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Molecular cloning, characterization and expression of the caffeic acid O-methyltransferase (*COMT*) ortholog from kenaf (*Hibiscus cannabinus*)

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Abstract

We cloned the full-length of the gene putatively encoding caffeic acid O-methyltransferase (*COMT*) from kenaf (*Hibiscus cannabinus* L.) using degenerate primers and the RACE (rapid amplification of cDNA ends) method. COMT is an important methylating enzyme in the phenylpropanoid pathway that belongs to the SAM (S-adenosyl L-methionine)-dependent methyltransferases family. We investigated the expression pattern of kenaf *COMT* during developmental stages in different tissues and organs as well as in response to diverse abiotic stresses [wounding, salicylic acid (SA), NaCl, cold, H_2O_2 and methyl jasmonate (MeJA)]. The full-length *COMT* ortholog is composed of a 1,098-bp open reading frame (ORF) encoding 365 amino acids. The deduced amino acid sequence indicated that kenaf COMT had the highest similarity (95%) with that of *Gossypium hirsutum*. Three-week-old stem tissues were used to analyze *COMT* ortholog expression upon abiotic stresses. The highest level of *COMT* transcript (32%, relative to *ACTIN*) was detected at an early stage (4-week old) during stem development. The transcript levels of *COMT* ortholog were almost constant during the developmental stages of leaves and flowers, while early induction of the *COMT* ortholog was detected following wounding, SA, cold and H_2O_2 treatments, and MeJA led late induction and NaCl led to intermediate induction of the *COMT* ortholog.

Keywords: kenaf (*Hibiscus cannabinus*), phenylpropanoid pathway, COMT (caffeic acid O-methyltransferase), lignification, abiotic stresses.
Abbreviations: ABA_abscisic acid; COMT_Caffeic acid 3-*O*-methyltransferase; MeJA_methyl jasmonate; SA_salicylic acid; Hc_ *Hibiscus cannabinus.*

Introduction

There is currently a great deal of interest in development of sustainable bioenergy using renewable resources for substitution of traditional energy sources. For bioethanol production, the biomass of diverse plant species, including corn stover, sugar cane bagasse, trees and grasses, has been exploited, and these are potential resources for lignocellulosic ethanol production (Wan et al., 1983; Lynd et al., 1991; Demirbas et al., 2005; Somerville et al., 2006; Somerville et al., 2007). Nonetheless, it is not easy to determine which plants have the greatest efficiency for biomass production. In addition, further studies are required to identify efficient methods of converting plant lignocellulose into bioethnaol. Such efforts can provide renewable and carbon-neutral energy sources for use as alternatives to traditional energy sources. Kenaf (*Hibiscus cannabinus* L.) is a rapidly growing herbaceous annual dicotyledonous plant that has a wide ecological habitat ranging from temperate to tropical areas (Dempsey et al., 1975). Kenaf is believed to have great potential for biomass production, though it has not yet been used for bioethanol production (Francois et al., 1992; Lam et al., 2002; Araki et al., 2005). Keanf is also an important crop in the pulp and paper industries due to the quality of the fibers it produces (Pande et al., 1996; Ahmed et al., 1998), which include long fibers produced in its outer stem and short fibers in its core (Anterola et al., 2002; Apel and Hirt, 2004). Vascular plant cell walls are composed of cellulose, hemicellulose, pectin and lignin (Popper et al., 2011), which give the cell walls their structural integrity. Cellulose is the most abundant biopolymer, while lignin is the second most abundant and gives the stem its stiffness and strength. Lignin imparts hydrophobic properties to the vascular system, and is closely related to plant defense systems against pathogens (Boerjan et al., 2003; Neutelings et

