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Comparative transcriptional analysis of caffeoyl-coenzyme A 3-*O*-methyltransferase from *Hibiscus cannabinus* L., during developmental stages in various tissues and stress regulation

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Abstract

We have cloned a full-length gene, putatively encoding for caffeoyl-coenzyme A 3-*O*-methyltransferase (CCoAOMT), an important enzyme involved in lignin biosynthesis, from kenaf (*Hibiscus cannabinus* L.). Herein, we investigated the expression pattern of a *CCoAOMT* orthologue from various tissues and organs during development, and in response to different environmental cues. The full-length *CCoAOMT* orthologue of kenaf consists of a 744 bp open reading frame (ORF), encoding for 247 amino acids of 27.91 kDa and an isoelectric point (pI) of 5.43. The deduced amino acids of CCoAOMT evidenced a high degree of identity (up to 84%) with other plant CCoAOMT sequences. Phylogenetic analysis demonstrated its close relationship with the CCoAOMT of *Gossypium hirsutum* (ACQ59096). Kenaf CCoAOMT harbors eight highly conserved motifs: A, B, and C are putative S-adenosylmethioine (SAM)-binding motifs and D, E, F, G, and H are CCoAOMT signature motifs. According to quantitative real-time reverse transcriptase polymerase chain reaction (q-PCR) analysis, the kenaf *CCoAOMT* transcript was detected in all plant tissues and organs, whereas the highest expression was noted in mature flower tissues, which indicates that it might be involved in the flower development or in the biosynthesis of flower specific compound. All the treatments highly induced the expression of *CCoAOMT* transcripts in the stems of 3-week-old kenaf, which indicates that it might have a role in stress regulatory pathway. Among the treatments, the cold and H_2O_2-treated samples evidenced the highest levels of expression at 6 and 24 h after treatment, respectively, whereas the wounded and NaCl-treated samples evidenced lower expression levels, which suggest that different signaling networks are involved for stress mediated up regulation of *HcCCoAOMT* transcripts. The highest transcript level of *CCoAOMT* was detected at either early (within 12 h of treatments) or intermediate (24 h after treatments) time points of treatments, except drought treated sample. Early induction was observed in the case of H_2O_2 and SA (salicylic acid), and intermediate induction occurring as the result of wounding, NaCl, cold and ABA (abscisic acid). Whereas drought treated sample showed highest expression at seven days after treatment. MeJA (methyl jasmonic acid) treatment showed a complex biphasic expression which is different from others. In summary, we have cloned and characterized a full-length gene putatively encoding for CCoAOMT, which also showed stress responsive differential expression.

Keywords: kenaf (*Hibiscus cannabinus* L.), caffeoyl-coenzyme A 3-*O*-methyltransferase (CCoAOMT), real-time PCR, abiotic stresses.
Abbreviations: ABA- abscisic acid; CCoAOMT - Caffeoyl-coenzyme A 3-*O*-methyltransferase; MeJA-methyl jasmonate; SA-salicylic acid; Hc- *Hibiscus cannabinus.*

Introduction

Kenaf (*Hibiscus cannabinus* L.) is an annual dicotyledonous plant that grows in temperate and tropical areas, although it is native to Africa (Seca et al., 1998; Kobaisy et al., 2001). Nowadays, in an environment in which wood resources are continuously decreasing, kenaf has gradually become a great potential source for biomass production as an alternative raw material for pulp, due to its broad ecological adaptability from temperate to tropical regions, high rate of growth, and growing ability under adverse environmental conditions (Kuroda et al., 2002). In addition to its inner core fiber (75-60%, short fiber), outer bast fiber (25-40%, long fiber) is a good source of fiber, which makes it an important source of

