time PCR (QPCR). QPCR results showed that the *Hc4CL* transcript was expressed ubiquitously in all tissues and organs examined (Fig. 4A). The highest expression level of *Hc4CL* was observed in stems, followed by roots and petioles in 16-w-old kenaf plants (Fig. 4A). The results of a previous study revealed that *Ii4CL* was expressed in all tissues of *Isatis indigotica* at different levels (Di et al., 2012). It was also reported that *Ri4CL1* and *Ri4CL2* transcripts were expressed in all tissues of raspberry (*Rubus idaeus*) (Kumar and Ellis, 2003). High expression of the *Hc4CL* transcript was observed in 20-w-old keanf stem, while low expression was observed in early stage stems (2-w old) (Fig. 4B). Similar results were observed in aspen (*Populus tremuloides*) *pt4CL*, where high expression was observed in lower internodes (older tissue) and moderate expression was detected in the top internodes (Hu et al., 1998). There was no significant difference in the expression pattern of the *Hc4CL* transcript examined in different stages of leaf tissues with low expression levels (Fig. 4C). It was also reported that the *pt4CL1* gene was expressed at low levels in leaf tissues of aspen (Hu et al., 1998). The highest expression of *Hc4CL* transcript was observed in immature flower and the lowest was observed in mature flower, indicating that this pathway plays a role in flower development (Fig. 4D).

Expression of 4CL ortholog in response to diverse stresses

Plants are unprotected in nature and have to face different environmental risks such as biotic and abiotic stresses. Lignification is an important mechanism by which plants protect themselves against various stresses. Lignin deposited in secondary cell walls of a plant is an important biopolymer that protects cell wall polysaccharides from microbial degradation. 4CL is involved in lignin biosynthesis and coordinately activated in response to developmental cues as well as non-developmental signals such as wounding, irradiation with UV light, or pathogen attack (Dixon and Paiva, 1995). It has also been reported that *4CL* was induced in parsley, potato, soybean and Arabidopsis in response to different biotic or abiotic stresses such as pathogens, wounding, UV-B and plant hormones (Schmelzer et al., 1989; Becker-André et al., 1991; Lindermayr et al., 2002). Stresses also induce the production of plant hormones such as ABA, ethylene, JA and SA, which can control gene expression (Mahajan and Tuteja, 2005; Shao et al., 2007). We reported that genes involved in lignin biosynthesis were controlled by diverse abiotic stresses and plant hormones in kenaf (Choi et al., 2012; Chowdhury et al., 2012; Kim et al., 2013). However, this is the first report that showed *Hc4CL* transcript was induced in response to diverse abiotic stresses and plant hormones in kenaf. In this experiment, we used

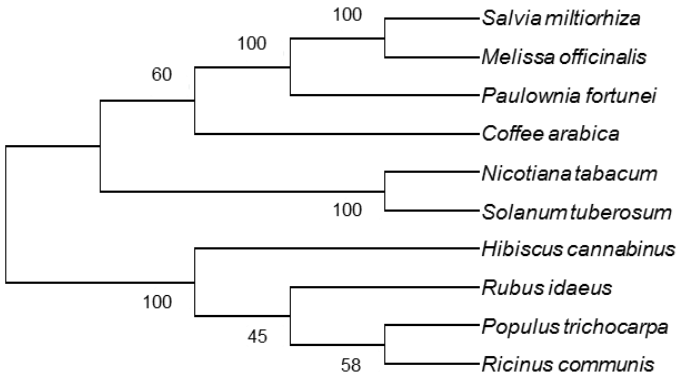

Fig 3. Phylogenetic analysis of kenaf 4CLwith other plant 4CL sequences. The phylogenetic tree was generated using Mega5. Node numbers indicate bootstrap values from 1,000 replications. The following 4CL sequences were used: *Populus trichocarpa* (XP002324477), *Ricinus communious* (XP002533186), *Hibiscus cannabinus* (JX548316), *Rubus idaeus* (AF239686), *Salvia miltiorrhiza* (AAP68991), *Melissa officinalis* (CBJ23825), *Paulownia fortune* (ACL31667), *Coffea arabica* (AFP49811), *Nicotiana tabacum* (BAA07828), and *Solanum tuberosum* (AAD40664.1).