al., 2011). However, lignin is one of the major obstructions to paper production, forage digestibility, and bioethanol production. Because lignin is adsorbed onto polysaccharides, the ability of hydrolytic enzymes to approach the cellulose polymers is decreased. In addition, the co-products produced during the lignin removal step inhibit the processes of saccharification and fermentation. Therefore, lignin must be reduced to enable successful biological conversion. It is less efficient to remove lignin from pulp or other biomass due to and the expense and associated environmental pollution. Accordingly, development of plants that produce less lignin or more easily degradable lignin would be useful (Boerjan et al., 2003; Vanholme et al., 2008). Lignin is a racemic aromatic heteropolymer commonly found in angiosperms that is derived from three hydroxycinnamyl alcohol monomers, *p*-coumaryl, coniferyl and sinapyl alcohols. These alcohol monomers are integrated into lignin polymers by peroxidase and laccase during polymerization and then form ρ-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively (Baucher et al., 2003; Boerjan et al., 2003). The efficiency of the pulping process can be affected by the amount of lignins in fibers. In kanef, bast fibers have less than 11% of lignin content with a high amount of cellulose, and the lignin consists of 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; Marques et al., 2010). 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), kenaf fiber is a good biomass for delignification. Diverse secondary metabolites are synthesized via the phenylpropanoid pathway (reviewed by Vogt, 2010), while monolignols such as ρ-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) are produced through the phenylpropanoid pathway. This pathway begins with cinnamic acid synthesized by deamination of phenylalanine, and is followed by a series of ring hydroxylations, *O*-methylations and side-chain modifications (Hisano et al., 2009). In plants, lignin production is conducted by two enzymes that convert cinnamic acid into hydroxylated and methylated monolignol derivatives. Two types of transmethylation reactions occur during monolignol formation. Among the two enzymes involved in transmethylation, caffeoyl-CoA-O-methyltransferase (CCoAO-MT) catalyzes synthesis of feruloyl-S-CoA from caffeoyl-S-CoA, while caffeic acid O-methyltransferase (COMT) catalyzes the formation of sinapyl aldehyde through transmethylation of 5-hydroxyconiferyl aldehyde (Barriere et al., 2004; Rastogi et al., 2008). COMT enzymes also convert caffeyl aldehyde into coniferaldehyde, which is a precursor of 5-hydroxyconiferyl aldehyde. COMT uses S-adensoylmethionine as a methyl donor to catalyze the transmethylation of a number of phenolic compounds, including monolignaol precursors. While COMT enzymes are usually encoded by multiple genes in plants (Zubieta et al., 2002; Lam et al., 2007; Rastogi et al., 2008), only a few genes are directly related to lignification (Bout et al., 2003; Chen et al., 2006; Palmer et al., 2008; Shen et al., 2009). Down-regulation of the *COMT* gene in tobacco, alfalfa and maize has been shown to lead to decreased total lignin content with a decreased S/G ratio (Li et al., 2008). Moreover, changes in lignin content and ratio were found to induce high digestibility of lignin and increased incorporation of 5-hydroxyconiferyl alcohol into lignin in both alfalfa and maize, indicating that 5-hydroxyconiferyl aldehyde may be the major native substrate involved in lignifying COMTs (Li et al., 2008). Here, a putative *COMT* ortholog in kenaf was cloned and characterized. We also observed the expression patterns of a *COMT* ortholog in various developmental stages and tissues and under different abiotic stresses including wounding, SA (salicylic acid), NaCl,

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ctcctgtcggaaaca atg ggttcaacgggtgaaacccaaatgactcccacccaagtctcc 61
                M  G  S  T  G  E  T  Q  M  T  P  T  Q  V  S
gacgaggaggccaacttattcgccatgcagctcgccagtgcctccgtcctccccatggtc 121
  D  E  E  A  N  L  F  A  M  Q  L  A  S  A  S  V  L  P  M  V
ctcaagtctgccattgagcttgacctgctcgagatcatggccaaagctggtcccggtgct 181
  L  K  S  A  I  E  L  D  L  L  E  I  M  A  K  A  G  P  G  A
ttcctttcccctatggaagtcgcttcccagctccccaccgccaaccccgatgcacccgtc 241
  F  L  S  P  M  E  V  A  S  Q  L  P  T  A  N  P  D  A  P  V
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accctccccgacggcagagtcgagagactctacggcctcggccctgtctgcaaattctta 361
  T  L  P  D  G  R  V  E  R  L  Y  G  L  G  P  V  C  K  F  L
accaagaatcaagatggtgttgctctttctgctctcagtcttatgaatcaagacaaggtt 421
  T  K  N  Q  D  G  V  A  L  S  A  L  S  L  M  N  Q  D  K  V
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  L  M  E  S  W  Y  Y  L  K  D  A  V  L  E  G  G  I  P  F  N
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  K  A  Y  G  M  T  A  F  E  Y  H  G  T  D  P  R  F  N  K  V
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  F  N  R  G  M  S  D  H  S  T  I  T  M  K  K  I  L  E  T  Y
gatggcttcgacgggctcaaaacactggtcgatgtcggcggtggtaccggagccacaatt 661
  D  G  F  D  G  L  K  T  L  V  D  V  G  G  G  T  G  A  T  I
aacatgatcgtctccaagcacccttccataaagggcattaacttcgatttgcctcatgtc 721
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  V  P  K  G  D  A  I  F  M  K  W  I  C  H  D  W  S  D  E  H
tgctcgaaatttttgaacaagtgctacgaagcgttgccagacaacggaaaagtaatcgtt 901
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  I  D  C  I  M  L  A  H  N  P  G  G  K  E  R  T  Q  K  E  F
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  E  A  L  A  K  G  A  G  F  Q  G  F  R  V  K  C  C  A  F  G
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  T  Y  I  M  E  F  L  K  R  V  *
ctcggaactattattttcgtgattgtcttgctcgaattatgttcc 1186
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Fig 1. The full-length cDNA and deduced amino acid sequence of kenaf *COMT* ortholog. The start codon (ATG) and stop codon (TAA) are underlined and in bold. The SAM binding ligand (LVDVGGGTG) is boxed.

