Plant Omics Journal POJ

POJ 6(1):65-72 (2013) ISSN:1836-3644

Expression analysis of kenaf cinnamate 4-hydroxylase (C4H) ortholog during developmental and stress responses

Jonggeun Kim[1], Bosung Choi[1], Savithiry Natarajan [2,]*, Hanhong Bae[1,]*

[1]School of Biotechnology, Yeungnam University, Gyeongsan 712-749, Republic of Korea
[2]US Department of Agriculture, Agricultural Research Service, Soybean Genomics and Improvement Laboratory, 10300, Baltimore Avenue, Beltsville, MD 20705, USA

***Corresponding author: savi.natarajan@ars.usda.gov, hanhongbae@ynu.ac.kr**

Abstract

This study was conducted to clone and analyze the expression pattern of a *C4H* gene encoding cinnamate 4-hydroxylase from kenaf (*Hibiscus cannabinus* L.). The full-length of the *C4H* ortholog was cloned using degenerate primers and the RACE (rapid amplification of cDNA ends) method. The full-length *C4H* ortholog contained a 1,518-bp open reading frame (ORF) encoding 505 amino acids. The deduced amino acid sequence showed that kenaf C4H had the highest similarity (95%) with that of *Gossypium arboretum*. We examined the expression patterns of the *C4H* ortholog in diverse tissues and at different developmental stages, as well as in response to abiotic stress conditions such as wounding, NaCl, cold, H_2O_2, ABA (abscisic acid) and SA (salicylic acid). Three-week-old stem tissues were used to examine the effects of abiotic stresses on *C4H* ortholog expression. While the highest transcript level of *C4H* ortholog was observed at an early stage in both stems and leaves, the transcripts were most abundant in the late stage flowers. In cases of wounding and SA, early induction of the *C4H* ortholog was observed. Conversely, H_2O_2 led to intermediate induction, while cold and ABA led to late induction. NaCl treatment showed different expression patterns such as complex biphasic expression. In summary, the *C4H* ortholog was expressed in all tissues and organs, as well as in response to various treatments.

Keywords: Kenaf (Hibiscus cannabinus); phenylpropanoid pathway; C4H (cinnamate 4-hydroxylase); lignification; abiotic stresses.
Abbreviations: C4H-cinnamate 4-hydroxylase; Hc-*Hibiscus cannabinus*; quantitative real-time PCR-QPCR.

Introduction

The excessive utilization has accelerated the exhaustion of energy resources during the global industrialization of the past century. Therefore, exploitation of alternative raw materials for bio-energy has gained increased attention. Kenaf (*Hibiscus cannabinus* L.) is an annual dicotyledonous plant that grows in tropical habitats and is thought to be native to Africa (Dempsey et al., 1975). Kenaf is an industrial plant used for pulp and paper production because its core and bark produce fibers (Pande et al., 1996; Ahmed et al., 1998). Due to its effective adaptability from temperate to tropical climates and its high growth rate, kenaf has great potential for biomass production (Francois et al., 1992; Lam et al., 2002; Araki et al., 2005).

Lignin is one of the main components of plant cell walls, but is recognized as a negative component in paper production, forage quality and cellulosic biofuel production (Boerjan et al., 2003; Vanholme et al., 2008). Vascular plant cell walls consist of cellulose, hemicellulose, pectin and lignin, which give the cell walls their structural integrity (Lee et al., 2011). Lignin plays an important role in defense mechanisms in plants against pathogens (Boerjan et al., 2003; Neutelings et al., 2011). Despite the potential to generate biofuels from plant biomass, there are major technical problems with this process, such as the need to reduce the interactions between lignin and the polysaccharides of plant cell walls to enable successful biological conversion (Vanholme et al., 2008). Lignin is an aromatic heteropolymer commonly found in angiosperms that is primarily derived from three hydroxycinnamyl alcohol monomers, *p*-coumaryl, coniferyl and sinapyl alcohols, which are incorporated into the lignin polymer during the polymerization process to form ρ-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively (Baucher et al., 2003; Boerjan et al., 2003). These monolignols are synthesized from phenylalanine ammonia-lyase (PAL) through a process known as the phenylpropanoid pathway, in which cinnamic acid is initially formed by deamination of phenylalanine followed by a series of ring hydroxylations, *O*-methylations and side-chain modifications (Hisano et al., 2009). The composition and amount of the three monolignols depend heavily on their degree of methoxylation. Lignin polymers found in dicotyledonous angiosperms are primarily composed of G, S and a small amount of H units. Conversely, lignin found in gymnosperms generally lacks S units and is composed of H units. However, lignin in monocotyledonous plants contains similar amounts of G and S units, but larger amounts of H units than that produced by dicotyledonous plants (Boerjan et al., 2003). Lignin containing high numbers of G units has more resistant linkages than lignin composed primarily of S units due to the number of carbon-carbon bonds (Del Río et al., 2004).

In kenaf, bast fibers contain less than 11% lignin and a high amount of cellulose, while the lignin has a high S/G ratio (5.4) and small amounts of H units (S 83.3%; G 15.4%; H 1.3%) (Gutiérrez et al., 2004). Additionally, the lignin content of kenaf is higher than that of other non-woody plants (Van Dam et al., 1994; Gutiérrez et al., 2004; Marques el al., 2010). Nevertheless, kenaf fiber is useful for delignification during the pulping process because the S units are relatively unbranched and have a lower degree of condensation than the G units (Adler et al., 1977; Nimz et al., 1974). Down-regulation of genes involved in the

early stages of the monolignol biosynthetic pathway such as *PAL*, *C4H* (cinnamate 4-hydroxylase), *HCT* (hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase) and *C3H* (ρ-coumarate 3-hydroxylase*)* is usually the most effective method of reducing lignin contents. C4H is a member of the CYP73 family of cytochrome P450 (CYP) enzymes (Teutsch et al., 1993) that controls the synthesis of *ρ*-coumaric acid (4-hydroxy *trans*-cinnamic acid) from *trans*-cinnamic acid. C4H enzymes in maize, alfalfa, periwinkle and aspen are encoded by a small gene family (Fahrendorf et al., 1993; Hotze et al., 1995; Potter et al., 1995; Kawai et al., 1996). However, arabidopsis, pea and parsley have only one gene encoding the C4H enzyme (Frank et al., 1996; Bell-Lelong et al., 1997; Koopmann et al., 1999). Down-regulation of *C4H* in alfalfa causes a decrease in total lignin contents with a reduced S/G ratio, which increases the digestibility of lignin and forage (Li et al., 2008).