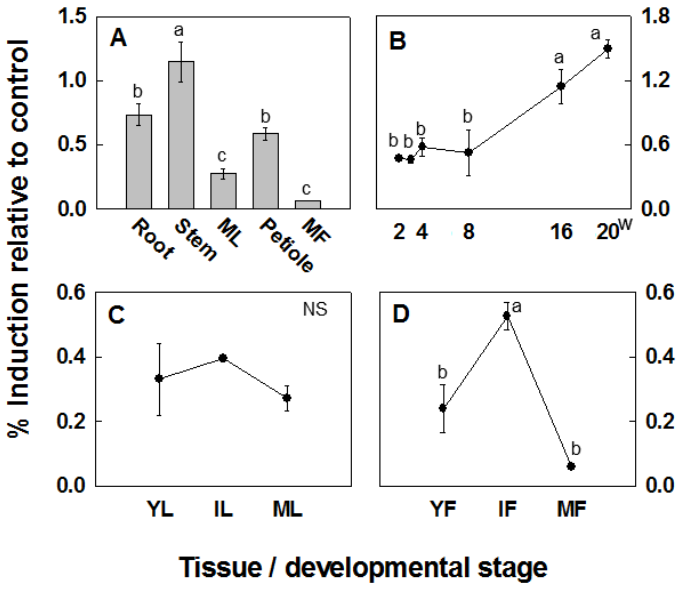

Fig 4. Transcript expression patterns of kenaf *4CL* ortholog in diverse tissues and organs during developmental stages. The relative transcript levels were measured using QPCR. *ACTIN* was used as an internal control. Transcript levels of kenaf *4CL* ortholog were adjusted by deduction of the control transcript level. A, B, C and D are the expression patterns of kenaf *4CL* ortholog in various tissues from 16-w-old kenaf plants during stem development (2, 3, 4, 8, 16, 20 weeks after sowing), leaf development (YU- young leaf, IL- immature leaf, ML- mature leaf), flower development (YF- young flower, IF- immature flower, MF- mature flower), and in various tissues from 16-w-old kenaf plants, respectively. The vertical bars represent the mean ± standard error of three biological replications. Different letters above each point indicate significant differences ($P \leq 0.05$) between the mean values, which were determined based on Duncan's multiple range test. NS, not significant.

QPCR to examine expression of the *Hc4CL* gene in stem tissues of 3-w-old kenaf in response to various stresses such as SA, MeJA, ABA, NaCl, wounding, cold, H_2O_2 and drought. *Hc4CL*was expressed differentially in stem tissues in response to all the treatments (Fig. 5), with the highest expression being observed at 24 h after treatment with ABA, followed by NaCl and cold, whereas down-regulation was observed in response to treatment with SA and MeJA. *Hc4CL* showed the highest expression levels at 24 h following treatments with ABA, NaCl and H_2O_2, while the greatest accumulation of *Hc4CL* transcripts was observed at the earliest time point (6 h) in cases of cold treatment. In cases of drought stress, levels of the *Hc4CL* transcript were high at 7 d after treatment and then decreased.

SA and MeJA

SA and MeJA function as elicitors that activate defense mechanisms such as cell wall strengthening (Desender et al., 2007). Plant hormones like SA, JA and ethylene share signaling pathways with wounding and pathogen responses (Maleck and Dietrich, 1999). The potential role of JA or its methyl ester jasmonates (MeJA) is formation of intracellular signaling molecules that help activate gene expression in response to wounding, elicitors and pathogen infection (Sembdner and Parthier, 1993; Farmer, 1994; Reinbothe et al., 1994; Creelman and Mullet, 1995). In previous studies, genes involved in lignin biosynthesis were responsive to SA and MeJA treatments (Choi et al., 2012; Chowdhury et al., 2012; Kim et al., 2013). In the present study, *Hc4CL* was down-regulated by SA and MeJA treatments when compared to the control level. The opposite result was observed following exogenous application of MeJA and JA, which resulted in *4CL* genes being induced in parsley and *Isatis indigotica* (Mary and Douglas, 1996; Di et al., 2012). The opposite expression pattern might be due to different classes of *4CL* cloned in kenaf, which is not positively responsive to SA and MeJA. In plants, there are multiple *4CL* genes (Guillaumie et al., 2007; Saballos et al., 2012); therefore, further study is required to determine if this is the case in kenaf.

