Plant Omics Journal POJ

POJ 5(3):305-313 (2012) ISSN:1836-3644

Transcriptional analysis of hydroxycinnamoyl transferase (HCT) in various tissues of *Hibiscus cannabinus* in response to abiotic stress conditions

Emran Md Chowdhury[1], Bo Sung Choi[1], Sang Un Park[2], Hyoun-Sub Lim[3], and Hanhong Bae[1,*]

[1]School of Biotechnology, Yeungnam University, Gyeongsan 712-749, Republic of Korea
[2]Department of Crop Science, Chungnam National University, Daejeon 305-754, Republic of Korea
[3]Department of Applied Microbiology, Chungnam National University, Daejeon 305-764, Republic of Korea

***Corresponding author: hanhongbae@ynu.ac.kr**

Abstract

We cloned a full-length gene from the kenaf plant putatively encoding hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase enzyme (HcHCT), which is involved in the lignin biosynthesis pathway. We examined the tissue and organ specific expression of an *HcHCT* ortholog during developmental stages and in response to abiotic stresses. The full-length of the *HcHCT* ortholog consisted of a 1,296 bp open reading frame (ORF) encoding 431 peptides. The molecular weight of deduced amino acids was 47.71 kDa, with an isoelectric point (pI) of 5.79. The deduced amino acid sequence showed 80-86% identities with HCTs of other plants. The deduced amino acid sequence of the *HcHCT* ortholog has a histidine containing motif (HHAAD), characteristic for acyl transfer catalysis. A second consensus sequence, a DFGWG block, is another acyl transferase of the BAHD family. Phylogenetic analysis showed the closest relationship (86%) with HCT of *Populus trichodcarpa* (ACC63882). According to quantitative real-time reverse transcription PCR (QPCR) analysis, *HcHCT* transcript was expressed in all the tissues and organs, but the highest expression was observed in roots and mature flowers. The expression of *HcHCT* transcript was also examined in stem tissues of 3-week-old kenaf plants in response to various abiotic stresses. The expression of *HcHCT* transcript was highly induced by all treatments, including wound, SA, NaCl, cold, H_2O_2, ABA, and drought. *HcHCT* was highly expressed in response to cold, SA, and H_2O_2 at 24 h, 6 h, and 6 h after treatment, respectively. Our results suggest that we have cloned the full-length gene putatively encoding for HCT, which is responsive to various abiotic stresses.

Keywords: kenaf (*Hibiscus cannabinus* L.), hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase enzyme (HCT), lignin biosynthesis, real-time PCR.
Abbreviations: ABA- abscisic acid; HCT-hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase enzyme; MeJA-methyl jasmonate; SA- salicylic acid; *Hc- Hibiscus cannabinus.*

Introduction

Kenaf (*Hibiscus cannabinus* L.), native to central Africa, is the third largest fiber crop of economic importance after cotton and jute. Kenaf is a rapid growing, short-day annual crop Rymsza, 1999). Kenaf stem mainly consists of an outer part called the bark, making up 35-40% of total stem weight, and an inner part called the core, making up the remaining 60-65% of total stem weight (Lin et al., 2004). The ratio of cork vs. bark fiber is ~3:1 (Dempsey, 1975). Kenaf fibers are used in making agricultural and industrial products such as paper pulp, thermoplastics, composites, geotextile, fabrics, industrial absorbents, etc. The importance of kenaf as a wood substitute has increased due to the increased consumption of paper and paperboard materials. The per unit area production of kenaf is 3 to 5-fold higher than that of pulpwood trees, and the products are equal or superior to those of other woods. The amount and quality of pulp depends on the plant cell wall composition. Plant cell walls are mainly made of cellulose, hemicellulose, pectin, proteins, and/or lignin, which provide mechanical support to individual cells as well as to the whole plant. The components of cell wall and their proportions vary according to cell type. Lignin is one of the most important limiting factors for conversion of plant biomass to pulps or biofuels (Vanholme et al., 2010). Lignin protects cell wall polysaccharides from microbial degradation and also inhibits saccharification enzymes due to the reduced accessibility to cellulose microfibrils and the adsorption of enzymes to lignin. In addition, the down-stream processes of saccharification and fermentation can be inhibited by the by-products produced during the process of removing lignin from biomass. Also, the removal of lignin from plant biomass is a costly process (Sticklen, 2008; Weng et al., 2008; Mansfield, 2009). These limitations prompted many researchers to design plants that either accumulate less lignin or produce lignins that are more amendable to chemical degradation. Lignin is an aromatic subunit polymer derived from phenylalanine (Fig 1). The main building blocks of lignin are hydroxycinnamyl alcohols (monolignols), including ρ-coumaryl, coniferyl, and sinapyl alcohols. These monolignols are synthesized through monolignol-specific pathways, undergo polymerization, and are incorporated into the lignin polymer (Weng et al., 2008). The resulting units of monolignols are called as guaiacyl (G), syringyl (S), and ρ-hydroxyphenyl (H) units (Vanholme et al., 2008). In gymnosperms, lignin is composed mainly of G units with a

small amount of H units, while the lignin from angiosperms is composed of G and S units (Novo Uzal et al., 2009). Bast fiber (outer stem) of kenaf is comprised of lignin with a high S/G ratio (5:4) and a low amount of H units (S 83.3, G 15.4, and H 1.3%) (Gutiérrez et al., 2004). Kenaf bast fiber is composed of < 11% lignin, which is higher than in other non-woody plants, and contains a great amount of cellulose (Van Dam, 1994; Gutiérrez et al., 2004; Marques, 2010). A series of well-defined enzymatic reactions occur in the lignin biosynthesis pathway. The initial step in lignin biosynthesis begins with deamination of phenylalanine by phenylalanine ammonia lyase (PAL), leading to the formation of CoA thioester from hydroxyl cinnamic acid (HCA) by 4-coumarate-coenzyme A ligase (4CL) utilizing the phenylpropanoid pathway in the cytosol (Fig 1). The genes involved in lignin biosynthesis can alter lignin composition by up- or down-regulation processes (Li Y et al., 2003; Ralph et al., 2006; Shadle et al., 2007). Down-regulation of the up-stream genes (*C3H*, *HCT*, or *4CL*) can reduce the lignin content and affect lignin composition (Li et al., 2008; Xu et al., 2008). *HCT* gene silencing causes a reduction of both G and S units and increases the H unit in *Nicotiana benthamiana* and *Medicago sativa*, which confirms that lignin biosynthesis can be controlled by HCT activity (Hoffmann et al., 2004; Chen et al., 2006). Hydroxycinnamoyltransferase (HCT) is a recently discovered enzyme of the monolignol-specific pathway, which catalyzes the reactions both immediately preceding and following insertion of the 3-hydroxyl group into monolignol precursors (Hoffmann et al., 2003 and 2004). HCT enzyme converts ρ-coumaroyl CoA and caffeoyl CoA to their corresponding shikimate or quinate esters, and also catalyzes the reverse reaction. The shikimate and quinate esters of ρ-coumaroyl CoA are the preferred substrates for hydroxylation by ρ-coumarate 3-hydroxylase (C3H), resulting in the conversion of their corresponding caffeoyl CoA esters (Fig 1) (Schoch et al., 2001; Hoffmann et al., 2003). In this study, the *HCT* gene putatively involved in lignin biosynthesis was cloned and characterized to investigate its expression pattern during the developmental stages of various tissues and organs, as well as in response to various abiotic stresses such as those produced by wounds, salicylic acid (SA), NaCl, cold, H_2O_2, ABA, and methyl jasmonic acid (MeJA) in 3-week-old stem tissues of *Hibiscus cannabinus*.