Here, we cloned and characterized *C4H* ortholog in kenaf to examine the expression pattern of *C4H* ortholog in different tissues and organs during the developmental process and in response to various abiotic stresses and elicitors. To accomplish this, three-week-old stem tissues were subjected to wounding, SA, NaCl, cold, H_2O_2 and ABA.

Results

Cloning of a full-length C4H ortholog in kenaf

Degenerate primers and RACE (rapid amplification of cDNA ends) were used to clone the full-length *C4H* ortholog of kenaf. Kenaf *C4H* ortholog (GenBank Accession No. JX524279) consists of a 1,518-bp open reading frame (ORF) that encodes 505 amino acids (Fig. 1). The predicted molecular weight of the deduced protein was 58.25 kDa, with an isoelectric point (pI) of 9.12, as calculated by the ExPASy Proteomics Server. BlastP analysis showed high sequence similarity with other C4H enzymes (Fig. 2). Specifically, the deduced kenaf *C4H* ortholog shared 95, 90, 90, 90 and 88% similarities with *C4H* from *Gossypium arboretum*, *Canarium album*, *Petunia* x *hybrid*, *Salvia miltiorrhiza* and *Rubus occidentalis*, respectively (Fig. 2 and 3). SignalP 4.0 analysis showed that the C4H ortholog may not have a signal peptide. TargetP V1.1 analysis also indicated the absence of a signal peptide for subcellular localization. These results strongly indicate that kenaf C4H ortholog is a cytoplasmic protein. MotifFinder analysis suggested the existence of a cytochrome P450 cysteine heme-iron ligand signature (FGVGRRSCPG, residues 440-449) in kenaf C4H ortholog (FGVGRRSCPG, amino acid position 440-449; Fig. 1 and 2), which matched the consensus sequence well [FW]-[SGNH]-x-[GD]-x-[RKHPT]-x-C-[LIVMFAP]-[GAD] (Lee et al., 2008). A phylogenetic tree was constructed from the C4H amino acid sequences of 13 plant species using Mega 5. Kenaf C4H ortholog showed the closest relationship to *Gossypium arboreum* (AAG10197; Fig. 3). Kenaf C4H ortholog was grouped into a sub-cluster of five proteins, *G. arboreum*, *C. album*, *Capsicum annuum* and *Leucaena leucocephala*. These results suggest that kenaf C4H ortholog belongs to a C4H enzyme; hence, kenaf *C4H* ortholog was designated as *HcC4H*.

HcC4H expression in diverse tissues and organs

Expression of *HcC4H* in diverse tissues and organs was analyzed by QPCR. The transcript level was examined in the roots, stems, leaves, petioles and flowers. *HcC4H* transcripts were detected in all tissues and organs tested (Fig. 4). *HcC4H* transcripts were highly accumulated in young stems (4-week-old), mature flowers and young leaves. During the stem developmental stage, *HcC4H* transcript increased significantly at four weeks, then decreased dramatically from eight weeks. During flower development, the highest level of *HcC4H* transcript was detected in mature flowers, while the lowest level was observed in immature flowers. Additionally, the transcript level decreased significantly during leaf development. During flower development, young leaves showed the highest transcript level of *HcC4H*. Among various tissues of 16-week-old kenaf plants, the highest expression level was observed in mature flower tissues.

```
aacttcaccgcc atg gaccttctccttctcgagaaagttctcatctccctcttcttcacc 61
            M  D  L  L  L  L  E  K  V  L  I  S  L  F  F  T
atcatcttcgccatcctagtcgccaaactccgcggcaagcgttacaagctacccccgggc 121
  I  I  F  A  I  L  V  A  K  L  R  G  K  R  Y  K  L  P  P  G
cctctccccgtccccatcttcggcaactggctccaagtcggcgatgacttgaaccaccgc 181
  P  L  P  V  P  I  F  G  N  W  L  Q  V  G  D  D  L  N  H  R
aacctcactgacttgaccaagaagttcggcgacatcttcttgctccgcatgggacaacgt 241
  N  L  T  D  L  T  K  K  F  G  D  I  F  L  L  R  M  G  Q  R
aacctcgtcgtcgtctcttccccggagctggccaaggaagtcctgcacacccaaggtgtc 301
  N  L  V  V  V  S  S  P  E  L  A  K  E  V  L  H  T  Q  G  V
gaattcggttccaggacgaggaacgtcgtctttgacatcttcaccggcaagggacaggac 361
  E  F  G  S  R  T  R  N  V  V  F  D  I  F  T  G  K  G  Q  D
atggtcttcaccgtctacggtgagcactggcgcaagatgaggaggatcatgaccgttcct 421
  M  V  F  T  V  Y  G  E  H  W  R  K  M  R  R  I  M  T  V  P
ttcttcaccaacaaggtcgtccaacagtaccgacacggatgggaggccgaggctgccagt 481
  F  F  T  N  K  V  V  Q  Q  Y  R  H  G  W  E  A  E  A  A  S
gtcgtcgaagatgtaagaaagaacccggaggcggcgaccaatgggattgttctgaggaga 541
  V  V  E  D  V  R  K  N  P  E  A  A  T  N  G  I  V  L  R  R
cgattacagcttatgatgtacaacaacatgtacaggatcatgttcgacaggagattcgag 601
  R  L  Q  L  M  M  Y  N  N  M  Y  R  I  M  F  D  R  R  F  E
agcgaagacgacccttgtttgtaaagctcaaggccttgaatggagagaggagtagattg 661
  S  E  D  D  P  L  F  V  K  L  K  A  L  N  G  E  R  S  R  L
gcacagagcttcgagtacaactatggcgatttcatccccattttgaggcctttcctgaga 721
  A  Q  S  F  E  Y  N  Y  G  D  F  I  P  I  L  R  P  F  L  R
ggatacttgaaattgtgcaaggaagtgaaggagatgagattgcagctttcaaggactat 781
  G  Y  L  K  L  C  K  E  V  K  E  M  R  L  Q  L  F  K  D  Y
ttcctcgaggaaaggaagaagcttgcaagcacaacgagaagcgacaacaatgctcttaaa 841
  F  L  E  E  R  K  K  L  A  S  T  T  R  S  D  N  N  A  L  K
tgtgccatcgatcatattcttgatgctcagcagaaaggagagatcaatgaagacaatgtt 901
  C  A  I  D  H  I  L  D  A  Q  Q  K  G  E  I  N  E  D  N  V
ctttacattgttgagaacattaatgttgctgccattgaaacaaccttatggtcaattgaa 961
  L  Y  I  V  E  N  I  N  V  A  A  I  E  T  T  L  W  S  I  E
tggggcattgctgagcttgttaaccatccccggatccagcagaagctccgcgatgagatc 1021
  W  G  I  A  E  L  V  N  H  P  R  I  Q  Q  K  L  R  D  E  I
gacaccgtactccgacccggtgtgcaggttaccgaacccgacacacataagcttccctat 1081
  D  T  V  L  G  P  G  V  Q  V  T  E  P  D  T  H  K  L  P  Y
ctgcaggcagtggtcaaggagacactcaggctccgaatggccatccctctattggttcct 1141
  L  Q  A  V  V  K  E  T  L  R  L  R  M  A  I  P  L  L  V  P
cacatgaacctccacgatgcgaagctcgcagggtacgacatccccgcggagagcaagatc 1201
  H  M  N  L  H  D  A  K  L  A  G  Y  D  I  P  A  E  S  K  I
ctcgtgaacgcgtggtggttagccaataacccggcacactggaagaacccggaagagttc 1261
  L  V  N  A  W  W  L  A  N  N  P  A  H  W  K  N  P  E  E  F
aggcccgagaggttcttcgaggaggaatcgaaggtcgaagcgaatgggaatgacttcagg 1321
  R  P  E  R  F  F  E  E  E  S  K  V  E  A  N  G  N  D  F  R
tatcttccgttcggtgtcggaaggaggagctgcccggggatcattcttgcgctgccgatc 1381
  Y  L  P  F  G  V  G  R  R  S  C  P  G  I  I  L  A  L  P  I
ctagggatcacgttgggacgaatggtgcagaacttcgagctgttgcctcctaacggacag 1441
  L  G  I  T  L  G  R  M  V  Q  N  F  E  L  L  P  P  N  G  Q
tctaagatcgatacgacggagaaaggaggacagttcagcttgcacattctgaagcattca 1501
  S  K  I  D  T  T  E  K  G  G  Q  F  S  L  H  I  L  K  H  S
actattgtcgcaaagccacgggtgttt tga atcaacgaaatttccttggaaaatctgggtt 1561
  T  I  V  A  K  P  R  V  F  *
tgatgaacttgaatattgctgtcctgg 1588
```