renewable and inexpensive raw material for biomass production (Kuroda et al., 2002; Gutiérrez et al., 2004). Higher plant cell walls are composed of cellulose, hemicelluloses, pectin, and lignin, which provides structural integrity to the cell wall. Among them, lignin, the second most abundant terrestrial biopolymer after cellulose, is crucial to the stiffness and strength of the stem. Additionally, lignin waterproofs the cell wall, provides hydrophobicity to the vascular system, and performs a defensive role in protecting plants against pathogens (Boerjan et al., 2003; Neutelings et al., 2011). Although wood is employed as an important industrial raw material and a renewable energy source year after year, but the field of biochemical and molecular biological investigation of wood formation is in its infancy. Although researchers have studied lignin for more than a century, many unresolved questions remain. Lignin is one of the major obstacles to high-quality paper production, forage digestibility, and processing of plant biomass into biofuels. Although several procedures are currently available to extract lignin from the pulp, these methods are expensive and environmentally hazardous. In this context, the production of wood with less lignin or altered chemical reactivity, which produces easily degradable lignin, may be the solution to this problem (Boerjan et al., 2003; Vanholme et al., 2008). Lignins are complex racemic aromatic heteropolymers derived principally from three hydroxycinnamyl alcohol monomers, including *p*-coumaryl, coniferyl, and sinapyl alcohols, which are incorporated into the lignin polymer during polymerization as *p*-hydroxyphenyl H, guaiacyl G, and syringyl S phenylpropanoid units, respectively (Baucher et al., 2003; Boerjan et al., 2003). These 3 monolignols differ only in regard to their degree of methoxylation and are generated within the cell, and are subsequently transported to the cell wall for polymerization (Baucher et al., 2003). The composition and amount of lignin varies among taxa and cell types, and is also affected by certain developmental changes and external environmental cues (Neutelings et al., 2011). Generally, in dicotyledonous angiosperms, lignins are composed principally of G and S units with less H units, but in gymnosperms they are primarily composed of G units with low levels of H units. Monocotyledonous plants like grasses are composed of similar amounts of G and S units, but contain more H units than dicotyledonous plants (Boerjan et al., 2003). Lignin rich in G units, such as gymnosperm lignin, harbors relatively more carbon-carbon bonds than lignin rich in S units, which causes more a resistant linkage of G lignins compared to lignin composed of relatively unbranched S units (Baucher et al., 2003; Gutiérrez et al., 2004; Rencoret et al., 2009). Fibers with the lignins of higher S/G ratio are a good source for pulping (Rencoret et al., 2009; Stackpole et al., 2011). Lignin composition (H/G/S) and contents can be altered by the upregulation or downregulation of genes involved in lignin biosynthesis pathway via genetic engineering. Monolignols are produced from phenylalanine ammonia-lyase (PAL) through the phenylpropanoid pathway (Fig.1). Many significant approaches have been pursued by altering the gene expression of this pathway, and these approaches have produced diverse effects (Baucher et al., 2003; Vanholme et al., 2008). Caffeoyl-coenzyme A 3-*O*-methyltransferase (CCoAOMT) is one of the key enzymes in the lignin biosynthesis pathway, which transfers the methyl groups of S-adenosyl-L-methionine (SAM) to position 3 on the aromatic ring of monolignols and catalyzes caffeoyl-coenzyme A to feruluyl-CoA (Ye et al., 2001; Zhao et al., 2004). CCoAOMT belongs to the Class I O-methyltransferases (OMTs), based on amino acid sequence and protein molecular weight, and requires the Mg^{2+} cation for its activity (Zhao et al., 2004; Widiez et al., 2011). Previous research demonstrated that the downregulation of CCoAOMT in *Nicotiana tabacum*, *Arabidopsis thaliana*, *Medicago sativa*, and *Populus tremula* × *alba* resulted in the reduction of lignin contents compared to the wild-type (Pinçon et al., 2001; Wagner et al., 2011). Its downregulation also altered the monolignol ratios in alfalfa (Boerjan et al., 2003). In this study, we cloned and characterized the *CCoAOMT* orthologue in kenaf. Additionally, we also evaluated the expression pattern of the *CCoAOMT* orthologue from various tissues and organs during developmental processes. We also evaluated the expression of the *CCoAOMT* orthologue in response to different environmental cues (abiotic stress) and elicitors, such as wounding, SA (salicylic acid), NaCl, cold, H_2O_2, MeJA (methyl jasmonic acid), and ABA (abscisic acid) in 3-week-old stem tissues of kenaf plants.

Results and discussion

Cloning of a CCoAOMT orthologue from kenaf

Degenerate primers and the RACE (rapid amplification of cDNA ends) system were used to clone a full-length *CCoAOMT* orthologue of kenaf. The *CCoAOMT* orthologue consists of a 744 bp open reading frame (ORF) encoding for 247 amino acids (GenBank Accession No. XXX, 957 bp; Fig. 2). The predicted molecular weight of the deduced protein was 27.91 kDa, with an isoelectric point (pI) of 5.43, as calculated by the ExPASy Proteomics Server. The superfamily results indicated that this putative protein belongs to the S-adenosyl-L-methionine-dependent methyltransferase superfamily. InterProScan and MOTIF search results suggested that it might be an *O*-methyltransferase (OMT) family-3 member. Protein blast analysis demonstrated a high degree of sequence identity with the CCoAOMT family. Superfamily, InterProScan, Motif search and BLASTX results demonstrated that our deduced protein sequence might be categorized within the Class-I OMTs (Widiez et al., 2011). The methylation reaction is very important for many biochemical pathways which alter the hydrophilicity, flavor, and colors of many compounds (Widiez et al., 2011). In plants, there are basically 3 methyltransferases: *O*-methyltransferases (OMTs), N-methyltransferases (NMTs), and C-methyltransferases (CMTs). Among them, CCoAOMT belongs to the class A OMTs, according to substrate specificity (Barakat et al., 2011). In the lignin biosynthesis pathway, two enzymes function as methyltransferases: CCoAOMT and caffeic acid *O*-methyltransferase (COMT) (Baucher et al., 2003; Bonawitz and Chapple, 2010). CCoAOMT involves an earlier step in the lignin biosynthesis pathway as compared to COMT. CCoAOMT transforms caffeoyl CoA to feruloyl CoA, whereas COMT functions at the end of the pathway to produce sinapyl alcohol, the main component of S-type lignin. Although the COMT enzymes principally methylate caffeic acid and 5-HFA (5-hydroxylferulic acid), they can also methylate a variety of other substrates (Barakat et al., 2011). The deduced kenaf CCoAOMT orthologue shared 95, 90, 85, and 84 % identity with the CCoAOMT from *Gossypium hirsutum*, *Dimocarpus longan*, *Solanum lycopersicum*, and *Bambusa oldhamii*, respectively (Fig. 3). Based on the results of SignalP 3.0 analysis, the CCoAOMT orthologue might be a non-secretory protein. TargetP V1.1