Results and Discussion

Cloning of kenaf HCT ortholog and sequence characterization

Degenerate primers and the RACE (rapid amplification of cDNA ends) system were used to clone a full-length *HCT* kenaf ortholog (GenBank Accession No. JQ779021). The cloned length of the kenaf *HCT* ortholog was 1,323 bp, with an open reading frame (ORF) of 1,296 bp, and its translation product was a 431 peptide residue (Fig 2). ExPASy Proteomic Server results indicated that the predicted molecular weight of the translated protein would be 47.71 kDa with an isoelectric point (pI) of 5.79. The deduced kenaf *HCT* ortholog shared 86, 84, 82, 82, 81, 81, 80, and 80% identities with other HCT proteins from *Populus trichocarpa*, *Arabidopsis thaliana*, *Coffea canephora*, *Cynara cardunculus*, *Cucumis sativus*, *Trifolium pretense*, *Ipomoea batatas,* and *Solenostemon scutellarioides*, respectively, which belong to a superfamily of plant acyltransferases (Fig 3 and 4). The kenaf HCT acyltransferase has a histidine containing motif (HHAAD, amino acids 153-157 in gray box; Fig 3) matching the highly conserved HXXXD motif, characteristic for acyltransfer proteins, as does the HCT isolated from *Cynara cardunculus* L. (Comino et al., 2007). A second consensus sequence is the DFGWG block (amino acids 378-382 in gray box; Fig 3) present in other acyltransferases of the BAHD family (St Pierre et al., 1998; Suzuki et al., 2003; D'Auria et al., 2006). SignalP 4.0 analysis indicates that there is no cleavage site and it might be a non-secretory protein. TargetP V1.1 analysis also indicates that there is no significant signal peptide for sub-cellular localization based on N-terminal amino acid sequence, which strongly suggests that the protein might be a cytoplasmic protein. A phylogenetic tree was constructed by the neighbor joining method of MEGA5 using HCT amino acid sequences of 13 plant species. Phylogenetic tree analysis showed that the closest relationship for the kenaf HCT ortholog was with *Populus trichodcarpa* HCT (ACC63882, 86% identity). The kenaf HCT enzyme belongs to a large family of acyltransferases in *Arabidopsis*, because it accepts ρ-coumaroyl CoA and caffeoyl CoA as substrates and transfers the acyl group on both shikimate and quinate acceptors (Fig 1) (Hoffmann et al., 2004). Repression of the *HCT* gene causes poor S-lignin synthesis in an *Arabidopsis* mutant and has a direct effect on lignin biosynthesis (Hoffmann et al., 2004). These results support that the kenaf *HCT* ortholog is similar to the *Arabidopsis HCT* gene and is involved in the lignin biosynthesis pathway. Therefore, the *HCT* ortholog of kenaf is from now on designated as *HcHCT*.

Tissue and organ specific expression of HcHCT gene

Total RNA was extracted from different tissues and organs (root, stem, petiole, and mature flower) of the kenaf plant to examine the expression pattern of the *HcHCT* transcript. Quantitative real-time reverse transcription PCR (QPCR) results indicated that expression pattern of *HcHCT* transcript was ubiquitous in all parts of a 4-week old plant, but was relatively high in roots and mature flowers (Fig 5A). *HcHCT* transcript was sharply increased in the stem development stage up to 4 weeks, and then abruptly decreased at 8 weeks and maintained its level for up to 20 weeks (Fig 5B). The highest level of *HcHCT* transcript was detected in young flowers and young leafs during stages of flowering and leaf development, respectively (Fig 5C and 5D). The previous result suggests that both the *HCT1* and *HCT2* of red clover plants were expressed in all tissues such as stems, leafs and flowers, but was high in flowers compared to expression in unexpanded leafs, mature leafs, and stems (Michael and Sullivan, 2009). *HCT* was also expressed in all tissues of *Arabidopsis*, with the strongest expression being in florescence stem tissue (Raes et al., 2003). Phenylpropanoid-derived compounds were highly detected in *Arabidopsis* flowers (Chapple et al., 1994), and *HcHCT* showed high levels of expression in flowers and roots, which suggests that *HcHCT* participates in the biosynthesis of secondary metabolites in floral and root tissues.

Expression of HcHCT gene in response to various abiotic stresses

Plants are exposed to various abiotic and biotic stresses in nature, and have evolved numerous mechanisms for cell wall modification to defend themselves. Lignin protects cell wall polysaccharides from microbial degradation and imparts resistance to decay. Several enzymes are involved in the lignin biosynthesis pathway. The genes involved in lignin biosynthesis respond to various biotic and abiotic stress