ABA

ABA is involved in the response to diverse stresses such as drought and salt (Zhu, 2002; Tuteja, 2007). The activities of different lignin biosynthesis enzymes including PAL, 4CL and POD are correlated with plant hormones and are highly dependent on the ratio of ABA/GA_3 (Luo Zi-sheng, 2006). ABA treatment reduced the *Hc4CL* transcript slightly at 1 h when compared to the control, while it reached its maximum at 24 h. The lignin content in tobacco callus decreased due to ABA treatment with low concentration (Li et al., 2006). *Ii4CL* of *Isatis tinctoria* was expressed at high levels for up to 12 h in response to ABA treatment, which was similar to the results of the present study (Di et al., 2012). Similar expression patterns like a reduction in early time point and subsequent induction at later time points were observed in other kenaf genes involved in lignin synthesis (*PAL*, *CCoAOMT*, *C3H*, *HCT* and *F5H*) in reponse to ABA (Choi et al., 2012; Chowdhury et al., 2012; Kim et al., 2013). Taken together, these results indicate that ABA can regulate expression of the *Hc4CL* gene in kenaf.

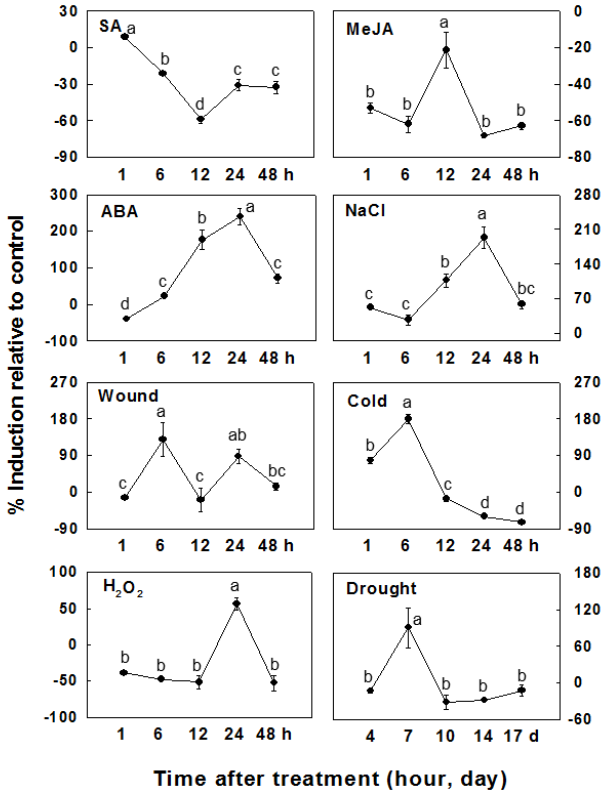

Fig 5. Transcript expression patterns of kenaf 4CL ortholog under different abiotic stress conditions. The abiotic stresses were as follows: SA, MeJA, ABA, NaCl, wounding, cold, H_2O_2 and drought. The vertical bars represent the mean ± standard error of three biological replications. Different letters above each point indicate significant differences ($P \leq 0.05$) between the mean values, which were determined based on Duncan's multiple range test. NS, not significant.

NaCl

Agricultural systems are greatly affected by salinity. To overcome this problem, plants undergo cell wall modifications such as lignification (Neves et al., 2010). For example, the lignin content was increased by about 72-90% in soybeen root due to NaCl. In this study, we found that *Hc4CL* was up-regulated by NaCl treatment, reaching its maximum level at 24 h after treatment. Previous studies indicated that different lignin biosynthesis genes such as *PAL*, *CCoAOMT, C3H, HCT* and *F5H* of kenaf (Choi et al., 2012; Chowdhury et al., 2012; Kim et al., 2013) were up-regulated in response to NaCl treatment. H_2O_2 also accumulated in response to NaCl treatment (Kovácik et al., 2009), and similar expression paterns of *Hc4CL* were observed by H_2O_2 treatment.