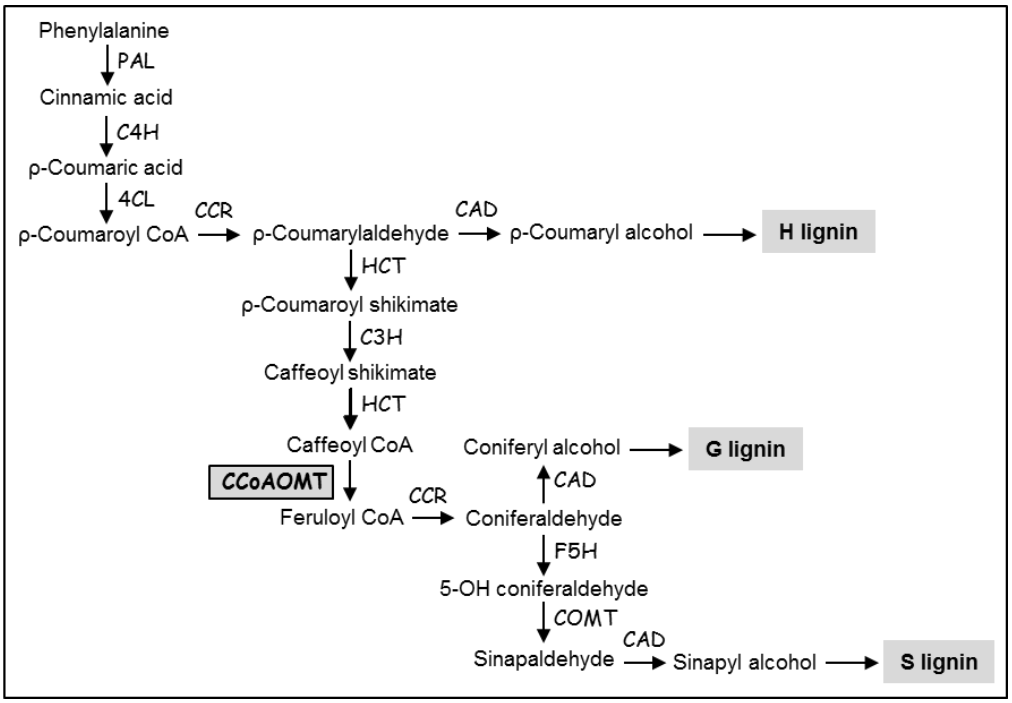

Fig 1. Monolignol biosynthetic pathway in dicotyledonous angiosperms. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; HCT, *p*-hydroxycinnamoyl-CoA: quinate shikimate *p*-hydroxycinnamoyltransferase; C3H, *p*-coumarate 3-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; COMT, caffeic acid *O*-methyltransferase; F5H, ferulate 5-hydroxylase. Modified from Neutelings, (2011)

analysis revealed that there is no significant signal peptide for subcellular localization which strongly suggests that the protein might be a cytoplasmic protein. This result is similar to the results of a previous report (Chen et al., 2000) in which it was demonstrated that CCoAOMT in the poplar tree is randomly distributed in the cytoplasm and not involved directly in membrane-bound multienzyme complexes. The CCoAOMT orthologue harbors 8 conserved motifs. Motifs A, B, and C are common in plant OMTs and known as a SAM binding site, whereas motifs D, E, F and G, H are CCoAOMT signature sequences (Joshi and Chiang, 1998; Zhao et al., 2004; Fig. 3). The consensus sequences for motifs A, B, and C were LVK(V/I/L)GGXX(G/A); V(A/L)P(P/A/D)DAP; and AL(A/P)(A/V)D (P/E/D/T)R(I/V)EI(C/S), respectively, which were very similar to the previous findings of Joshi and Chiang (1998). A phylogenetic tree was constructed using the CCoAOMT amino acid sequences of 19 plant species using Mega5. The kenaf CCoAOMT orthologue evidenced the closest relationship to *Gossypium hirsutum* (ACQ59096; Fig. 4). The kenaf CCoAOMT orthologue was grouped in a sub-cluster of 4 proteins: *Gossypium hirsutum*, *Corchorus capsularis*, and *Leucaena leucocephala*. According to the data, the kenaf CCoAOMT orthologue probably belongs to a CCoAOMT enzyme. From now on, the kenaf *CCoAOMT* orthologue is designated as *HcCCoAOMT*.

Ubiquitous expression of HcCCoAOMT

The expression pattern of *HcCCoAOMT* was analyzed in different plant tissues (root, stem, petiole, mature leaf, and mature flower) via q-PCR (Fig. 5). Although *HcCCoAOMT* was expressed in all plant parts, but the transcripts were detected at relatively high levels in mature flowers (open white flower; Fig. 5A). *HcCCoAOMT* transcripts in the developing stems gradually increased in level for up to 4 weeks, and then declined sharply at 8 weeks and maintained their lower expression levels for up to 20 weeks (Fig. 5B). Day et al. (2001) demonstrated that the expression levels of flax *CCoAOMT* in stem tissues were highest in the flowering stage, which was contradictory to our results. During flower and leaf development, the highest levels of *HcCCoAOMT* transcripts were noted in young flowers and intermediate leaves, respectively (Fig. 5C & 5D). Previous studies also showed that *CCoAOMT* was expressed in all tissues, including the anthers of rice, stems of ginger and alfalfa, rhizomes of ginger, leaves of *Nicotiana* and *Arabidopsis*, roots of *Arabidopsis*, and flowers and siliques of *Arabidopsis* (Maury et al., 1999; Day et al., 2001; Parvathi et al., 2001; Imin et al., 2006; Ramirez-Ahumada et al., 2006; Do et al., 2007). High levels of phenylpropanoid-derived compounds were detected in *Arabidopsis* flowers (Chapple et al., 1994). Northern blot analysis revealed that some members of the *CCoAOMT* gene family preferably expressed in tobacco flowers, specifically in pistil, stamen and petal (Martz et al., 1998). AtTSM1 encoding a CCoAOMT-like enzyme specifically expressed in tapetum of *Arabidopsis* flowers. It is plausible that the gene products might be involved in anther maturation or pollen grain development and biosynthesis of polyamine conjugates (Fellenberg et al., 2008). All of these findings indicate that *CCoAOMT* may play a vital role during flower development. This differential expression pattern in