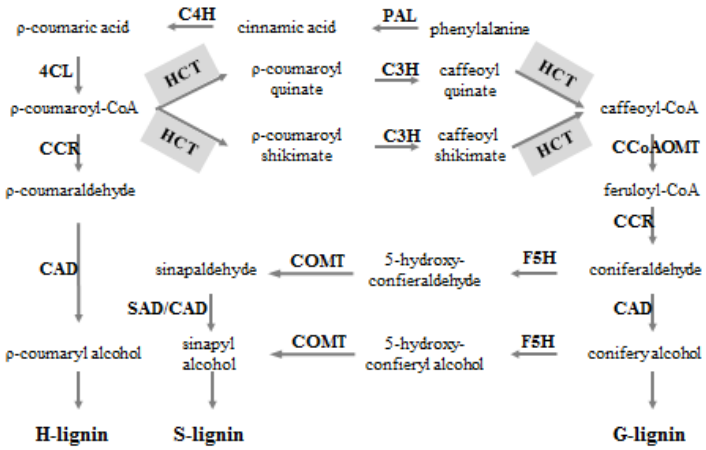

Fig 1. A current view of the lignin biosynthesis pathway in angiosperms. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, ρ-coumarate 3-hydroxylase; 4CL, 4- hydroxycinnamoyl-CoA ligase; HCT, hydroxycinnamoyltransferase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; CAD, cinnamylalcohol dehydrogenase; COMT, caffeic/5-hydroxyferulic acid O-methyltransferase; SAD, sinapylalcohol dehydrogenase.

conditions, such as wounding, pathogen infection, metabolic stress, and perturbations in cell wall structure (Caño-Delgado et al., 2003; Tronchet et al., 2010). Many researchers are also involved in investigating the crucial roles of different enzymes in the lignin biosynthesis pathway during stress conditions. In this experiment, we also tried to elucidate the role of *HcHCT* in response to various treatments and stresses (wound, SA, NaCl, cold, H_2O_2, ABA, MeJA, and drought) using QPCR. Stem tissues of 3-week-old kenaf plants were used to investigate the expression pattern of *HcHCT* transcript. QPCR results showed that *HcHCT* transcript was induced by all treatments at the early, intermediate or late stage of treatment (Fig 6). Among the treatments, the highest expression of *HcHCT* transcript was observed after 24 h of cold treatment, followed by the treatment with H_2O_2 (6 h after treatment), while the lowest expressions were found following treatments with drought and wound (7 d and 24 h after treatment, respectively). MeJA treatment did not increase the level of *HcHCT* transcript, but rather reduced the transcript. Depending on the time of maximum expression, the expression can be divided into 3 groups: i) early expression (6-12 h) by SA, H_2O_2 and wound; ii) intermediate expression (24 h) by cold and ABA, and iii) late expression (48 h) by NaCl. The expression pattern of *HcHCT* in response to drought and MeJA was little different than others. *HcHCT* transcript was highly expressed at 7 days after treatment by drought, decreased significantly up to 10 days, and then increased again up to 17 days after treatment. In the case of MeJA treatment, *HcHCT* expression was decreased at 24 h after treatment.

Wound

Lignification is a frequent process demonstrated by plants in response to infection and wounding (Raes et al., 2003). Similar responses, such as rapid accumulation of phenolic compounds, defense related gene expression, and lignin deposition at injury or invasion sites, were observed by wounding and pathogenic infection (Chen et al., 2000). It has been reported that a lignin biosynthesis gene resembling *HCT1* was up-regulated locally in the bark of Norway spruce (*Picea abies*) trees in response to pathogens and wounding (Nadeem et al., 2012). Another study stated that the gene (GH618817) of tea leaf, which has high sequence identity with *HCT* of *Populus trichocarpa* (XP002329671), was up-regulated by wounding following green leaf hopper infestation (Yanga et al., 2011). It has been stated that several genes of the lignin biosynthesis pathway were induced by wounding and microbial elicitors (Moura et al., 2010).

ABA

Abscisic acid (ABA) plays important signaling and regulatory roles that enable plants to survive under various conditions of abiotic stresses, such as drought, salinity and cold (Finkelstein and Rock, 2002). ABA is a senescence-triggering plant hormone which acts as an important signal transducer in response to wounding and microbial infection in plants. The *PAL1* gene of *Salvia miltiorrhiza* was found to be induced by various treatments including ABA, wounding, and dehydration (Song et al., 2009). Research on strawberry fruit with ABA treatment proved that ABA could up-regulate PAL activity during fruit ripening (Jiang and Joyce, 2003). From the previous findings, it is clear that ABA triggers the expression of lignin biosynthesis genes, which supports our findings.

Drought

In contrast to the lignification induced by stress, lignin has negative effects on plant growth (Osakabe, 2011). Lignification and biosynthesis of lignin precursors are induced by biotic and abiotic stresses such as wounding, pathogen attack, and drought (Vance et al., 1980; Dixon and Paiva, 1995; Hu et al., 1999; Alvarez et al., 2008). The 2 genes *CCR1* and *CCR2*, encoding cinnamoyl-CoA reductase 1 and 2 activity, which are involved in lignin biosynthesis, were increased by drought stress (Fan et al., 2006). A recent report showed that the level of caffeate O-methyltransferase (CCR), an enzyme associated with lignin biosynthesis, can be increased in the immature elongating tissues of maize leaf due to water deficit (Vincent et al., 2005). Maize *CCR* transcript was up-regulated early in a root elongation zone by water deficit. We found that drought treatment caused the biphasic expression of *HcHCT* with early induction.

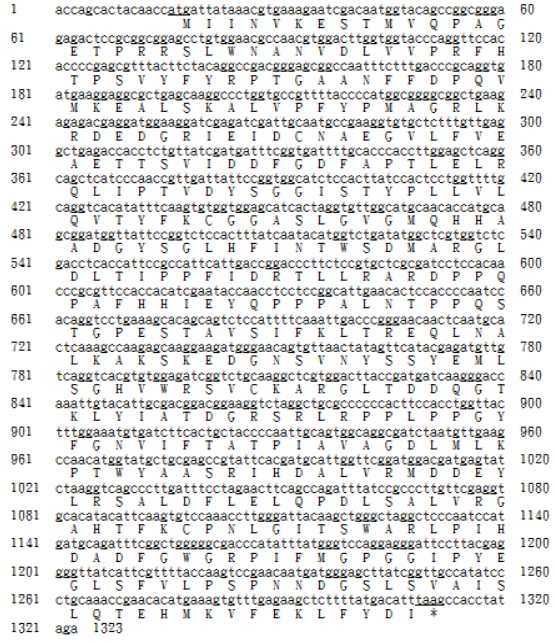

Fig 2. Full-length coding and deduced amino acid sequence of kenaf HCT ortholog. The start codon (ATG) and stop codon (TAA) are underlined.

NaCl

Salinity is a major problem in agriculture and posses a threat to plant life, and plants have developed several mechanisms to meet this threat. Accumulation of lignin or alteration of the monomeric composition of lignin in the cell wall are the important mechanisms plants use to overcome salinity stress (Neves et al., 2010). Proteome analysis of xylem sap of *Brassica oleraceae* demonstrated that the accumulation of hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase, acid peroxidase, and cysteine protein, involved in lignification, was induced by salt stress (Fernandez-Garcia et al., 2009). It was also observed that the root of *B. oleraceae* was lignified under salt treatment (Fernandez-Garcia et al., 2009); thus lignin biosynthesis genes are induced by salt stress, which affects the lignification process.