Fig 1. The full-length cDNA and deduced amino acid sequence of *C4H* ortholog from kenaf. The start codon (ATG) and stop codon (TAA) are underlined and in bold. The heme-binding sequence (FGVGRRSCPG) is boxed.

HcC4H expression in stem tissues in response to various abiotic stresses

HcC4H expression patterns were analyzed using 3-week-old kenaf stem tissues after exposure to various abiotic stresses such as wounding, SA, NaCl, cold, H_2O_2 and ABA (Fig. 5). All treatments induced the expression of *HcC4H*. Among treatments, SA showed the highest induction of *HcC4H* at 6 h after treatment, with a sharp decrease being observed thereafter. In the wound and H_2O_2 treatments, *HcC4H* transcript reached its

maximum level at 6 h and 12 h after treatment, respectively, and then gradually decreased. Conversely, in the cold and ABA treatments, the *HcC4H* transcript level was highest during later time points (48 h for cold and 24 h for ABA). The expression pattern of *HcC4H* subjected to NaCl treatment showed the lowest induction among treatments, which differed greatly from that of the other treatments. In the NaCl treatment, induction was observed for up to 12 h after treatment, followed by a decrease at 24 h after treatment, and a subsequent increase at 48 h after treatment.

Discussion

Sequence characteristics of HcC4H

C4H is a CYP-dependent monooxygenase that converts cinnamic acid to ρ-coumaric acid (Teutsch et al., 1993). Similar to the other two hydroxylases in the phenylpropanoid pathway (C3H and F5H), C4H catalyzes the first step of oxygenation in the general phenylpropanoid metabolism in higher plants (Teutsch et al., 1993). The amino acid sequence of the HcC4H ortholog shares conserved domains with other C4H amino acid sequences (Fig. 2 and 3). HcC4H contains the CYP cysteine heme-iron ligand signature (FGVGRRSCPG), which is highly homologous with the heme-binding domain that is well conserved in the CYPs of higher plants (Lee et al., 2008). In a previous study, alignment of seven proteins of the CYP73 gene family showed similar results (Gravot et al., 2004). While 3 *C3H* genes exist, only one *C4H* gene has been reported in Arabidopsis (Bell-Lelong et al., 1997). However, multiple family members have been reported in other plants (Betz et al., 2001). Specifically, there are two classes of *C4H* genes present in plants (Nedelkina et al., 1999; Betz et al., 2001). These two classes might have originated before the divergence of angiosperms and gymnosperms, which suggests that members of class II was present in the previous period of evolution of plant lineages (Raes et al., 2003). While no signal peptide was predicted in HcC4H, a previous study predicted an endoplasmic reticulum (ER)-targeting peptide in C4H based on TargetP analysis (Emanuelsson et al., 2000). HcC4H also contains a conserved hinge region consisting of (P/I)PGPx(G/P)xP (Chapple et al., 1998). The HcC4H amino acid sequence showed 95% and 89% similarities with C4H sequences of *Gossypium arboreum* and *Capsicum annuum*, respectively, and belongs to class I, while Arabidopsis C4H belongs to class II. HcC4H showed low homology with Arabidopsis C4H (86%) when compared to other species. The phylogenetic tree also showed that HcC4H was more closely related to *G. arboreum* and *C. annuum* than Arabidopsis (Fig. 3). Taken together, these results suggest that *HcC4H* belongs to class I.

Expression of HcC4H ortholog

HcC4H transcripts were detected in all tissues and organs examined (Fig. 4), which suggests the importance of C4H as a second enzyme in the phenylpropanoid pathway. C4H enzyme regulates the carbon flux of various phytoalexins in response to pathogens and is involved in flavonoid biosynthesis as well as that of other important secondary metabolites (Teutsch et al., 1993; Singh et al., 2009). C4H regulates biosynthesis and flux control in the *Scutellaria baicalensis* flavone pathway (Xu et al., 2010). High levels of *HcC4H* transcripts were detected in roots and flowers, especially in mature flowers. While the transcript level was highest in mature flowers, the expression level was lowest in stem tissues, which is similar to the results of previous studies. The highest level of *Angelica gigas C4H* transcripts was detected in roots and mature flowers, while the lowest level