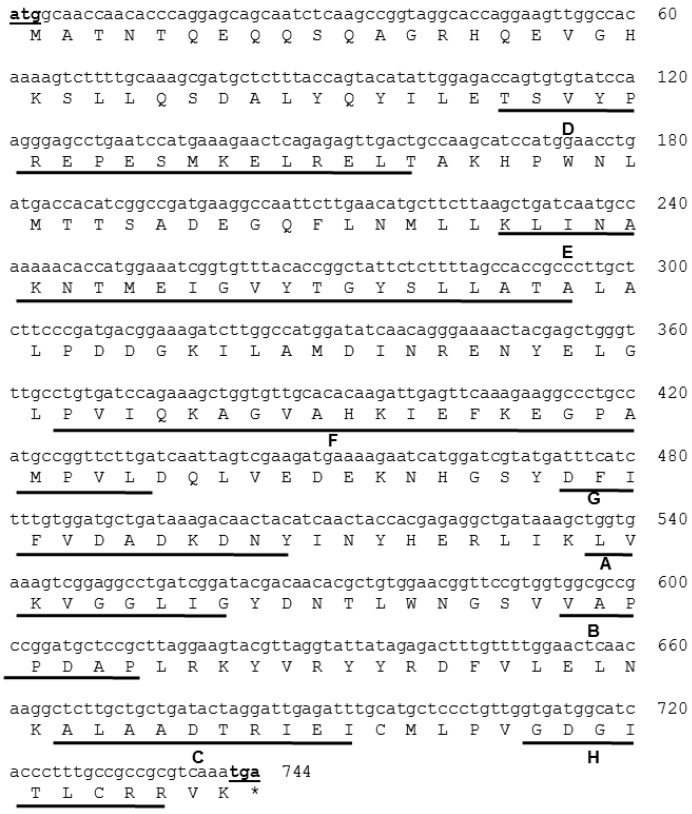

Fig 2. Full-length coding and deduced amino acid sequences of kenaf CCoAOMT orthologue. The start codon (ATG) and stop codon (TGA) are underlined and in bold. Putative SAM-binding motifs (A, B, C) and CCoAOMT signature motifs (D, E, F, G, H) are underlined.

the developing stem, flower and leaf indicated that *HcCCoAOMT* expression was spatio-temporal.

Induction of HcCCoAOMT in stem tissues in response to various abiotic stresses

The lignin biosynthesis pathway is an intricate network, in which several enzymes and proteins are involved, responding to various biotic and abiotic stresses and environmental cues (Moura et al., 2010). The plant cell wall is the first line of defense against microbial attack and adverse environmental cues, such as drought, cold, heat, etc. Lignin is the most important biopolymer present in the cell wall and creates a hydrophobic physiochemical barrier (Chen et al., 2000). Cell wall reinforcement and strengthening by lignin deposition at the affected site is a common mechanism of the stress-affected defense procedure (Chen et al., 2000; Desender et al., 2007). Various abiotic stresses are responsible for the ROS generation, which helps to random cross-linking of subunits and formation of lignin (Karuppanapandian et al., 2011). Many researchers have attempted to determine the roles of different enzymes involved in the lignin biosynthesis pathway under stress conditions. Herein, we also evaluated the role of *HcCCoAOMT* in response to different stresses and treatments such as wounding, SA, NaCl, cold, H_2O_2, MeJA, ABA, and drought stress using q-PCR. The stem tissues of 3-week-old kenaf plants were used.

We determined that *HcCCoAOMT* transcripts were upregulated by all treatments at the early or intermediate stage of treatments (Fig. 6). Among them, the cold and H_2O_2 treated samples evidenced the highest expression levels (24 h after treatment), and the wounding, NaCl, and MeJA-treated samples evidenced lower expression (24 h and 12/48 h after treatment, respectively) compared to other treatments. These treatments could be divided into 2 groups based on the timing of the maximum induction: i) early induction (6-12 h) by H_2O_2, SA; and ii) intermediate induction (24 h) by wounding, NaCl, cold, and ABA. Drought induced the *HcCCoAOMT* transcripts at 7 days after treatment and reduced them at 10 days after treatment. The expression pattern of *HcCCoAOMT* after MeJA treatment differs profoundly from the others. MeJA treatment evidenced the highest expression levels at 12 h after treatment and was significantly decreased at 24 h after treatment, although it was again expressed at higher levels at 48 h after treatment.

Wound

Mechanical wounding and pathogenic attack frequently evidence similar responses, such as the rapid accumulation of phenolic compounds, phytoalexins production, the expression of defense-related genes, hydrolytic enzyme synthesis, and lignin deposition at the injury or invaded site (Chen et al., 2000). It has been reported that several enzymes of lignin biosynthesis pathway were induced via wounding or microbial elicitors (Moura et al., 2010). Wounding induced the expression of different genes of the phenylpropanoid pathway, such as *PAL*, *C4H*, *F5H*, *CAD*, *CCR*, and *4CL* (Fig. 1) were already reported (Soltani et al., 2006; Moura et al., 2010). Chen et al. (2000) reported that chimeric *CCoAOMT* genes were strongly induced at the wound site of a transgenic poplar tree, followed by the deposition of lignin or lignin-like material at that site. Soltani et al. (2006) evaluated the expression pattern of the *Arabidopsis 4CL* (*At4CL*) family, an important member of the lignin biosynthesis pathway in response to injury induced by the perforation of the leaf with a pipette tip. Phytohormones such as SA, JA, ethylene, and ABA can be produced by a variety of stresses, and they are directly or indirectly interconnected with each other in different steps of the signaling cascade which resulted in the efficient perception, propagation, and control of signals (Mahajan and Tuteja, 2005; Shao et al., 2007; Bari and Jones, 2009). All of these phytohormones are related to changes in cell wall composition, structure, and ectopic lignin production during different biotic and abiotic stresses (Denness et al., 2011).