cold, H_2O_2, ABA (abscisic acid), methyl jasmonate (MeJA) and drought.

Results

Cloning of a full-length kenaf COMT ortholog

Degenerate primers and RACE (rapid amplification of cDNA ends) were used to clone the full-length of the kenaf *COMT* ortholog. Kenaf *COMT* ortholog (GenBank Accession No. JX524278) is composed of a 1,098-bp open reading frame (ORF) encoding 365 amino acids (Fig. 1). ExPASy analysis indicated that the expected molecular weight of the deduced protein was 39.98 kDa with an isoelectric point (pI) of 5.42. PROSITE analysis suggested that the putative protein may belong to the Class I SAM (S-adenosyl L-methionine)-dependent methyltransferases family. Methyltransferases can be divided into five classes based on the structure of their catalytic domain (Schubert et al., 2003). ExPASy analysis also predicted Gly (residue 208), Glu (residue 231), Asp (residue 251), Met (residue 252), Lys (residue 265), and S-adenosyl-L-methionine binding sites and a His (residue 269) proton acceptor active site (Fig. 1 and 2). Nine previously described amino acid residues (LVDVGGGTG) including the SAM binding site matched the sequences of the putative COMT protein (residues 204-212; Fig. 1 and 2) (Ma et al., 2008). Alignment analysis using BlastP indicated that the deduced kenaf COMT ortholog shared 95, 86, 85 and 85% similarities with COMT from *Gossypium hirsutum*, *Eucalyptus camaldulensis*, *Jatropha curcas* and *Boehmeria nivea*. TargetP V1.1 analysis showed the absence of signal peptide and

SignalP 4.0 analysis also suggested the absence of signal peptide for subcellular localization. Based on these results, the COMT ortholog is thought to be a cytoplasmic protein. COMT protein sequences of the 13 plant species were used to generate a phylogenetic tree using Mega 5. The Kenaf COMT ortholog showed the closest relationship to *Gossypium hirsutum* (ACT32029), and belonged to a sub-cluster of three proteins that also included *G. hirsutum* and *Eucalyptus camaldulensis* (ACY66932; Fig. 3). These findings strongly suggest that kenaf COMT ortholog is a COMT enzyme; therefore, it was designated as *HcCOMT*.

Levels of HcCOMT transctipts in various tissues and organs

To observe *HcCOMT* expression in various tissues and organs, the transcript level was analyzed in roots, stems, leaves, petioles and flowers using QPCR. *HcCOMT* transcripts were detected in all tissues and organs tested and found to be highly accumulated in young stems (4-week old), leaves and flowers (Fig. 4). The *HcC4H* transcript level was highest in 4-week-old stems, after which it sharply decreased. Similar levels of *HcCOMT* transcript were observed during the flower and leaf developmental stages. The lowest transcript level was observed in root and petiole tissues. Duncan's multiple range test for determining the statistical significance indicates the transcript values during flower and leaf development (Fig.4 B and C) were not significant ($P > 0.05$), while those during stem development and in different tissues/organs (Fig. 4 A and D) were significant ($P \leq 0.05$).