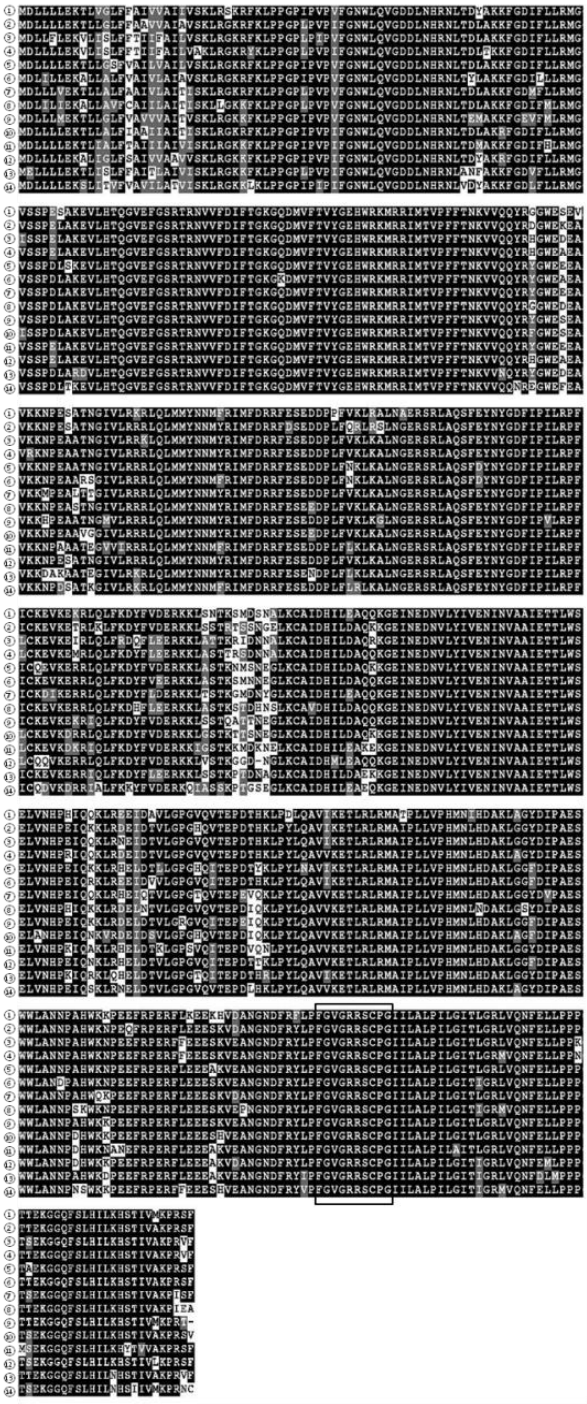

Fig 2. Multiple alignment of the deduced C4H amino acid sequences. The alignment was conducted using the ClustalW and BOXSHADE sequence alignment programs in Biology WorkBench. Identical amino acids are shaded dark and the heme-binding ligand (FGVGRRSCPG) is boxed. The C4H sequences used were as follows: (1) *Capsicum annuum* (AAG43824), (2) *Leucaena leucocephala* (AEM63594), (3) *Gossypium arboreum* (AAG10197), (4) *Hibiscus cannabinus* (JX524279), (5) *Populus trichocarpa* x *Populus deltoides* (AAG50231), (6) *Canarium album* (ACR10242), (7) *Camptotheca acuminata* (AAT39513), (8) *Parthenocissus henryana* (ABA59555), (9) *Rubus occidentalis* (ACM17896), (10) *Astragalus mongholicus* (AEH68208), (11) *Gynura bicolor* (BAJ17666), (12) *Salvia miltiorrhiza* ABC75596), (13) *Allium cepa* (AAS48416), (14) *Arabidopsis thaliana* (CAP08828).

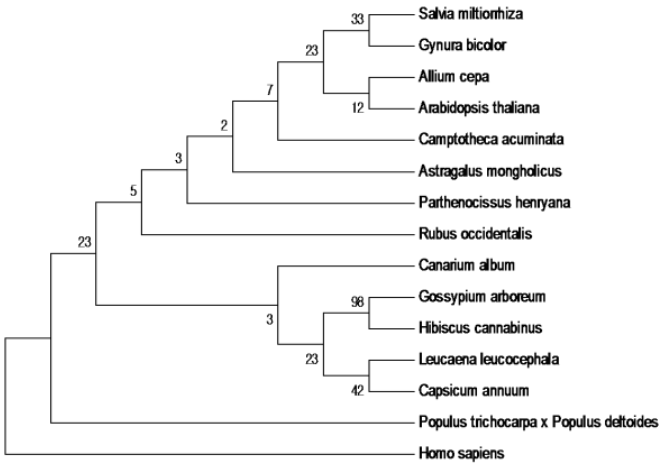

Fig 3. Neighbor-joining tree of C4H sequences. The phylogenetic tree was constructed by the neighbor-joining method using Mega5. The numbers at the nodes indicate boostrap values from 1,000 replications and weighted amino acid substitutions. The plant species and GenBank accession numbers of C4H sequences were as follows: *Salvia miltiorrhiza* (ABC75596), *Gynura bicolor* (BAJ17666), *Allium cepa* (AAS48416), *Arabidopsis thaliana* (CAP08828), *Camptotheca acuminata* (AAT39513), *Astragalus mongholicus* (AEH68208), *Parthenocissus henryana* (ABA59555), *Rubus occidentalis* (ACM17896), *Canarium album* (ACR10242), *Gossypium arboreum* (AAG10197), *Hibiscus cannabinus* (JX524279), *Leucaena leucocephala* (AEM63594), *Capsicum annuum* (AAG43824), *Populus trichocarpa* x *Populus deltoides* (AAG50231). *Homo sapiens* (ACJ38232) sequence was used as an outgroup.

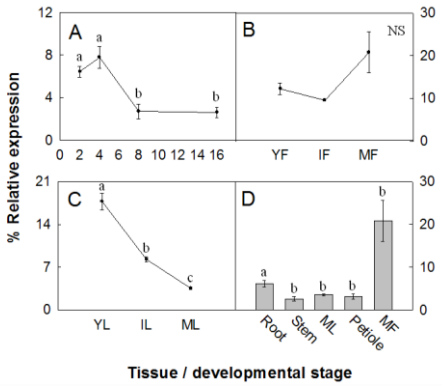

Fig 4. Transcript accumulation of kenaf *C4H* ortholog in various tissues and organs during developmental stages. Quantitative real-time PCR was used to measure *C4H* ortholog and the transcript level was calculated with respect to *ACTIN*. The percent induction relative to the control was calculated after deduction of the control transcript level. (A) expression pattern of *C4H* ortholog during stem development (2, 4, 8, 16 weeks after sowing), (B) expression pattern of *C4H* ortholog during flower development (YF, young flower; IF, immature flower; MF, mature flower), (C) expression pattern of *C4H* ortholog during leaf development (YL, young leaf; IL, immature leaf; ML, mature leaf) and (D) expression pattern of *C4H* ortholog in various tissues and organs from 16-week-old kenaf plants. Values were the average of three biological replications ± the standard error. Letters above each point indicate significant differences at the 5% level between the mean values. NS, not significant.