SA

SA plays a crucial role in the regulation of physiological and biochemical processes of plants, even in stress conditions such as drought, chilling, heavy metal toxicity, heat, and osmotic stress (Vicente and Plasencia, 2011). In our experiment, *HcHCT* transcript was up-regulated by SA. Previous results showed that genes encoding enzymes in general the phenylpropanoid pathways of phytoalexin, anthocyanin, and lignin synthesis were coordinately induced by treatments with SA and MeJA (Salzman et al., 2005). The promoter activity of *IbCAD1* encoding cinnamyl alcohol dehydrogenase of sweet potato was strongly induced by SA, jasmonic acid (JA), and ABA (Kim, 2010).

MeJA

Jasmonic acid (JA) and its methyl ester (MeJA), collectively termed as jasmonates, function as key signalling molecules in the wound signal transduction pathway (Seo et al., 1997). Wound signals are transmitted to JA. Defense gene expression is controlled by either a JA-dependent or JA-independent pathway in response to wounding (Perez-Amador et al., 2002; Soltani et al., 2006). It has been reported that the lignin biosynthesis gene (*HCT1*) was up- regulated locally in the bark of Norway spruce (*Picea abies*) trees in response to MeJA (Nadeem et al., 2012). In our experiment, *HcHCT* transcript was repressed at 24 h after treatment with MeJA. The results suggest that multiple signaling routes might exist for phenylpropanoid gene transcriptional activation (Batard et al., 2000). Therefore, the different expression patterns of *HCT* in response to MeJA may be due to multiple JA signaling pathways (Soltani et al., 2006).

Cold

During cold acclimation, a number of physiological and biochemical changes occur in plants, including changes in gene expression, plant cell wall composition, membrane structure, and primary/secondary metabolism (Guy, 1990; Thomashow, 1999; Wisniewski et al., 2003; Kaplan et al., 2004). It has been reported that *C3H* (CYP98A3) was up-regulated 47-fold in cold acclimated leaf tissues of *Rhododendron* (Wei et al., 2006). A recent study showed a general increase of the *Arabidopsis* leaf metabolome, as reflected in the pool size of phenylpropanoid pathway intermediates due to cold stress (Kaplan et al. 2004). Phenylalanine ammonia-lyase (PAL) was up-regulated after cold treatment in rice seedlings (Cui et al., 2005). These findings support that cold stress can up-regulate genes involved in the lignin biosynthesis pathway.

H_2O_2

As an ultimate electron acceptor, peroxidases require H_2O_2, and H_2O_2 synthesis may be the rate-limiting factor for lignin polymerization (Nose et al., 1995; Gabaldón et al., 2006). Previous experiments showed that *IbCAD1* promoter activity was highly induced by H_2O_2 treatment (Kim et al., 2010).

In a brief, we have cloned a full-length gene from *Hibiscus cannabinus* putatively encoding hydroxycinnamoyl CoA: shikimate/quinate hydroxycinnamoyl transferase (HCT) enzyme. However, it is not clear whether there may be other copies of the *HCT* gene in kenaf plants which may act differently to various treatments applied. HCT is an important enzyme in the lignin biosynthesis pathway, and lignin content can be regulated by down-regulation or up-regulation of the up-stream *HCT* gene. Further investigation is required to understand the relationship between stresses and expression of the genes related to lignin modification.

Materials and methods

Plant materials

Kenaf seeds (*Hibiscus cannabinus* L., C-9) originated from Russia, and were provided by Dr. Si-Yong Kang (Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup 580-185, Korea) (GeneBank of Korea Rural Development Administration IT No. 202789). Seeds were sown in pots filled with a sterile non-soil mixture (TOBIETEC, Chungbuk, Korea) and seedlings were grown up to 4 weeks while being maintained using a 16-h light/8-h dark photoperiod at 22°C with watering twice a week in a growth room. Photosynthetically active radiation (PAR)