Levels of HcCOMT transcripts in stem tissues in response to different abiotic stresses

We used 3-week-old kenaf stem tissues to analyze the transcript levels of *HcCOMT* in response to diverse abiotic stresses (Fig. 5). The results revealed that *HcCOMT* was upregulated in response to all treatments. The highest induction of *HcCOMT* was observed in response to wounding at 6 h after treatment, after which the level decreased gradually. The expression patterns of *HcCOMT* subjected to wounding, SA, H_2O_2 and cold treatments showed the maximum levels at 6 h after treatment, after which they decreased. Conversely, MeJA treatment reduced the *HcCOMT* transcript level at early time points after treatment, which was followed by an increase at 48 h after treatment. In the case of NaCl, the transcript reached its maximum level at 12 h after treatment, decreased at 24 h after treatment, and then increased significantly 48 h after treatment. It was determined that all transcript values in response to various abiotic stresses were statistically significant based on Duncan's multiple range test ($P \leq 0.05$).

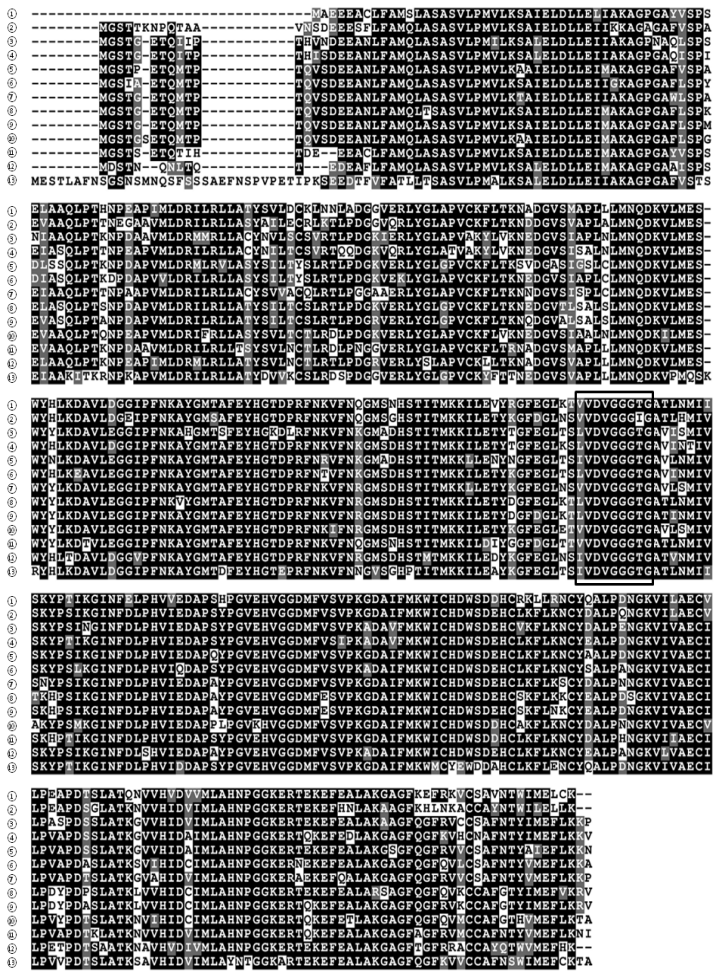

Fig 2. Multiple alignment of deduced amino acid sequences of COMT ortholog with other COMT sequences. The alignment was conducted using ClustalW and BOXSHADE sequence alignment programs in Biology WorkBench. Amino acid sequences shaded in black were identical to those of other COMT sequences. The box indicates the SAM binding domain (L/V/I-VDVGGGTG). The COMT sequences used were as follows: (1) *Coffea canephora* (AAN03726), (2) *Salvia miltiorrhiza* (AEO14871), (3) *Leucaena leucocephala* (ABS57468), (4) *Medicago truncatula* (XP003602396), (5) *Malus* x *domestica* (ABI54117), (6) *Jatropha curcas* (ACT87981), (7) *Boehmeria nivea* (ABG27066), (8) *Gossypium hirsutum* (ACT32029), (9) *Hibiscus cannabinus* (JX524278), (10) *Eucalyptus camaldulensis* (ACY66932), (11) *Camellia sinensis* (ADN27527), (12) *Capsicum annuum* (AAG43822), (13) *Vitis vinifera* (AAF44672).