MeJA (methyl jasmonate)

Wounding and JA signaling are interlinked with one another. Wound-mediated defense gene expression is controlled by either a JA-dependent or a JA-independent pathway (Perez-Amador et al., 2002; Soltani et al., 2006). In our experiment, MeJA-treated kenaf stem evidenced biphasic expression of *HcCCoAOMT*, peaking at 12 h and again at 48 h. A similar type of coordinate biphasic expression pattern was noted for *At4CL1* and *At4CL2* during wound response (Soltani et al., 2006). The biphasic wounding response of genes involved in the phenylpropanoid pathway has also been reported in parsley and the Jerusalem artichoke (*Helianthus tuberosus*) (Soltani et al., 2006). The biphasic expression pattern of

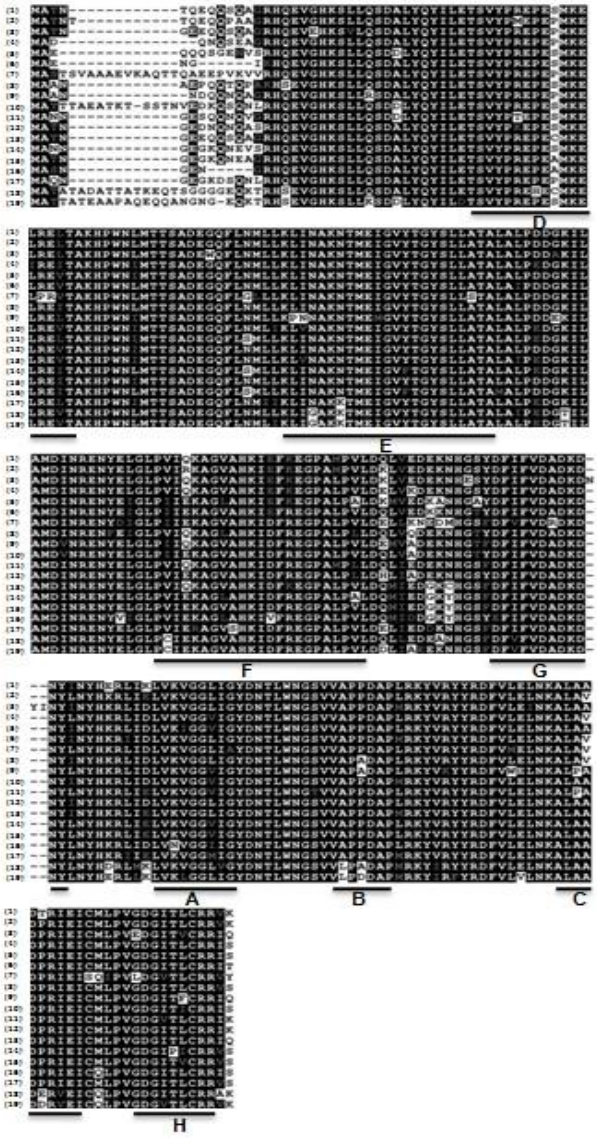

Fig 3. Multiple alignment of the deduced amino acid sequences of the kenaf CCoAOMT orthologue. Alignment with other plants was conducted using the ClustalW and BOXSHADE sequence alignment programs from Biology WorkBench. Identical amino acids are highlighted in black. Conserved SAM-binding motifs (A, B, C) and CCoAOMT signature motifs (D, E, F, G, H) are underlined. GenBank accession numbers are represented as follows: 1, *Hibiscus cannabinus* (JQ779020); 2, *Gossypium hirsutum* (ACQ59096); 3, *Corchorus capsularis* (AAR91504); 4, *Leucaena leucocephala* (ABE60812); 5, *Linum usitatissimum* (AAY89237); 6, *Nicotiana tabacum* (AAB80931); 7, *Pinus taeda* (AAD02050); *8, Eucalyptus camaldulensis* (ACY66929); 9, *Jatropha curcas* (ACV50428); 10, *Brassica rapa subsp. Pekinensis* (ABE41833); 11, *Dimocarpus longan* (AEK52482); 12, *Betula luminifera* (ACJ38669); 13, *Populus trichocarpa* (XP002313125); 14, *Boehmeria nivea* (AAT75320); 15, *Broussonetia papyrifera* (AAT37172); 16, *Solanum lycopersicum* (ABV90868); 17, *Coffea canephora* (ABO77959); 18, *Bambusa oldhamii* (ABO26812); 19, *Zea mays* (CAB45149).

HcCCoAOMT may be attributable to the involvement of multiple JA signaling pathways (Soltani et al., 2006).

Drought

CCoAOMT transcripts were induced via drought stress in the seedlings of 2-year-old maritime pine, maize leaves, *Nicotiana benthamiana*, and roots of *Arabidopsis* (Costa et al., 1998; Vincent et al., 2005; Senthil-Kumar and Mysore, 2010). Knock-down by RNA interference and mutants of *CCoAOMT* of *Nicotiana tabacum* and *Arabidopsis* showed the roles of *CCoAOMT* in drought stress (Senthil-Kumar et al., 2010). In the soybean primary root, an increasing abundance of *CCoAOMT* occurred under both water-stressed and well-watered roots (Yamaguchi et al., 2010).

Salt

Salinity is one of the biggest obstacles to agriculture around the world. Extensive research into salinity stress has identified several genes that responded to this stress. Increased lignification or altered monomeric composition of lignin is one of the most important mechanisms of plant cell wall to overcome salinity stress (Neves et al., 2010). Treatment with 150 - 200 mM NaCl induced a 72 -90% increase of lignin contents in soybean roots (Neves et al., 2010). Several genes involved in the phenylpropanoid pathway evidenced a high degree of responsiveness toward NaCl stress. Upregulation of SAMS in tomato and *Tamarix hispida* and *COMT* genes in *T. hispida* were noted during salt stress (Sánchez-Aguayo et al., 2004; Li et al., 2009). The overexpression of *IbLEA14* (*Ipomoea batatas late embryogenesis abundant 14*) that is responsive to dehydration, NaCl, and ABA in transgenic sweet potato calli evidenced increased *CAD* expression when treated with 300 mM NaCl (Park et al., 2011).