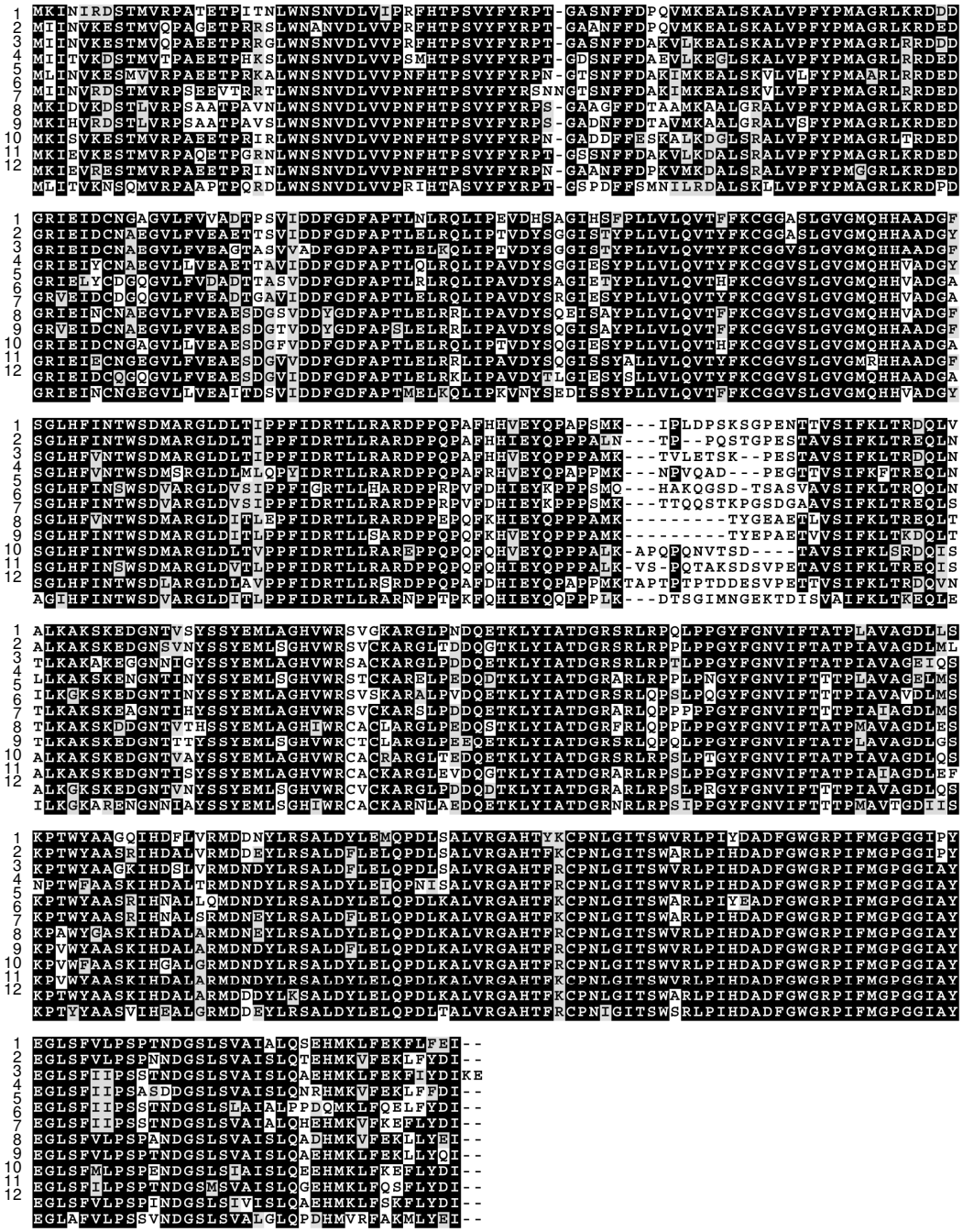

Fig 3. Multiple alignment of kenaf HCT ortholog. Alignment with members of other species ofhydroxycinnamoyl transferase family was generated using ClustalW and BOXHADE sequence alignment program. The same amino acids are highlighted in black. Gray boxes indicate the structural motifs conserved in the acyltransferase family. GenBank accession numbers are represented as follows: 1, *Arabidopsis thaliana* (NP199704); 2, *Hibiscus cannabinus* (JQ779021); 3, *Populus trichocarpa* (ACC63882); 4, *Cucumis sativus* (AEJ88365); 5, *Medicago Truncatula* (XP003637275); 6, *Trifolium pretense* (ACI16630); 7, *Salvia miltiorrhiza* (ACA64049); 8, *Solenostemon scutellarioides* (CBI83579); 9, *Ipomoea batatas* (BAJ14794); 10, *Coffea canephora* (ABO47805); 11, *Cynara cardunculus* (AAZ80046); 12, *Pinus radiate* (ABO52899).

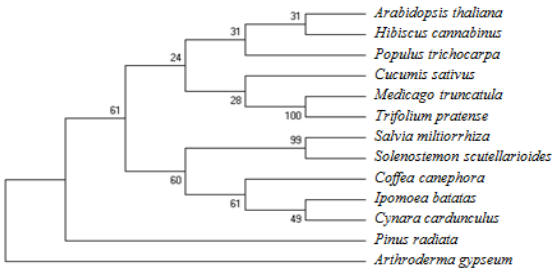

Fig 4. Phylogenetic analysis of kenaf HCT amino acid sequence with other plant species of the acyltransferase family. The phylogenetic tree was generated by the neighbor-joining method using ClustalW and Mega5. Boostrap values from 1000 replications are at the nodes. GenBank accession numbers are given below: *Arabidopsis thaliana* (NP199704), *Hibiscus cannabinus* (JQ779021), *Populus trichocarpa* (ACC63882), *Cucumis sativus* (AEJ88365), *Medicago truncatula* (XP003637275), *Trifolium pretense* (ACI16630), *Salvia miltiorrhiza* (ACA64049), *Solenostemon scutellarioides* (CBI83579), *Ipomoea batatas* (BAJ14794), *Coffea canephora* (ABO47805), *Cynara cardunculus* (AAZ80046), and *Pinus radiate* (ABO52899). *Streptomyces violaceusniger* (YP004810992) sequence was used as an outgroup for rooting the tree.

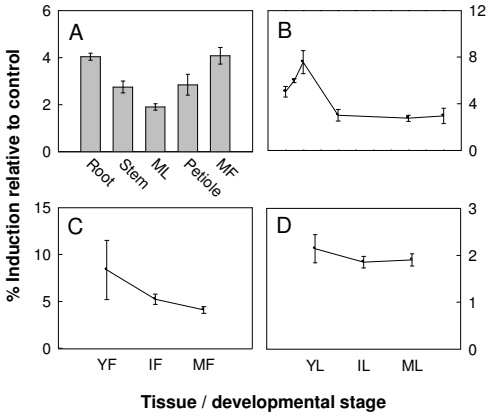

Fig 5. Expression pattern of kenaf *HCT* ortholog in various tissues and organs during developmental stage. The relative transcript levels were measured by QPCR and *ACTIN* was used as an internal control. *HCT* transcript level was adjusted after deduction of the control transcript level. (A) Expression pattern of kenaf *HCT* ortholog in various tissues and organs from 16-week-old kenaf plants, (B) expression pattern of kenaf *HCT* ortholog during stem development (2, 3, 4, 8, 16, 20 weeks after sowing), (C) expression pattern of kenaf *HCT* ortholog during flower development (YF-young flower, IF-immature flower, MF-mature flower), and (D) expression pattern of kenaf *HCT* ortholog during leaf development (YU-young leaf, IL-immature leaf, ML-mature leaf). *ACTIN* (DQ866836) was used as an expression control. Bar shows the average ± standard error of 3 biological replications.

irradiance was 100 µmol m^{-2} s^{-1} during the growth period. After 4 weeks, Kenaf seedlings were transplanted into the 20-cm pots filled with the same potting mixtures and transferred to a greenhouse with natural sunlight. The seedlings were maintained up to 20 weeks with watering twice a week under greenhouse conditions. After 16 weeks, various tissue samples (root, stem, petiole, leaf, and flower) were collected from kenaf plants grown in the greenhouse. For transcriptional analysis, leaf samples were harvested at the following 3 developmental stages: i) young leaf (YL, < 2 cm long), ii) immature leaf (IL, 3-5 cm long), and iii) mature leaf (ML > 9 cm long). Flower samples were also harvested for transcriptional analysis at the following 3 developmental stages: i) young flower (YF, unopened green flower, < 2 cm long with green sepal), ii) immature flower (IF, unopened white flower, > 3 cm long with green sepal), and iii) mature flower (MF, open white flower). The expression of *HcHCT* transcript was also examined in stem tissues of 3-week-old kenaf plants in response to various abiotic stresses.