Discussion

Kenaf COMT

Two structurally distinct methyltransferases, COMT and CCoMT, are responsible for the methylation of lignin precursors. COMT catalyzes methylation of caffeic acid and 5-hydroxyferulic acid, whereas CCoMT catalyzes methylation of caffeoyl-CoA and 5-hydroxyferuloyl-CoA (Zubieta et al., 2002; Dixon and Reddy, 2003; Ferrer et al., 2005; Davin et al., 2008). COMT was originally postulated to be a bifunctional enzyme that methylates both caffeic and 5-hydroxyferulic acids. According to in vitro and transgenic studies, the major role of COMT is the methylation of 5-hydroxyconiferaldehyde and/or 5-hydroxyconiferyl alcohol to sinapaldehyde and/or sinapyl alcohol (Raes et al., 2003). Nevertheless, COMT catalyzes caffeic acid, but not 5-hydroxyferulic acid in gymnosperms (Ma et al., 2008). While only one COMT gene was detected in the Arabidopsis genome (Raes et al., 2003), two classes of *COMT* transcripts were found in tobacco leaves infected by tobacco mosaic virus (TMV) (Legrand et al., 1978; Jaeck et al., 1992; Pellegrini et al., 1993). In a normal lignified tissue, class I *COMT* transcript was highly expressed, but class II *COMT* transcript was rarely expressed in healthy tissues. However, class II *COMT* transcript was highly expressed in response to viral infection or elicitor, which suggests that COMT II might produce ferulic derivatives whose deposition within the cell wall builds up a mechanical barrier, thus restricting pathogens (Nicholson and Hammerschmitt, 1992; Matern et al., 1995).

These findings indicate that the specific role of class I enzyme is for lignin biosynthesis and that of class II COMT is for the production of defense-related compounds. In general, one class of COMTs presents in plants. We also detected and cloned one putative COMT gene in kenaf. The amino acid sequence of HcCOMT showed high similarity to other COMTs, and phylogenic analysis indicated that HcCOMT was closely related to COMTs of different species (Fig. 2 and 3). The amino acid sequence (LVDVGGGTG, residues 204-212) of the HcCOMT ortholog shares the conserved domains with amino acid sequences of other COMTs (Fig. 1 and 2). This conserved domain (L/V/I-VDVGGGTG) is thought to be the SAM binding domain, which has sequences highly similar to those of the SAM binding domains of other COMTs (Ma et al., 2008). Therefore, it is thought that HcCOMT may have similar function to the COMT enzyme related to monolignol biosynthesis.

Expression of kenaf COMT ortholog in various tissues and organs

HcCOMT transcripts were expressed in all tested tissues and organs, and were highly upregulated in young stems (4-week), leaves and flowers (Fig. 4). *HcCOMT* were constantly expressed during all developmental stages of leaves and flowers, while comparatively lower expression levels were observed in roots and petioles. Similar results have been reported in previous studies in which tobacco *COMT* was highly expressed in stem tissues (Atanassova et al., 1995; Jaeck et al., 1996). Additionally, down-regulation of *COMT* caused a reduction in lignin (17% reduction) with extremely less S-units (Jouanin et al., 2000). These results indicate that *COMT* expression in the developmental stage of stems is important for lignification. COMT is not only involved in lignification, but also the biosynthesis of flavonoids and sinapoyl malate in Arabidopsis (Do et al., 2007). High levels of phenylpropanoid-derived compounds including si-napate and flavonoids were observed in Arabidopsis flower (Chapple et al., 1994), indicating that COMT may play an important role in flower development. Taken together, these studies support our gene expression data in which *HcCOMT* transcripts were highly expressed during flower development. The detection of *HcCOMT* transcripts in various tissues and organs strongly suggests that *HcCOMT* is essential for synthesis of monolignols and other phenylpropanoid derivatives (Anterola et al., 2002). Overall, the *HcCOMT* expression patterns indicate that this putative gene has a COMT function.