Wounding

Lignification occurs at injury or invasion sites in response to wounding and pathogenic infection (Chen et al., 2000; Raes et al., 2003). Wounding helps to initiate profound changes in plant transcriptomes (Soltani et al., 2006). Several genes and enzymes involved in lignin biosynthesis have been found to be induced by wounding and microbial elicitors (Lee et al. 1995; Bell-Lelong et al. 1997; Mizutani et al. 1997; Meyer et al. 1998; Moura et al., 2010). Moreover, *Hc4CL* showed biphasic expression in response to wounding. Specifically, the transcript was highly expressed at 6 h and then decreased sharply at 12 h with a second induction at 24 h. Similar results were obtained for *At4CL1* and *At4CL2* of Arabidopsis in response to wounding (Soltani et al., 2006). Additionally, wounding increased *Cs4CL* transcript from tea at 12 h after treatment, after which it decreased to the same levels as the controls after 48 h (Rani et al., 2009). Genes involved in the phenylpropanoid pathway of parsley and Jerusalem artichoke (*Helianthus tuberosus*) tubers showed biphasic expression by wounding (Logemann et al., 1995; Batard et al., 2000). Taken together, these results suggest the existence of multiple signaling routes for transcriptional activation of phenylpropanoid genes (Batard et al., 2000).

Cold

Exposure to cold triggers regulated cascades of cold-acclimation processes (Christie et al., 1994). Genes involved in biochemical and physiological activities are expressed during cold acclimatization (Guy, 1990; Thomashow, 1999; Kaplan et al., 2004). *Hc4CL* was accumulated at the maximum level 6 h after treatment and then decreased. In previous studies, genes involved in lignin biosynthesis such as *PAL, CCoAOMT, C3H, HCT, COMT1* and *F5H* were found to be induced by cold in kenaf (Choi et al., 2012; Chowdhury et al., 2012; Kim et al., 2013). These results indicate that cold treatment facilitates *Hc4CL* gene expression and might cause lignification in kenaf stem tissues.

H_2O_2

Hydrogen peroxide (H_2O_2) performs a dual role as a toxic by-product of normal cell metabolism and as a regulatory molecule in stress perception and signal transduction in plants (Vanderauwera et al., 2005). H_2O_2 diffuses to the cytosol from peroxisomes and contributes to interorganellar communication within a cell, leading to nuclear gene activation (Corpas et al., 2001). H_2O_2 functions as a developmental signal in secondary cell wall deposition and promotes secondary wall formation in young fiber of cotton (Potikha et al., 1999). Our results showed that *Hc4CL* gene was down-regulated at early time points and then up-regulated 24 h after H_2O_2 treatment, after which the transcript level returned to a negative level relative to the control. Similar expression patterns were observed in previous studies. Genes involved in lignin synthesis of kenaf stem tissues (*PAL, CCoAOMT, C3H* and *HCT*) were slightly down-regulated at 1 h and then reached amaximum level 6-12 h after treatment (Choi et al., 2012; Chowdhury et al., 2012).

Drought

The relationship between drought and lignin biosynthesis as well as their gene expression has not yet been thoroughly characterized. In this study, *Hc4CL* transcript was up-regulated at early time points after water withholding, and then decreased at later time points. Similar expression patterns were observed in *HcCCoAOMT* and *HcHCT* in kenaf stem tissues after drought stress (Chowdhury et al., 2012). The changes in lignin content and gene expression may be dependent on tissues and the time of exposure to drought (Fan et al. 2006; Lee et al., 2007; Vincent et al. 2005; Yang et al. 2006; Yoshimura et al. 2008). Additionally, drought may cause reduced growth with lignin deposition and

increased expression of lignin genes such as *PAL*, *C3H*, *4CL*, *CCoAOMT*, *COMT*, *CAD* and *POD*. Increased lignin deposition stiffens cell wall extensibility, decreases cell wall expansion and reduces growth, which might be beneficial for water availability (Fan et al., 2006).

In conclusion, we cloned the full length *4CL* gene from *Hibiscus cannabinus*. *Hc4CL* was expressed differentially in all tissues during the developmental stages of kenaf, and showed differential responses to various abiotic stresses applied. Further investigation is needed to investigate the presence of other copies of the *4CL* gene in kenaf.