ABA (abscisic acid)

Many internal phytohormone levels fluctuate in response to different stresses which help to amplify the initial signal (Mahajan and Tuteja, 2005). Among them, ABA, SA, JA, and ethylene are the most important, and act within an intricate network via both synergistic and antagonistic interactions (Ton et al., 2009). ABA plays a prominent role in both the biotic and abiotic signaling cascade, although the internal ABA level fluctuates primarily as the result of drought and salt stress in plants (Seki et al., 2007; Ton et al., 2009; Urano et al., 2010). Detached leaves of *Camptotheca acuminate* evidenced increased expression of ferulate 5-hydroxylase (*F5H*), when treated with 10 μM 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, 2005). The bioinformatic analysis of promoter sequences of the *Populus CAD* genes identified the ABA-responsive motif in the promoter sequence (Barakat et al., 2009). Although our result indicated that ABA induced *HcCCoAOMT* expression, some previous studies have demonstrated that ABA inhibited lignin biosynthesis and *PAL1* and *4CL* expression (Mohr and Cahill, 2007).

Cold

A great deal of evidence has been found to suggest that lignin contents can change due to low temperatures, although little is currently known regarding the role of lignin metabolism during the cold acclimation process (Moura et al., 2010).

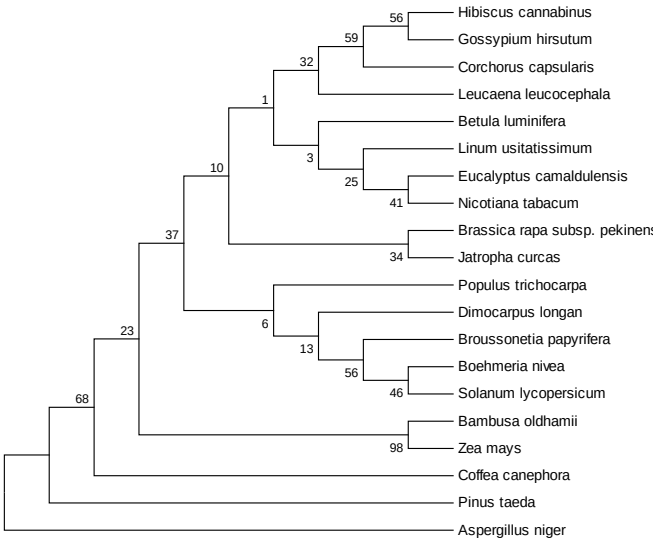

Fig 4. Phylogenetic analysis of the deduced amino acid sequences of kenaf CCoAOMT orthologue and other plants. The tree was constructed via the neighbor-joining method using ClustalW and Mega5. The numbers at the nodes indicate bootstrap values from 1000 replications. GenBank accession numbers are represented as follows: *Hibiscus cannabinus* (JQ779020); *Gossypium hirsutum* (ACQ59096); *Corchorus capsularis* (AAR91504); *Leucaena leucocephala* (ABE60812); *Linum usitatissimum (*AAY89237); *Nicotiana tabacum* (AAB80931); *Pinus taeda* (AAD02050); *Eucalyptus camaldulensis* (ACY66929); *Jatropha curcas* (ACV50428); *Brassica rapa subsp. Pekinensis* (ABE41833); *Dimocarpus longan* (AEK52482); *Betula luminifera* (ACJ38669); *Populus trichocarpa* (XP002313125); *Boehmeria nivea* (AAT75320); *Broussonetia papyrifera* (AAT37172); *Solanum lycopersicum* (ABV90868); *Coffea canephora* (ABO77959); *Bambusa oldhamii* (ABO26812); *Zea mays* (CAB45149). *Aspergillus niger O*-methyltransferase (XP001398534) sequence was used as an outgroup for rooting the tree.

Low temperature has been shown to exert a miscellaneous effect on plant lignin content. The induction of genes involved in lignin biosynthesis has been reported in previous studies: the induction of *PAL* in *Brassica napus* and *Glycine max*; *PAL*, *4CL*, *HCT*, *CCR* and *CAD* in the leaf of winter barley cv. Luxor; *C3H* in *Rhododendron* and the *IbCAD1* gene in the sweet potato (Wei et al., 2006; Kim et al., 2010; Moura et al., 2010; Janská et al., 2011). Additionally, an increase in lignin content under low-temperature conditions was noted *ex vitro* in poplar (*Populus tremula* × *P.tremuloides* L. cv. Muhs1) seedlings grown at 10°C (Moura et al., 2010). Although *COMT* and *CCoAOMT* were downregulated in the leaves of winter barley cv. Luxor, *CCoAOMT* was shown to be upregulated after 4 days of cold treatment in the anthers of rice (Imin et al., 2006; Janská et al., 2011). In summary, we have cloned a full-length gene putatively encoding for Caffeoyl-coenzyme A *O*-methyltransferase (CCoAOMT), an important enzyme involved in lignin biosynthesis, derived from *Hibiscus cannabinus*. In order to alter lignin content and composition, *CCoAOMT* downregulation may be an excellent target for genetic manipulation (Moura et al., 2010). Although *CCoAOMT* evidenced differential upregulation when exposed to various stresses, further investigation will clearly be necessary.