Kenaf COMT ortholog expression in response to abiotic stresses in stem tissues

Plants develop effective defense mechanisms against various environmental conditions through evolutionary processes. These environmental stresses, which are primarily biotic and abiotic, can actually activate and affect the defense mechanisms, including reinforcement of cell walls via lignin deposition and induction of diverse defensive enzymes and proteins (Hano et al., 2006; Desender et al., 2007; Hamann et al., 2009). The lignin biosynthesis pathway is a complex process that involves many enzymes that sensitively respond to various environmental conditions (Moura et al., 2010). Stresses can also activate the production of phytohormones such as ABA, SA, JA and ethylene. The expression of various genes involved in lignin biosynthesis might be controlled by these phytohormones (Yu et al., 2006; Desender et al., 2007; Jiang et

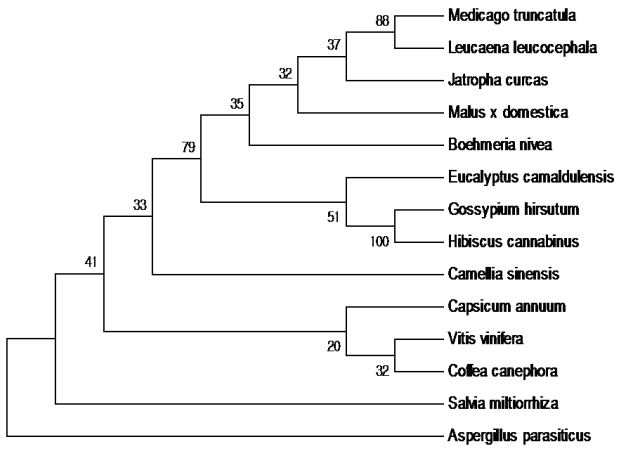

Fig 3. Phylogenetic analysis of the partial amino acid sequences of kenaf COMT ortholog with the sequences from other plants. The tree was constructed by the neighbor-joining method of ClustalW and Mega5. The numbers at the nodes indicate bootstrap values from 1,000 replications. The COMT sequences used were as follows: *Coffea canephora* (AAN03726), *Salvia miltiorrhiza* (AEO14871), *Leucaena leucocephala* (ABS57468), *Medicago truncatula* (XP003602396), *Malus* x *domestica* (ABI54117), *Jatropha curcas* (ACT87981), *Boehmeria nivea* (ABG27066), *Gossypium hirsutum* (ACT32029), *Hibiscus cannabinus* (JX524278), *Eucalyptus camaldulensis* (ACY66932), *Camellia sinensis* (ADN27527), *Capsicum annuum* (AAG43822), and *Vitis vinifera* (AAF44672). The sequence of *Aspergillus parasiticus* (ACJ38232) was used as an outgroup.

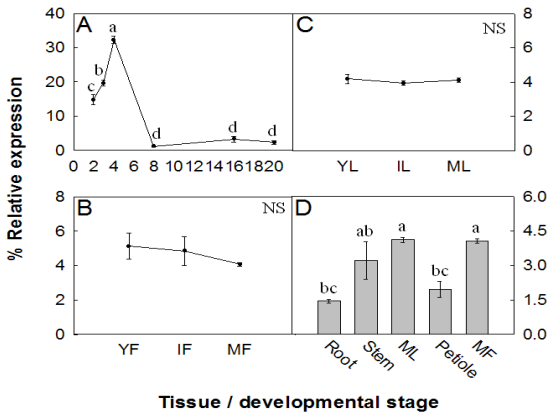

Fig 4. Kenaf *COMT* ortholog expression pattern during developmental stages of various tissues and organs. Relative transcript levels were measured using QPCR with respect to *ACTIN* transcripts. The levels of kenaf *COMT* transcript were adjusted after deduction of the control transcript level. (A) expression pattern of kenaf *COMT* ortholog during stem development (2, 3, 4, 8, 16, 20 weeks after sowing), (B) expression pattern of kenaf *COMT* ortholog during flower development (YF, young flower; IF, immature flower; MF, mature flower), (C) expression pattern of kenaf *COMT* ortholog during leaf development (YL, young leaf; IL, immature leaf; ML, mature leaf), and (D) expression pattern of kenaf *COMT* ortholog in various tissues and organs from 16-week-old kenaf plants. Vertical bars show means ± standard error of three biological replications. Different letters above each point indicate significant differences between the mean values at the 5% level. NS, not significant.