Abiotic stress treatments

Different abiotic stresses were imposed on 3-week-old kenaf seedlings grown in the growth room. Stem tissues of kenaf seedlings were harvested at different times after treatment and samples were used for QPCR analysis. Kenaf plants were exposed to 10°C for 1, 6, 24, and 48 h for cold treatment. Kenaf seedlings were watered with ABA (100 µM), H_2O_2 (10 mM), SA (5 mM), or NaCl (200 mM) to investigate the effects of various stresses on *HCT* transcript (Gao et al., 2009; Zheng et al., 2010). Kenaf seedlings, watered with distilled water, were used as control specimens. Kenaf stems were cut longitudinally twice with scissors for wounding treatment. In the case of MeJA treatment, 100 µM MeJA dissolved in 0.004% ethanol was sprayed onto kenaf seedlings and the treated seedlings were covered with vinyl bags. Kenaf seedlings, spraying with 0.004% ethanol, were used as control specimens. The stem tissues of plants receiving the respective treatments, including control specimens, were harvested for analysis at 1, 6, 24, and 48 h after treatment. All plant samples were frozen in liquid nitrogen and stored at -80°C.

RNA isolation

A protocol was designed based on modification of published methods (Bailey et al., 2005; Yang et al., 2008) to obtain RNA using a Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, USA). Harvested samples were ground in liquid nitrogen and sea sand using a mortar and pestle. Two types of extraction buffer were used for RNA extraction. Extraction buffer-I was prepared with 2% (w/v) CTAB, 0.1 M Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), and 2% (w/v) PVP. Extraction buffer II was prepared with 2% (w/v) CTAB, 0.1 M Tris-HCL (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH8.0), and 2% (w/v) PVPP. DEPC-treated water (0.1%) was used to make the solutions needed for RNA extraction**.** Extraction buffers-I, II and 2-mercaptoethanol (2%, w/v) were mixed and incubated at 65°C prior to use. The mixed extraction buffer solution was added to the ground samples, mixed vigorously, and incubated at 65°C for 20 min. An equal volume of chloroform was added, mixed vigorously by vortex, and then centrifuged at 8,000 × g for 30 min. After centrifugation, the supernatant was transferred into a new tube and the chloroform extraction step was repeated one more time. A one-third volume of DEPC-treated 8 M LiCl was added and incubated at 4°C overnight to

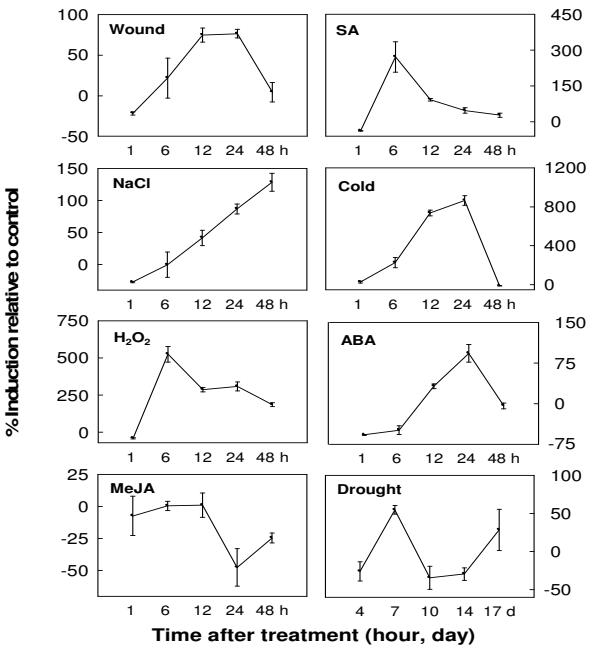

Fig 6. Expression pattern of kenaf *HCT* ortholog in response to various abiotic stress treatments. The level of *HcHCT* transcript in response to various abiotic stresses was examined in stem tissues of 3-week-old kenaf plants. *ACTIN* (DQ866836) was used as an expression control. Bar shows the average ± standard error of 3 biological replications.

precipitate RNA and then centrifuged at 8,000 × g, at 4°C for 1 h. The supernatant was discarded and 500 µL Qiagen buffer RLT with 1% (w/v) 2-mercaptoethanol was added to dissolve the pellet (Christensen et al., 2001). Pure ethanol (250 µL) was added to precipitate RNA, mixed well, and then transferred to a Qiagen RNeasy mini-column, followed by centrifugation of the column at 14,000 × g for 1 min. Then, the solution was removed, and 700 µL of Qiagen RW1 buffer was added to wash by centrifugation at 14,000 × g for 15 sec. Qiagen RPE buffer (500 µL) was added to the column to wash 2 times following centrifugation at 14,000 × g for 15 sec. The washing step was repeated following centrifugation at 14,000 × g for 2 min. RNA was eluted twice with 50 µL water, then treated with 80 µL DNase I (10 µL DNase I and 70 µL buffer; Roche Applied Science, Indianapolis, IN, USA), and incubated for 15 min at room temperature. RNA was treated with an equal volume of 3:1 phenol:$CHCl_3$ and centrifuged at 14,000 × g, at 4°C for 10 min. The supernatant was transferred into a new tube. RNA was precipitated by adding 20 µL of 3 M NaOAc (pH 5.5) and 600 µL of 100% ethanol, followed by incubation overnight at -80°C. RNA was collected by centrifugation at 14,000 × g, 4°C for 10 min, and the supernatant was discarded. The RNA pellet was washed with 80% ethanol. After washing and air-drying, RNA was suspended in 50 µL water.

Cloning

According to the manufacturer's instructions, 2 µg of total RNA was used for cDNA synthesis of the transcriptionally active *HCT* ortholog using Superscript® III First-Strand Synthesis Supermix (Invitrogen, Carlsbad, CA, USA). Gene specific degenerate primers [HCT-F, 5'- CG(T/A/C/G)A C(G/A/C)CT(A/C)CTCCG-3' and HCT-R, 5'-CCAAA(A/G)TC(T/A)GCATCAT-3'] were used and the primers were designed based on the consensus sequences of the *HCT* orthologs of *Clitoria ternatea* (AB185953), *Arabidopsis thaliana* (124270.3), *Coffea canephora* (EF137954), *Coffea arabica* (EF143341), and *Capsicum annuum* (EU616565). Both 5' and 3' RACE (rapid amplification of cDNA ends) were performed using the RACE system (Invitrogen) to clone a full-length of the *HCT* ortholog. Electrophoresis was conducted using the PCR product through 1.2% agarose gel for confirmation, and the PCR product was purified using the Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI, USA). After purification, the product was cloned into pGEM® -T easy Vector (Promega) and prepared for sequencing. DNA sequences were determined by Cosmogenetech Co. (Seoul, Korea).