was detected in the stems (Park et al., 2010). Therefore, it is believed that C4H plays an important role during flower development, especially during the mature phase. In addition, high levels of phenylpropanoid-derived compounds such as sinapate and flavonoids were detected in Arabidopsis flower (Chapple et al., 1994). The *C4H* transcript levels in purple kale were found to be about 5-fold higher than those in white kale, indicating that C4H might be involved in anthocyanin accumulation (Zhang et al., 2012). Kenaf flowers used in this study are pale yellow with red centers. The red color is probably due to the accumulation of anthocyanin. Accordingly, the high expression level of *C4H* might be required for anthocyanin accumulation. The ubiquitous expression of *HcC4H* in diverse tissues and organs suggests that it is important to the production of monolignols and other phenylpropanoid compounds (Anterola et al., 2002). Overall, the expression patterns confirm the putative function of HcC4H as C4H.

HcC4H ortholog expression in response to abiotic stresses

The expression patterns of *HcC4H* were studied after exposure to diverse abiotic stresses. According to QPCR analysis, *HcC4H* transcripts were up-regulated by all treatments (Fig. 5). Defense mechanisms such as cell wall reinforcement can be activated by stresses via the induction of defense enzymes and deposition of lignin compounds (Fujita et al., 2006; Hano et al., 2006; Desender et al., 2007; Hamann et al., 2009). Phytohormones such as ABA, SA, JA (jasmonic acid) and ethylene can be produced by stresses. These phytohormones might regulate the expression of various genes including those involved in lignin biosynthesis. For gene expression, the initial signals can be amplified by the phytohormones that produce a second round signaling pathway (Mahajan et al., 2005; Shao et al., 2007).

Wounds

It is well known that wounding activates specific defense mechanisms, such as cuticle formation, lignification and toxic compound production (e.g., alkaloids and tannins) (Delessert et al. 2004). Genes related to lignin biosynthesis, such as *PAL*, *C4H*, *F5H* (ferulate 5-hydroxylase), *CAD* (cinnamyl alcohol dehydrogenase), *CCR* (cinnamoyl-CoA reductase) and *4CL* (4-coumarate-CoA ligase), are regulated by wounding, which leads to the accumulation of lignin compounds surrounding the wound sites (Delessert et al., 2004; Soltani et al., 2006; Moura et al., 2010). Similar expression patterns of *C4H* were observed in *Scutellaria baicalensis* (*SbC4H*) (Xu et al., 2010). *SbC4H* transcript was sharply increased within 1 h of wounding and then gradually decreased to the control level. Up-regulated gene expression after wounding has also been reported in soybean and alfalfa (Creelman et al., 1992; Junghans et al., 1993).

NaCl

Salinity is a serious problem in agriculture, and many investigators have found multiple genes regulated by salt stress. Increasing lignification or changing the monomeric composition of lignin in plant cell walls is one of the most important mechanisms by which plants overcome salt stress (Neves et al., 2010). Treatment with 150 - 200 mM NaCl increased the accumulation of lignin in soybean roots by 72-90% (Neves et al., 2010). Moreover, several genes involved in the phenylpropanoid pathway showed a high response to salt stress. *SAMS* encoding S-adenosyl-L-methionine synthase in tomato and *COMT* encoding caffeic acid *O*-methyltransferase in *Tamarix hispida*

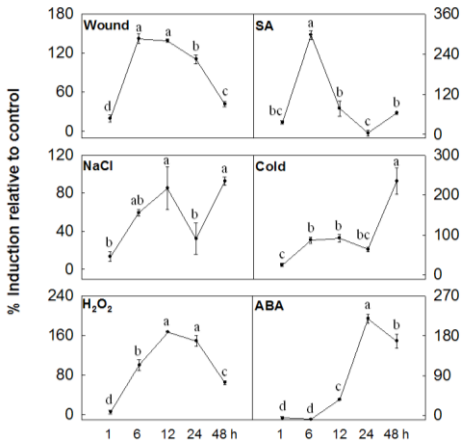

Fig 5. Transcript accumulation of kenaf C4H ortholog in response to various abiotic stresses. Three-week old stem tissues were used for stress treatments, which included wounding, SA (salicylic acid), NaCl, cold, H_2O_2 and ABA (abscisic acid). Quantitative real-time PCR was used to measure C4H ortholog and the transcript level was calculated with respect to ACTIN. The percent induction relative to the control was calculated after deduction of the control transcript level. Values were the average of three biological replications ± the standard error. Letters above each point indicate significant differences among mean values at the 5% level. NS, not significant.

were up-regulated by salt treatment (Sánchez-Aguayo et al., 2004; Li et al., 2009).

ABA

ABA is well known as an important phytohormone involved in responses to abiotic stresses, such as drought, cold and osmotic stress. While the genes involved in ABA biosynthesis were induced by both drought and cold treatments, they did not respond to exogenous ABA treatment (Zhu et al., 2002). These findings indicate that there are two types of ABA signal transduction pathways, ABA-independent and ABA-dependent. The *C4H* gene in tea was down-regulated in response to ABA treatment, indicating that *C4H* might be categorized into an ABA-independent cascade (Singh et al., 2009). In the present study, the transcript level of *HcC4H* was induced by ABA treatment, peaking at 24 h (Fig. 5). Therefore, *HcC4H* is believed to be categorized as an ABA-dependent cascade. In other studies, several genes were induced by ABA treatment as well as by drought, salt, osmotic stress and cold treatment (Shinozaki et al., 1996). Induced expression of *F5H* was observed in the detached leaves of *Camptotheca acuminate* treated with 10μM ABA and 10 mM H_2O_2 (Kim et al., 2006). H_2O_2 also regulated the expression of genes involved in lignin biosynthesis (Ros et al., 2005). These findings support those of the present study, in which the transcript level of *HcC4H* was induced by H_2O_2 treatment, peaking at 12 h (Fig. 5). Phytohormones such as SA, JA and ethylene are widely involved in the signaling pathway in response to pathogenic infection. Application of exogenous SA might induce defense mechanisms, including the activation of PAL (Yu et al., 2006). A similar expression pattern was also observed after treatment with SA and wounding.