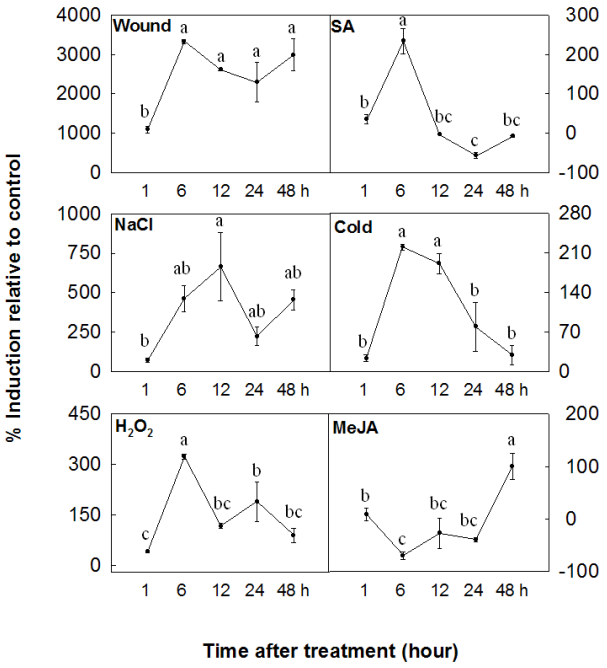

Fig 5. Kenaf *COMT* ortholog expression pattern in response to various abiotic stresses. Three-week old stem tissues were used for the stress treatments. Relative transcript levels were measured using QPCR with respect to *ACTIN* transcripts. The levels of kenaf *COMT* transcript were adjusted after deduction of the control transcript level. Vertical bars show the means ± standard error of three biological replications. Different letters above each point indicate significant differences between the mean values at the 5% level.

al., 2011; Choi et al., 2012; Ghosh et al., 2012). The produced phytohormones can generate the initial signals during the gene expression process, and the initial signals sequentially induce a second round signaling pathway (Mahajan et al., 2005; Shao et al., 2007). Many scientists have attempted to identify gene expression patterns and enzyme activities related to lignin biosynthesis in response to diverse abiotic stresses. However, this is the first report of the *COMT* expression pattern in kenaf. In this study, we examined the *HcCOMT* expression patterns in response to various abiotic stresses using QPCR and found that *HcCOMT* transcripts were up-regulated in response to all treatments applied in this study (Fig. 5).

Wounding

Mechanical injuries activate specific defense mechanisms, which range from physical barriers such as cuticle formation, lignification, spines and trichomes, to the biosynthesis of toxic compounds including alkaloids and tannins (Delessert et al., 2004). Several genes involved in the lignin biosynthesis pathway were induced by wounding or microbial elicitors (Moura et al., 2010). Plants show similar responses against mechanical wounding and pathogen attack, including the accumulation of phenolic compounds and phytoalexins, expression of defense related genes, synthesis of hydrolytic enzymes and lignin deposition at the injury or invaded site (Chen et al., 2000). We found similar responses in kenaf stem tissues (Fig. 5). The extractable activity of COMT enzyme in alfalfa cells showed an initial rapid increase following bacterial elicitor treatment, followed by a rapid decrease and then a second increase (Dalkin et al., 1990). Comparable results have been observed in other plant species (Gowri et al., 1991; Chiron et al., 2000).

Salt

The process of acclimatization to salt stress occurs through many physiological adjustments that can be observed as alterations in the levels of numerous transcripts (Singh et al., 1985). Increasing lignification or changing the monolignol composition in plant cell walls can be an efficient method of surmounting salt stress (Neves et al., 2010). In previous studies, salt treatment was found to induce the transcription of *SAMS* (S-adenosyl-L-methionine synthase) in tomato and *COMT* in *Tamarix hispida* (Sánchez-Aguayo et al., 2004; Li et al., 2009). We found similar responses of *HcCOMT* transcription to salt stress (Fig. 5).

Cold

Low temperature causes various changes in plants such as gene expression, metabolite production, membrane structure, and cell wall composition (Thomashow, 1999; Kaplan et al., 2004; Cui et al., 2005). The phenypropanoid pathway can be activated by cold stress, resulting in gene induction and metabolite accumulation (Wei et al., 2006; Moura et al., 2010). Additionally, COMT protein has been shown to increase in response to low temperature in bark tissues of peach, which supports our findings (Fig. 5) (Renaut et al., 2008). Although the role of lignin in response to cold stress is not clear, the accumulation of phenolic compounds may protect plants from free phenolic compounds (Whetten and Sederoff, 1995; Moura et al., 2010).