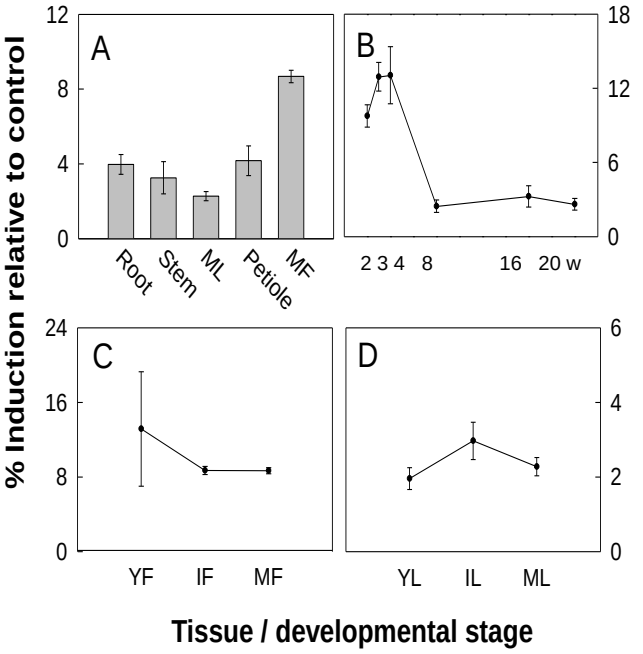

Fig 5. Transcript expression patterns of the kenaf *CCoAOMT* orthologue in various tissues and organs during developmental stages. Relative transcript levels were measured via q-PCR, and *ACTIN* transcripts were used as internal controls. The transcript levels of the kenaf *CCoAOMT* orthologue were adjusted after deduction of the control transcript level. (A) expression pattern of kenaf *CCoAOMT* orthologue during stem development (2, 3, 4, 8, 16, 20 weeks after sowing), (B) expression pattern of kenaf *CCoAOMT* orthologue during flower development (YF, young flower; IF, immature flower; MF, mature flower), (C) expression pattern of the kenaf *CCoAOMT* orthologue during leaf development (YL, young leaf; IL, immature leaf; ML, mature leaf), and (D) expression pattern of the kenaf *CCoAOMT* orthologue in various tissues and organs from 16-week-old kenaf plants. Bars show the means ± standard error of 3 biological replications.

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 provided by Dr. Si-Yong Kang (Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup 580-185, Korea). Seeds were germinated in a flat system consisting of a precisely arranged array of 32 individual 58 × 58 × 63 mm pots in a cavity tray format. Plants were grown in a sterile non-soil mixture (TOBIETEC, Chungbuk, Korea) in a growth room for up to 4 weeks with watering twice a week under the following conditions: 16-h light /8-h dark photoperiod, 22°C, and 100 μmol m^{-2} s^{-1} light intensity. Four weeks later, kenaf seedlings were transplanted into 20-cm pots, filled with the non-soil mixture and allowed to grow in natural sunlight for up to 20 weeks under greenhouse conditions, with watering twice a week. Sixteen-week-old kenaf plants grown in the greenhouse were used for the harvesting of a variety of tissue samples (root, petiole, leaf and flower). Leaf and flower samples were harvested at 3 developmental stages. Three developmental stages of leaves

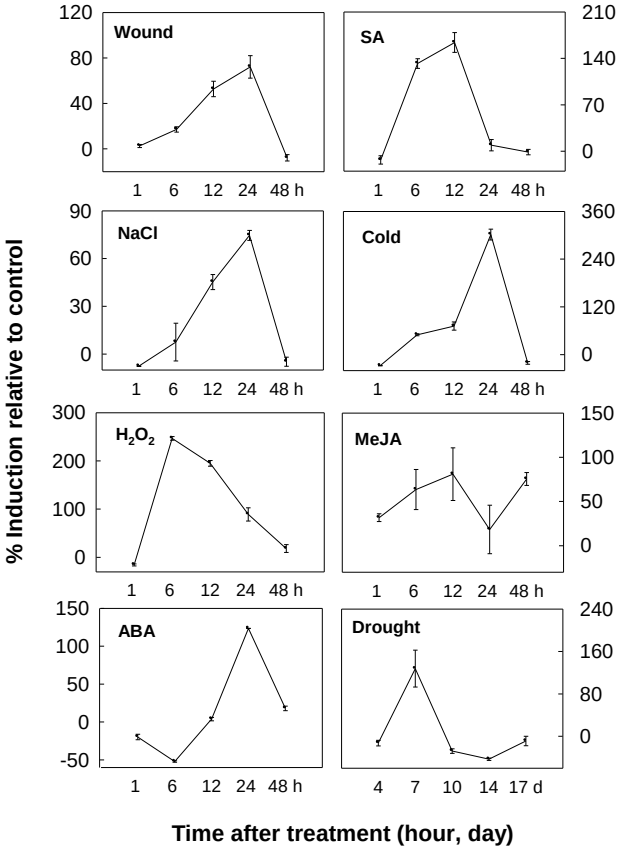

Fig 6. Transcript expression patterns of the kenaf *CCoAOMT* orthologue in response to various abiotic stresses. Relative transcript levels were measured via q-PCR, and *ACTIN* transcripts were used as internal controls. The transcript levels of the kenaf *CCoAOMT* orthologue were adjusted after deduction of the control transcript level. Three-week-old stem tissues were treated with different stresses, such as wounding, SA, NaCl, cold, H_2O_2, MeJA, ABA, and drought. Bars show the means ± standard error of 3 biological replications.

were as follows: 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 separated into 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 (open white flower).

Various treatments

Three-week-old kenaf seedlings grown in the growth room were treated with various abiotic stresses, and stem tissues were harvested for q-PCR analysis. For various chemical treatments kenaf seedlings were watered with ABA (100 µM), H_2O_2 (10 mM), SA (5 mM), or NaCl (200 mM) to evaluate the effects of various stresses on *CCoAOMT* expression (Gao et al., 2009; Zheng et al., 2010). For experimental control, kenaf seedlings watered with distilled water were used. For cold treatment, 3-wk-old kenaf seedlings were exposed to 10°C for 1, 6, 24, and 48 h in the dark. Kenaf stems, cut twice longitudinally with scissors, were employed as wound-treated samples.100 µM MeJA dissolved in 0.004% ethanol was sprayed on kenaf seedlings. MeJA treated seedlings were covered with a vinyl bag immediate after spraying and stem tissues were harvested at 1, 6, 24, and 48 h after treatments. Plants sprayed with 0.004% ethanol were used as controls for MeJA treatment. Drought treatment was applied by stopping to water 3-week-old plants and samples were harvested at 4,7,10, and 14 days. Control for drought samples was grown in normal condition with watering once in every 3 days interval. Untreated and treated plant samples were collected, frozen in liquid nitrogen, and stored at -80 °C for RNA purification.