Cold

It is still unclear how lignin functions in the acclimation to cold, but there is a great deal of evidence that low temperature can cause changes in plant lignin contents (Moura et al., 2010). It was previously reported that several genes belonging to lignin biosynthesis were induced by cold treatment. The induction by cold was reported in several genes including *PAL* in *Brassica napus* and *Glycine max*, *PAL*, *4CL*, *HCT*, *CCR* and *CAD* in the leaves of winter barley cv. Luxor, *C3H* in *Rhododendron* and *CAD1* in sweet potato (Wei et al., 2006; Kim et al., 2010; Moura et al., 2010; Janská et al., 2011). Moreover, some of the genes involved in anthocyanin biosynthesis in purple kale were up-regulated in response to low temperature conditions (Zhang et al., 2012).

In summary, a full-length *C4H* gene putatively encoding cinnamate 4-hydroxylase, which is an enzyme involved in the second step of the phenylpropanoid pathway, was cloned from *Hibiscus cannabinus* (*HcC4H*). We found that the expression of *HcC4H* was regulated by developmental stages and diverse abiotic stresses.

Materials and methods

Plant materials

Kenaf seeds (*Hibiscus cannabinus* L., C-9) originally from Russia (GenBank of Korea Rural Development Administration IT No. 202789) were germinated in pots (arrays of 32 individuals) filled with a sterile non-soil mixture (Tobietec, Chungbuk, Korea). Plants were grown in the growth room for up to four weeks, during which time they were watered twice a week and maintained under the following conditions: 16-h light /8-h dark, 22°C and 100 μmol m^{-2} s^{-1} light intensity. Kenaf plants were transplanted into 20-cm pots filled with non-soil mixture and grown in a greenhouse with natural sunlight for up to 20 weeks with watering twice a week. The roots, stems, petioles, leaves and flowers were harvested from 16-week-old kenaf plants grown in the greenhouse. Leaf and flower tissues were harvested at different developmental stages: for leaf tissues [1) young leaf (YL), < 2 cm long; 2) immature leaf (IL), 3-5 cm long; 3) mature leaf (ML), > 9 cm long] and for flower tissues [1) young flower (YF), unopened green flower, < 2 cm long with green sepal; 2) immature flower (IF), unopened white flower, > 3 cm long with green sepal; 3) mature flower, open white flower].

Stress treatments

Various abiotic stresses were applied to three-week-old kenaf seedlings grown in the growth room as previously described (Ghosh et al., 2012). After treatment, the stem tissues were harvested for QPCR analysis. Plants were treated with ABA (100 μM), H_2O_2 (10 mM), SA (5 mM), NaCl (200 mM), or distilled water as a control (Gao et al., 2009; Zheng et al., 2010). For the wounding treatment, stem tissues were cut twice longitudinally using scissors. Plants were treated with 100 μM jasmonate (MeJA) dissolved in 0.004% ethanol using an air-sprayer and then covered with a plastic bag. For a control, 0.004% ethanol without MeJA was applied. Stem tissues of the treated plants were harvested 1, 6, 24 and 48 h after treatments and then stored at -80°C after being frozen in liquid nitrogen. Total RNA was isolated from various kenaf tissues as previously described (Ghosh et al., 2012).

Cloning

Two micrograms of RNA from kenaf stems were used to synthesize cDNA using a Superscript III First-strand synthesis kit (Invitrogen, Carlsbad, CA, USA). Degenerate primers were used to synthesize C4H fragments (C4H-2F, 5'-

ATGAC(C/T/G)GT(C/T/A)CC(T/C/A/G)TT(C/T)TTCAC-3'; C4H-3R, 5'-AT(G/A)TTCTC(G/A)AC(G/A)ATGTA-3'). The degenerate primers were designed based on the conserved *C4H* sequences of *Gossypium arboreum* (AF286648), *Capsicum annuum* (EU620574), *Citrus sinensis* (AF255014), *Ruta graveolens* (AF548370), *Camptotheca acumina* (AY621152), *Zinnia elegans* (ZEU19922), *Brassica napu* (DQ485132), *Arabidopsis thaliana* (NM128601.2), *Rubus coreanus* (EU123531) and *Humulus lupulus* (FJ617541). The PCR product was cloned into the pGEM®-T easy Vector for sequencing (Promega, Madison, WI, USA). Both 5' and 3' RACE kits were used to clone a full length *C4H* ortholog (Invitrogen).

QPCR analysis

QPCR was conducted as previously described (Bae et al., 2008) using the Mx3000P QPCR System (Agilent, Santa Clara, CA, USA) with SYBR Green QPCR Master Mix (LPS Solution, Daejeon, Korea). QPCR primers were designed using the Biology WorkBench Primer 3 program. The QPCR primer sequences of *C4H* ortholog were as follows: forward primer, 5'-ATCCCTCTATTGGTTCCTCACAT-3'; reverse primer, 5'-AAGATACCTGAAGTCAT TCCCATTC-3'. *ACTIN* (DQ86-6836) was used as an expression control: forward primer, 5'-ATGGACAAGTCATTACTATTGGAGC-3'; reverse primer, 5'-AGTGATTTCCTTGCTCATACGGT-3'.

Data analyses

The following software programs were used to analyze DNA and protein sequences: NCBI Blast (http://blast.ncbi.nlm.nih.gov/), Biology WorkBench (http://workbench.sdsc.edu/), ExPASy Proteomics Server (http://expasy.org/tools/pi_tool.html), SignalP 4.0 (http://www.cbs.dtu.dk/services SignalP/) and TargetP V1.1 (http: //www.cbs.dtu.dk/services/TargetP/). A phylogenetic tree was generated from the amino acid sequences by the neighbor joining method using Mega5 (http://www/megasoftware.net/). Duncan's multiple range test was used to analyze the statistical significance of the differences among means at a significance of $P \leq 0.05$ using SASS (SASS Inc., Cary, NC, USA).

Acknowledgements

This research was supported by the Yeungnam University Research Grant in 212-A-380-256.