QPCR analysis

QPCR conditions and data analysis were performed according to the previously described method (Bae et al., 2008). The Mx3000P QPCR system (Agilent, Santa Clara, CA, USA) with SYBR Green QPCR Master Mix (Agilent) was used to perform QPCR. Primer 3 software of Biology Workbench (http://workbench.sdsc.edu/) was used for designing the primers. The forward primer for the *HCT* ortholog was 5'-CTGGTTACTTTGGAAATGTGATCTT-3' and reverse primer was 5'-AAGGTTTGGACACTTGAA-TGTATGT-3'. As an expression control, a housekeeping gene, *ACTIN* (DQ866836), was used with the primer sequences: forward primer, 5'-ATGGACAAGTCATTACT--ATTGGAGC-3' and reverse primer, 5'-AGTGATTTCCTT-GCT CATACGG T-3'.

Data analyses

NCBI Blast (http://blast. ncbi.nlm.nih.gov/), Biology WorkBench, 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/) were used to analyze DNA and protein sequences. On the basis of amino acid sequences, a phylogenetic tree was constructed following the neighbor joining method in Mega5 (http://www.megasoftware.net/).

Acknowledgement

This work was supported by a grant from the Next-Generation Biogreen 21 Program (PJ00806302), Rural Development Administration, Republic of Korea.

References

Alvarez S, Marsh EL, Schroeder SG, Schachtman DP (2008) Metabolomic and proteomic changes in the xylem sap of maize under drought. Plant Cell Environ 31:325-340

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

Batard Y, Hehn A, Nedelkina S, Schalk M, Pallett K, Schaller H, Werck-Reichhart D (2000) Increasing expression of P450 and P450 reductase protein from monocots in heterologous systems. Arch Biochem Biophys 379:161-169

Caño-Delgado A, Penfield S, Smith C, Catley M, Bevan M (2003) Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. Plant J 34:351-362

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

Chen C, Meyermans H, Burggraeve B, De Rycke RM, Inoue K, De Vleesschauwer V, Steenackers M, Van Montague MC, Engler GJ, Boerjan WA (2000) Cell-specific and conditional expression of caffeoyl-coenzyme A-3-O-methyltransferase in Poplar. Plant Physiol 123:853-867

Chen F, Reddy MSS, Temple S, Jackson L, Shadle G, Dixon RA (2006) Multi-site genetic modulation of monolignol biosynthesis suggests new routes for formation of syringyl lignin and wall-bound ferulic acid in alfalfa (*Medicago sativa* L.). Plant J 48:113-124

Christensen JH, Overney S, Rohde A, Diaz WA, 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

Comino C, Lanteri S, Portis E, Acquadro A, Romani A, Hehn A, Larbat R, Bourgaud F (2007) Isolation and functional characterization of a cDNA coding a hydroxylcinnamoyl transferase involved in phenylpropanoid biosynthesis in *Cynara cardunculus* L. BMC Plant Biol 7:14

Cui S, Huang F, Wang J, Ma X, Cheng Y, Liu J (2005) A proteomic analysis of cold stress responses in rice seedlings. Proteomics 5:3162-3172

D'Auria J (2006) Acyltransferases in plants: a good time to be BAHD. Curr Opin Plant Biol 9:331-340

Dempsey JM (1975) Fiber crops, Rose printing company, Tallahase, FL.

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085-1097

Fan L, Linker R, Gepstein S, Tanimoto E, Yamamoto R, Neumann PM (2006) Progressive inhibition by water deficit of cell wall extensibility and growth along the elongation zone of maize roots is related to increased lignin metabolism and progressive stelar accumulation of wall phenolics. Plant Physiol 140: 603-612

Fernandez-Garcia N, Lopez-Perez L, Hernandez M, Olmos E (2009) Role of phi cells and the endodermis under salt stress in *Brassica oleracea*. New Phytol 181:347-360

Finkelstein RR, Rock CD (2002) Abscisic acid biosynthesis and response. In: CR Somerville, EM Meyerowitz (eds). The Arabidopsis book. American Society of Plant Biologists, Rockville, DOI 10.1199/tab.0058, http://www.aspb.org/publications/arabidopsis/

Gabaldón C, LópezSerrano M, Pomar F, Merino F, Cuello J, Pedreño MA, Ros Barceló A (2006) Characterization of the last step of lignin biosynthesis in *Zinnia elegans* suspension cell cultures. FEBS Lett 580:4311-4316

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

Gutiérrez A, Rodríguez IM, Del Río JC (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

Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol 41:187-223

Hoffmann L, Maury S, Martz F, Geoffroy P, Legrand M (2003) Purification, cloning, and properties of an acyltransferase controlling shikimate and quinate ester intermediates in phenylpropanoid metabolism. J Biol Chem 278:95-103

Hoffmann L, Besseau S, Geoffroy P, Ritzenthaler C, Meyer D, Lapierre C, Pollet B, Egrand M (2004) Silencing of hydroxycinnamoyl-Coenzyme A shikimate/quinate hydroxycinnamoyl- transferase affects phenylpropanoid biosynthesis. Plant Cell 16:1446-1465

Hu WJ, Harding SA, Lung J, Popko JL, Ralph J, Stokke DD, Tsai CJ, Chiang VL (1999) Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. Nat Biotechnol 17:808-812

Jiang Y, Joyce DC (2003) ABA effects on ethylene production, PAL activity, anthocyanin and phenolic contents of strawberry fruit. Plant Growth Regul 39:171-174

Kaplan F, Kopka J, Haskell DW, Zhao W, Schiller KC, Gatzke N, Sung DY, Guy CL (2004) Exploring the temperature stress metabolome of Arabidopsis. Plant Physiol 136:4159-4168

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

Lin P, Lin L, Wu J, Lin N (2004) Breeding of FuHong 4, a kenaf variety with high yielding and resistance. Plant Fiber Prod 26:1-4

Li Y, Kajita S, Kawai S, Katayama Y, Morohoshi N (2003) Down-regulation of an anionic peroxidase in transgenic aspen and its effect on lignin characteristics. J Plant Res 116:175-182

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

Mansfield SD (2009) Solutions for dissolution-engineering cell walls for deconstruction. Curr Opin Biotechnol 20:286-294