RNA isolation

The RNA extraction method used herein was a modified version of previously published methods (Bailey et al., 2005; Yang et al., 2008); a Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, USA) was used. To prepare all solutions DEPC-treated (0.1%) water was used. Before using, extraction buffers-I [2% (w/v) CTAB, 0.1 M Tris-HCl (pH 8), 1.4 M NaCl, 20 mM EDTA (pH 8), 2% (w/v) PVP], extraction buffer-II [% (w/v) CTAB, 0.1 M Tris-HCl (pH 8), 1.4 M NaCl, 20 mM EDTA (pH 8), 2% (w/v) PVPP] and 2-mercaptoethanol (2%, w/v) were mixed well, and incubated at 65°C. The kenaf samples were ground in liquid nitrogen with sea sand using a mortar and pestle. The pre-warmed extraction buffers were added immediately to the ground samples, mixed vigorously, and incubated for 20 min at 65°C. An equal volume of chloroform was added, mixed very well, and centrifuged for 30 min at 8,000 × g. The supernatant was transferred into a new tube and the chloroform extraction step was repeated one more time. For the precipitation of RNA, a one-third volume of DEPC-treated 8 M of LiCl was added and incubated overnight at 4°C and subsequently centrifuged for 1 h at 8,000 × g and 4°C. After removing the supernatant pellet was dissolved in 500 µL Qiagen buffer RLT with 1% (w/v) 2-mercaptoethanol (Christensen et al., 2001). Then, 250 µL of 100% ethanol was added, mixed well, and transferred to a Qiagen RNeasy mini-column. The column was centrifuged at 14,000 × g for 1 min, washed with 700 µL of Qiagen RW1 buffer, and again centrifuged at 14,000 × g for 15 sec. To wash the column, 500 µL of Qiagen RPE buffer was added to the column and centrifuged at 14,000 × g for 15 sec two times. Additional centrifugation was conducted at 14,000 × g for 2 min. After elution of RNA (twice with 50 µL water), the RNA was treated with 80 µL DNase I (10 µL DNase I and 70 µL buffer; Roche Applied Science, Indianapolis, IN, USA) and incubated at room temperature for 15 min. RNA was treated with an equal volume of 3:1 ratio of phenol: chloroform, centrifuged for 10 min at 14,000 × g at 4°C, and the supernatant was transferred into a new tube. RNA was precipitated overnight by adding 20 µL of 3 M NaOAc (pH 5.5) and 600 µL of 100% ethanol at -80°C. Supernatant was removed after 10 min of centrifugation at 14,000 × g, at 4°C. The RNA pellets were washed in 80% ethanol, and then air-dried for 10 min in laminar air flow. After dissolving the pellet in 50 µL of water, the RNA integrity was checked by running in agarose gel.

Cloning

Two micrograms of RNA were used for cDNA synthesis using Superscript® III First-strand synthesis supermix (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Gene-specific primers were used to amplify cDNA (CCoAOMT-F, 5'-GAA(G/A)G CTGGTGT(T/C)G-3'; CCoAOMT-R, 5'-CACTAGCTTC-

ATTAG(T/C)CTCTCG-3'). The degenerate primers were designed based on the consensus sequences of the *CCoAOMT* orthologues of *Arabidopsis thaliana* (NM105469), *Raphanus sativus* (FD940747), and *Brassica napus* (CX193510). The PCR product was confirmed by running in a 1.2% agarose gel and then purified using Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI, USA), and cloned into pGEM®-T easy Vector (Promega). Further DNA sequencing was performed and sequences were analyzed by Cosmogenetech Co. (Seoul, Korea). For the cloning of the full-length *CCoAOMT* orthologue, both 5' and 3' RACE were used in accordance with the manufacturer's instructions (Invitrogen).

q-PCR analysis

q-PCR was performed as described previously (Bae et al., 2008). Mx3000P q-PCR System (Agilent, Santa Clara, CA, USA) with SYBR Green q-PCR Master Mix (Agilent) were used. Primers were designed using Primer 3 software from Biology Workbench (http://workbench.sdsc. edu/). The forward and reverse primers of the *CCoAOMT* orthologue were as follows: forward primer, 5'-TCCGCTTAGGAAG-TACGTTAGGTAT-3'; reverse primer, 5'-ACATATAAT-CTTGTCAACCTTGGGC-3'. *Actin* (DQ866836) transcript was used to quantify the expression levels of *HcCCoAOMT* under various treatments with the primer sequences: forward primer, 5'-ATGGACAAGTC ATTACTATTGGAGC -3'; reverse primer, 5'-AGTGATTTCCTTGCTCATACGGT-3'.

Data analyses

DNA and protein sequences were analyzed using Superfamily (http://supfam. org/ SUPERFAMILY/index. html), InterProScan (http://wwwdev.ebi.ac.uk/interpro/), MOTIF (http://www.genome.jp/tools/motif/), NCBI Blast (http://blast. ncbi.nlm.nih.gov/), Biology WorkBench, ExPASy Proteomics Server (http://expasy.org/tools/ pi_tool.html), SignalP 3.0 (http://www.cbs.dtu.dk/services/ SignalP/), and TargetP V1.1 (http: //www.cbs.dtu.dk /services/ TargetP/). Based on amino acid sequences, a phylogenetic tree was generated via the neighbor joining method with1000 bootstrap values in Mega5 (http://www.megasoftware.net/).

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