References

Adler E (1977) Lignin chemistrys past, present and future. Wood Sci Technol. 11:169-218

Ahmed A, Scott G, Akhtar M, Myers (1998) Biokraft pulping of kenaf and its bleachability. North American Nonwood Fiber Symposium. 231-238

Anterola A, Jeon J, Davin L, Lewis N (2002) Transcriptional control of monolignol biosynthesis in *Pinus taeda*: Factors affecting monolignol ratios and carbon allocation in phenylpropanoid metabolism. J Biol Chem. 277:18272-18280

Araki T, Kubota F (2005) Comparison of growth feature and drought tolerance between two high productive species, kenaf (*Hibiscus cannabinus*, C3-plant) and napier grass (*Pennisetum purpureum*, C4-plant). J Faculty Agr. 50:521-532

Bae H, Kim S-H, Kim MS, Sicher RC, Strem MD, Natarajan S, Bailey BA (2008) The drought response of *Theobroma cacao* (cacao) and the regulation of genes involved in polyamine biosynthesis by drought and other stresses. Plant Physiol Biochem. 46:174-188

Bailey B, Bae H, Strem M, De Mayolo A, Guiltinan M (2005) Gene expression in leaves of *Theobroma cacao* in response to mechanical wounding, ethylene, and/or methyl jasmonate. Plant Sci. 168:1247-1258

Baucher M, Halpin C, Petit-Conil M, Boerjan W (2003) Lignin: genetic engineering and impact on pulping. Crit Rev Biochem Mol. 38:305-350

Bell-Lelong D, Cusumano J, Meyer K, Chapple C (1997) Cinnamate-4-hydroxylase expression in Arabidopsis. Regulation in response to development and the environment. Plant Physiol. 113:729-738

Betz C, McCollum T, Mayer R (2001) Differential expression of two cinnamate 4-hydroxylase genes in 'Valencia' orange (*Citrus sinensis* Osbeck). Plant Mol Biol. 46:741-748

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol. 54:519-46

Chapple C (1998) Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. Annu Rev Plant Physiol Plant Mol Biol. 49:311-343

Chapple C, Shirley B, Zook M, Hammerschmidt R, Somerville S (1994) Secondary metabolism in Arabidopsis, In Arabidopsis (Meyerowitz, E.M., ed.), Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press. 989-1030

Christensen J, Overney S, Rohde A, Diaz W, Bauw J, Simon P, Van Montagu M, Boerjan W (2001) The syringaldazine-oxidizing peroxidase PXP 3-4 from poplar xylem: cDNA isolation, characterization and expression. Plant Mol Biol. 47: 581-593

Creelman R, Tierney, M, Mullet J (1992) Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. P Natl Acad Sci USA. 89:4938-4941

Del Río JC, Gutiérrez A, Hernando M, Landín P, Romero J, Martínez AT (2004) Correlation between lignin syringyl/guaiacyl ratio in eucalypt wood and paper pulp yield. Presented at the 16th International Symposium on Analytical and Applied Pyrolysis. Alicante, Spain. 165

Delessert C, Wilson I, Van Der Straeten D, Dennis E, Dolferus R (2004) Spatial and temporal analysis of the local response to wounding in Arabidopsis leaves. Plant Mol Biol. 55:165-181

Dempsey J (1975) Fiber Crops. The University Presses of Gainesville, Gainesville, FL. 203-302

Desender S, Andrivon D and Val F (2007) Activation of defence reactions in Solanaceae: where is the specificity. Cell Microbiol. 9:21-30

Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol. 300: 1005-1016.

Fahrendorf T, Dixon R (1993) Stress responses in alfalfa (Medicago sativa L.). XVIII: Molecular cloning and expression of the elicitor-inducible cinnamic acid 4-hydroxylase cytochrome P450. Arch Biochem Biophys. 305:509-515

Francois L, Donovan T and Maas E (1992) Yield, vegetative growth, and fibre length of kenaf grown on saline soil. Agron J. 84:592-598

Frank M, Deyneka J, Schuler M (1996) Cloning of wound-induced cytochrome P450 monooxygenases expressed in pea. Plant Physiol. 110:1035-1046

Fujita K, Komatsu K, Tanaka K, Ohshima S, Asami Y, Murata E, Akita M (2006) An *in vitro* model for studying vascular injury after laser microdissection, *Histochem*. Cell Biol. 125:509-514

Gao Q, Liu Y, Wang M, Zhang J, Gai Y, Zhu C, Guo X (2009) Molecular cloning and characterization of an inducible RNA-dependent RNA polymerase gene, GhRdRP, from cotton (*Gossypium hirsutum* L.). Mol Biol Rep. 36:47-56

Ghosh R, Choi B, Jeong M, Bae D, Shin S, Park S, Lim H, Kim J, Bae H (2012) Comparative transcriptional analysis of caffeoyl-coenzyme A 3-O-methyltransferase from Hibiscus cannabinus L., during developmental stages in various tissues and stress regulation. Plant Omics J. 5:184-193

Gravot A, Larbat R, Hehn A, Lièvre K, Gontier E, Goergen JL, Bourgaud F (2004) Cinnamic acid 4-hydroxylase mechanism-based inactivation by psoralen derivatives: cloning and characterization of a C4H from a psoralen producing plant-*Ruta graveolens*-exhibiting low sensitivity to psoralen inactivation. Arch Biochem Biophys. 422:71-80

Gutiérrez A, Rodríguez I, Del Río J (2004) Chemical characterization of lignin and lipid fractions in kenaf bast fibers used for manufacturing high-quality papers. J Agric Food Chem. 52:4764-4773

Hamann T, Bennett M, Mansfield J, Somerville C (2009) Identification of cell-wall stress as a hexose-dependent and osmosensitive regulator of plant responses. Plant J. 57:1015-1026

Hano C, Addi M, Bensaddek L, Croˆnier D, Baltora-Rosset S, Doussot J, Maury S, Mesnard F, Chabbert B, Hawkins S, Lainé E, Lamblin F (2006) Differential accumulation of monolignol-derived compounds in elicited flax (*Linum usitatissimum*) cell suspension cultures. Planta. 223:975-989

Hisano H, Nandakumar R, Wang Z (2009) Genetic modification of lignin biosynthesis for improved biofuel production. In Vitro Cell Dev Biol Plant. 45:306-313

Hotze M, Schröder G, Schröder J (1995) Cinnamate 4-hydroxylase from *Catharanthus roseus*, and a strategy for the functional expression of plant cytochrome P450 proteins as translational fusions with P450 reductase in *Escherichia coli*. FEBS Lett. 374:345-350

Janská A, Aprile A, Zámečník J, Cattivelli L, Ovesná J (2011) Transcriptional responses of winter barley to cold indicate nucleosome remodelling as a specific feature of crown tissues. Funct Integr Genomics. 11:307-325

Junghans H, Dalkin K, Dixon R (1993) Stress responses in alfalfa (*Medicago-Sativa* L). 15. Characterization and expression patterns of members of a subset of the chalcone synthase multigene family. Plant Mol Biol. 22:239-253

Kawai S, Mori A, Shiokawa T, Kajita S, Katayama Y, Morohoshi N (1996) Isolation and analysis of cinnamic acid 4-hydroxylase homologous genes from a hybrid aspen, *Populus kitakamiensis*. Biosci Biotechnol Biochem. 60:1586-1597

Kim Y, Bae J, Huh G (2010) Transcriptional regulation of the cinnamyl alcohol dehydrogenase gene from sweetpotato in response to plant developmental stage and environmental stress. Plant Cell Rep. 29:779-791