Marques G, Rencoret J, Gutiérrez A, del Río JC (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 JC, Bonine CA, de Oliveira Fernandes Viana J, Dornelas MC, Mazzafera P (2010) Abiotic and biotic stress and changes in the lignin content and composition in plant. J Integr Plant Biol 52:360-376

Nadeem Y, Igor AY, Krokene P, Kvaalen H, Halvor S, Carl GF (2012) Defence-related gene expression in bark and sapwood of Norway spruce in response to *Heterobasidion parviporum* and methyl jasmonate. Physiol Mol Plant Pathol 77:10-16

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

Nose M, Bernards MA, Furlan M, Zajicek J, Eberhardt TL, Lewis NG (1995) Towards the specification of consecutive steps in macromolecular lignin assembly. Phytochem 39:7179

Osakabea Y, Kajitab S, Osakabec K (2011) Genetic engineering of woody plants: current and future targets in a stressful environment. Physiol Plant 142:105-117

Perez-amador MA, Leon J, Green PJ, Carbonell J (2002) Induction of arginine decarboxylase *ADC2* gene provides evidence for the involvement of polyamines in the wound response in Arabidopsis. Plant Physiol 130:1454-1463

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

Ralph J, Akiyama T, Kim H, Lu F, Schatz PF, Marita JM, Ralph SA, Reddy MS, Chen F, Dixon RA (2006) Effects of coumarate 3-hydroxylase down-regulation on lignin structure. J Biol Chem 281:8843-8853

Rymsza TA (1999) Utilization of kenaf raw materials. Paper presented to the Forest Products Society. Boise, Idaho.

Salzman RA, Brady JA, Finlayson SA, Buchanan CD, Summer EJ, Sun F, Klein PE, Klein RR, Pratt LH, Cordonnier-Pratt MM, Mullet JE (2005) Transcriptional profiling of sorghum induced by methyl jasmonate, salicylic acid, and aminocyclopropane carboxylic acid reveals cooperative regulation and novel gene responses. Plant Physiol 138:352-368

Schoch G, Goepfert S, Morant M, Hehn A, Meyer D, Ullmann P, Werck-Reichhart D (2001) CYP98A3 from *Arabidopsis thaliana* is a 3'-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. J Biol Chem 276:36566-36574

Seo S, Sano S, Ohashi Y (1997) Jasmonic acid in wound signal transduction pathways. Physiol Plant 101:740-745

Shadle G, Chen F, Srinivasa Reddy MS, Jackson L, Nakashima J, Dixon RA (2007) Down-regulation of hydroxycinnamoyl CoA: Shikimate hydroxycinnamoyl transferase in transgenic alfalfa affects lignification, development and forage quality. Phytochem 68:1521-1529

Soltani BM, Ehlting J, Hamberger B, Douglas CJ (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

Song J, Wang Z (2009) Molecular cloning, expression and characterization of a phenylalanine ammonia-lyase gene (*SmPAL1*) from *Salvia miltiorrhiza*. Mol Biol Rep 36:939-952

Sticklen MB (2008) Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. Nat Rev Genet 9:433-443

St Pierre B, Laflamme P, Alarco A, De Luca V (1998) The terminal oacetyltransferase involved in vindoline biosynthesis defines a new class of proteins responsible for Coenzyme A dependent acyl transfer. Plant J 14:703-713

Sullivan M (2009) A novel red clover hydroxycinnamoyl transferase has enzymatic activities consistent with a role in phaselic acid biosynthesis. Plant Physiol 150:1866-1879

Suzuki H, Nakayama T, Nishino T (2003) Proposed mechanism and functional amino acid residues of malonyl-CoA: anthocyanin 5-O-glucoside-6-O-malonyltransferase from flowers of *Salvia splendens*, a member of the versatile plant acyltransferase family. Biochem 42:1764-1771

Theisen AA, Knox EG, Mann FL (1978) Feasibility of introducing food crops better adapted to environmental stress. Natl Sci Found, Div Appl Res NSF/RA-780289. U.S. Govt. Print. Office, Washington, DC

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50:573-599

Tronchet M, Balagué C, Kroj T, Jouanin L, Roby D (2010) Cinnamyl alcohol dehydrogenases C and D, key enzymes in lignin biosynthesis, play an essential role in disease resistance in Arabidopsis. Mol Plant Pathol 11:83-92

Uzal EN, Gómez Ros LV, Pomar F, Bernal MA, Paradela A, Albar JP, Ros Barceló A (2009) The presence of sinapyl lignin in *Ginkgo biloba* cell cultures changes our views of the evolution of lignin biosynthesis. Physiol Plant 135:196-213

Vance CP, Kirk TK, Sherwood RT (1980) Lignification as a mechanism of disease resistance. Annu Rev Phytopathol 18:259-288

Van Dam JEG, Van Vilsteren GET, Zomers FHA, Shannon WB, Hamilton IT (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, pp. 249

Vanholme R, Morreel K, Ralph J, Boerjan W (2008) Lignin engineering. Curr Opin Plant Biol 11:78-285

Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W (2010) Lignin biosynthesis and structure. Plant Physiol 153:895-905

Vicente MR, Plasencia J (2011) Salicylic acid beyond defence: its role in plant growth and development. pp. 1-18

Vincent D, Lapierre C, Pollet B, Cornic G, Negroni L, Zivy M (2005) Water deficits affect caffeate O-methyltransferase, lignification, and related enzymes in maize leaves: a proteomic investigation. Plant Physiol 137:949-960

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

Weng JK, Li X, Bonawitz ND, Chapple C (2008) Emerging strategies of lignin engineering and degradation for cellulosic biofuel production. Curr Opin Biotechnol 19:166-172

Wisniewski M, Bassett C, Gusta LV (2003) An overview of cold hardiness in woody plants: seeing the forest through the trees. Hortscience 38:952-959

Xu F, Sun RC, Zhai MZ, Sun JX, Jiang JX, Zhao GJ (2008) Comparative study of three lignin fractions isolated from mild ball-milled *Tamarix austromogoliac* and *Caragana sepium*. J Appl Polym Sci 108:1158-1168

Yang G, Zhou R, Tang T, Shi S (2008) Simple and efficient isolation of high-quality total RNA from *Hibiscus tiliaceus*, a mangrove associate and its relatives. Prep Biochem Biotechnol 38:257-64

Yanga H, Xiea S, Wanga L., Jing S, Zhua X, Li X, Zenga W, Yuana H (2011) Identification of up-regulated genes in tea leaves under mild infestation of green leafhopper. Sci Hortic 130:476-481

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