Materials and Methods

Plant materials and abiotic stress treatments

Kenaf seeds (*Hibiscus cannabinus* L., C-9, Gene Bank of Korea Rural Development Administration IT No. 202789) were sown in pots and seedlings were grown for up to 20 weeks as previously described (Chowdhury et al., 2012). For tissue specific expression, various tissue samples from roots, stems, petioles, leaves and flowers were collected from 16-w-old kenaf plants. Different abiotic stresses such as SA, methyl jasmonate (MeJA), abscisic acid (ABA), NaCl, wounding, cold, H_2O_2 and drought were imposed on 3-w-old kenaf seedlings, and stem tissues were used for transcriptional analysis as previously described (Chowdhury et al.,2012). For SA, ABA, NaCl and H_2O_2 treatments, plants were watered with SA (5 mM), ABA (100 µM), NaCl (200 mM) and H_2O_2 (10 mM). Kenaf seedlings that were watered with distilled water were used as control samples. For MeJA treatment, seedlings were sprayed with 100 µM MeJA that was dissolved in 0.004% ethanol. The treated seedlings were covered with a plastic bag. For control samples, plants sprayed with 0.004% ethanol without MeJA. For wounding treatment, stem tissues were cut twice longitudinally using clean scissors. For cold treatment, plants were exposed to 10 °C (16-h light /8-h dark and 100 µmol m^{-2} s^{-1} light intensity). Stem tissues were harvested 1, 6, 12, 24, 48 h after treatments (SA, MeJA, ABA, NaCl and H_2O_2). For drought treatment, watering was stopped for 4,7,10, and 14 days. For control samples, plants were watered once in every 3 days. The harvested stems were frozen in liquid N_2 and stored at -80°C.

RNA isolation and cloning

Total RNA was extracted from various kenaf tissues as described previously (Choi et al., 2012). Degenerate primers for *4CL* [4CL-F, 5'-ATGAC(A/G/T)GA(G/A)GC(A/T)GGACCAGT-3'and 4CL-R, 5'-AC(A/C)GG(G/A)ACTTC(C/T/A)CCAGC-3'] were used to synthesize the cDNA of transcriptionally active *4CL* ortholog based on the consensus sequences of *4CL* genes of *Gossypium hirsutum* (EV496462), *Gossypium raimondii* (CO122311), *Raphanus sativus* (EY949516), *Raphanus raphanistrum* (FD971519), *Arabidopsis thaliana* (NM105180), and *Lithospermum erythrorhizon* (D49367). Both 5' and 3' RACE (rapid amplification of cDNA ends) systems were applied to clone a full length *4CL* ortholog (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time PCR (QPCR) analysis

QPCR was performed using the Mx3000P QPCR system (Agilent, Santa Clara, CA, USA) with SYBR Green Master Mix (LPS Solution, Daejeon, Korea) as previously described (Bae et al., 2008). QPCR primers were designed using the Primer 3 software of Biology Workbench (http://workbench.sdsc.edu/). The forward and reverse primers of *4CL* ortholog were 5'-AACATCACTGAGGAT-GAGATCAAG-3' and 5'-AGAAGCTATTACACATCTG-TGGACC-3', respectively. A housekeeping gene *(ACTIN*, DQ866836) was used as an expression control (forward primer, 5'-ATGGACAAGTCATTACTATTGGAGC-3'; reverse primer, 5'-AGTGATTTCCTTGCTCATACGGT-3').

Data analyses

cDNA and protein sequences were analyzed using NCBI Blast (http://blast. ncbi.nlm.nih.gov/), Biology WorkBench (http://workbench.sdsc.edu/), ExPASy Proteomics Server (http://expasy.org/tools/pi_tool.html),SignalP4.0 (http://www.cbs.dtu.dk/services/SignalP/) and TargetP V1.1 (http://www.cbs.dtu.dk/services/TargetP/). The neighbor joining method in Mega5 (http://www.megasoftware.net/) was used to construct the phylogenetic tree. Statistical analyses were conducted using SASS (SASS Inc., Cary, NC, USA). The statistical significance was determined based on Duncan's multiple range test at a significance of $P \leq 0.05$ using MSTATC program.

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