Kim Y, Kim D, Lee S, Lee I (2006) Wound-induced expression of the ferulate 5-hydroxylase gene in *Camptotheca acuminate*. Biochim Biophys Acta. 1760:182-190

Koopmann E, Logemann E, Hahlbrock K (1999) Regulation and functional expression of cinnamate 4-hydroxylase from parsley. Plant Physiol. 119:49-56

Lam T, Hori K, Iiyama K (2002) Structural characteristics of cell walls of kenaf (*Hibiscus cannabinus L.*) and fixation of carbon dioxide. J Wood Sci. 49:255-261

Lee KJ, Marcus SE, Knox JP (2011) Cell wall biology: perspectives from cell wall imaging. Mol Plant. 4:212-219

Lee S, Lee E, An B, Barampuram S, Kim J, Cho J, Chung B (2008) Molecular cloning and characterization of *cinnamate-4-hydroxylase* gene from *Rubus coreanus*. Open Plant Sci J. 2: 31-36

Li H, Wang Y, Jiang J, Liu G, Gao C, Yang C (2009) Identification of genes responsive to salt stress on *Tamarix hispida* roots. Gene. 433:65-71

Li X, Weng J, Chapple C (2008) Improvement of biomass through lignin modification. Plant J. 54:569-581

Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: an overview. Arch Biochem Biophys. 444:139-158

Marques G, Rencoret J, Gutiérrez A, del Río J (2010) Evaluation of the Chemical Composition of different non-woody plant fibers used for pulp and paper manufacturing. Open Agri J. 4:93-101

Moura J, Bonine C, de Oliveira Fernandes Viana J, Dornelas M, Mazzafera P (2010) Abiotic and biotic stresses and changes in the lignin content and composition in plants. J Integr Plant Biol. 52:360-376

Nedelkina S, Jupe S, Blee K, Schalk M, Werck-Reichhart D, Bolwell G (1999) Novel characteristics and regulation of a divergent cinnamate 4-hydroxylase (CYP73A15) from French bean: engineering expression in yeast. Plant Mol Biol. 39:1079-1090

Neutelings G (2011) Lignin variability in plant cell walls: Contribution of new models. Plant Sci. 181:379-386

Neves G, Marchiosi R, Ferrarese M, Siqueira-Soares R, Ferrarese-Filho O (2010) Root growth inhibition and lignification induced by salt stress in soybean. J Agron Crop Sci.196:467-473

Nimz H (1974) Beech lignins proposal of a constitutional scheme. Angew Chem Int Ed. 13: 313-321

Pande H, Roy D (1996) Delignification kinetics of soda pulping of kenaf. J Wood Chem Technol. 16:311-325

Park J, Park N, Xu H, Park S (2010) Cloning and characterization of phenylalanine ammonia-lyase and cinnamate 4-hydroxylase and pyranocoumarin biosynthesis in *Angelica gigas*. J Nat Prod. 73:1394-1397

Potter S, Moreland D, Kreuz K, Ward E (1995) Induction of cytochrome P450 genes by ethanol in maize. Drug Metabol Drug Interact. 12:317-327

Raes J, Rohde A, Christensen J, de Peer Y, Boerjan W (2003) Genome-wide characterization of the lignification toolbox in Arabidopsis, Plant Physiol. 133:1051-1071

Ros Barceló A (2005) Xylem parenchyma cells deliver the H_2O_2 necessary for lignification in differentiating xylem vessels. Planta. 220:747-756

Sánchez-Aguayo I, Rodríguez-Galán J, García R, Torreblanca J, Pardo J (2004) Salt stress enhances xylem development and expression of S-adenosyl-L-methionine synthase in lignifying tissues of tomato plants. Planta. 220:278-285

Shao H, Guo Q, Chu L, Zhao X, Su Z, Hu Y, Cheng J (2007) Understanding molecular mechanism of higher plant plasticity under abiotic stress. Colloids Surface B. 54:37-45

Shinozaki K, Yamaguchi-Shinozaki K (1996) Molecular responses to drought and cold stress. Curr Opin Biotechnol. 7:161-167

Singh K, Kumar S, Rani A, Gulati A, Ahuja P (2009) Phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) and catechins (flavan-3-ols) accumulation in tea. Funct Integr Genomics. 9:125-34

Soltani B, Ehlting J, Hamberger B, Douglas C (2006) Multiple *cis*-regulatory elements regulate distinct and complex patterns of developmental and wound induced expression of *Arabidopsis thaliana 4CL* gene family members. Planta. 224:1226-1238

Teutsch H, Hasenfratz M, Lesot A, Stoltz C, Garnier J, Jeltsch J, Durst F, Werck-Reichhart D (1993) Isolation and sequence of a cDNA encoding the *Jerusalem artichoke* cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the

general phenylpropanoid pathway. Proc Natl Acad Sci USA. 90:4102-4107

Van Dam J, Van Vilsteren G, Zomers F, Shannon W, Hamilton I (1994) Increased application of domestically produced plant fibres in Textiles, Pulp and Paper Production and Composite Materials, European Commission, Directorate-General XII. Science, Research and Development. 249

Vanholme R, Morreel K, Ralph J, Boerjan W (2008) Lignin engineering, Curr Opin Plant Biol. 11:278-285

Wei H, Dhanaraj AL, Arora R, Rowland LJ, Fu Y, Sun L (2006) Identification of cold acclimation responsive *Rhododendron* genes for lipid metabolism, membrane transport and lignin biosynthesis: importance of moderately abundant ESTs ingenomic studies. Plant Cell Environ. 29:558-570

Xu H, Park N, Li X, Kim Y, Lee S, Park S (2010) Molecular cloning and characterization of phenylalanine ammonia-lyase, cinnamate 4-hydroxylase and genes involved in flavone biosynthesis in *Scutellaria baicalensis*. Bioresour Technol. 101:9715-9722

Yu Z, Fu C, Han Y, Li Y, Zhao D (2006) Salicylic acid enhances jaceosidin and syringin production in cell cultures of *Saussurea medusa*. Biotechnol Lett. 28:1027-1031

Zhang B, Hu Z, Zhang Y, Li Y, Zhou S, Chen G (2012) A putative functional MYB transcription factor induced by low temperature regulates anthocyanin biosynthesis in purple kale (*Brassica Oleracea* var. acephala f. tricolor). Plant Cell Rep. 31:281-289

Zheng H, Lin S, Zhang Q, Lei Y, Hou L, Zhang Z (2010) Functional identification and regulation of the *PtDrl02* gene promoter from triploid white poplar. Plant Cell Rep. 29:449-460

Zhu J (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol. 53:247-273