H_2O_2

The expression of genes involved in lignin biosynthesis is also controlled by H_2O_2 (Ros et al., 2005). We found that the level of *HcCOMT* transcription was up-regulated upon H_2O_2 treatment, reaching the highest level at 6 h (Fig. 5). The phenylpropanoid pathway is activated by pathogen infection to induce cell wall reinforcement (Kostyn et al., 2012), which is associated with H_2O_2 production at the site of fungal infection (Thordal-Christensen et al., 1997; Wei et al., 1998). In addition, H_2O_2 is essential to cross-linking during lignin polymerization (Levine et al., 1994; Christensen et al., 2000), while silencing of wheat COMT has been shown to increase the penetration rate of pathogens (Bhuiyan et al., 2009). Taken together, these results indicate that COMT plays an important role in lignin deposition during pathogen infection, which is associated with H_2O_2 production at the site of pathogen infection.

MeJA

Jasmonic acid (JA) and methyl jasmonic acid (MeJA) can act as signals related to the wound response for the plant defense mechanism (Seo et al., 1997). There are two pathways involved in defense gene expression in response to wounding, the JA-dependent and JA-independent pathway (Perez-Amador et al., 2002; Soltani et al., 2006). In the present study, MeJA treatment induced the level of *HcCOMT* transcripts (Fig. 5), which is comparable to the results of previous studies. For example, barley *COMT* transcript was induced by MeJA treatment (Lee et al., 1997), while exogenous application of MeJA induced lignin deposition in the cell wall and accumulation of phenolic compounds and defense enzymes in eggplant roots (Mandal, 2010). In conclusion, we cloned a full-

length of *COMT* gene putatively encoding caffeic acid O-methyltransferase from kenaf. We found that COMT is an important methylating enzyme involved in lignin biosynthesis, and that *HcCOMT* expression was controlled by developmental stages as well as different abiotic stresses.

Materials and methods

Plant materials, stress treatment and RNA isolation

Kenaf seeds (*Hibiscus cannabinus* L., C-9) were kindly provided by Dr. Si-Yong Kang (Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup 580-185, Korea). The kenaf used in this study originated from Russia (GenBank of Korea Rural Development Administration IT No. 202789). Plant growth conditions, stress treatment and the RNA isolation method were described in a previous study (Ghosh et al., 2012).

Cloning

cDNA synthesis was conducted with a Superscript III First-strand synthesis kit (Invitrogen, Carlsbad, CA, USA) using 2 μg of RNA isolated from kenaf tissues. We designed degenerate primers (COMT-F, 5'-GT(T/A/G/C)GATGT(C/T/A)GGTGG-3'; COMT-R, 5'-TG(G/A)CA (A/T)ATCCACTTCAT-3') based on the conserved COMT sequences of *Chrysosplenium americ* (U16793), *Capsicum annuum* (AF212316), *Mangifera indica* (FJ645928), *Medicago sativa* (M63853), *Stylosanthes humilis* (L36109), *Prunus dulcis* (X83217), *Betula pendula* (FJ667539), *Clarkia_breweri* (AF006009), *Isatis tinctoria* (DQ115905) and *Arabidopsis thalia* (NM124796) for synthesis of COMT fragments. To sequence the PCR amplicon, it was cloned into the pGEM®-T easy Vector (Promega, Madison, WI, USA). A full-length of the *C4H* ortholog was then cloned using both 5' and 3' RACE kits (Invitrogen).

Quantitative real-time polymerase chain reaction (QPCR) analysis

SYBR Green QPCR Master Mix (LPS Solution, Daejeon, Korea) was used for QPCR, which was conducted using the Mx3000P QPCR System (Agilent, Santa Clara, CA, USA) as previously described (Bae et al., 2008). The Biology WorkBench Primer 3 program was used to design QPCR primers (forward primer, 5'-CATGTTCGAAAGTGTTCC CAAAG-3'; reverse primer, 5'- CAACATGATGCAAT-CTATATGGACC-3'). *ACTIN* (DQ866836) was used as a reference gene for normalization (forward primer, 5'-ATGGACAAGTCATTACTAT TGGAGC-3'; reverse primer, 5'-AGTGATTTCCTTGCTCATACGGT-3').

Data analyses

The following software programs were used in this study: 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/), TargetP V1.1 (http: //www.cbs.dtu.dk/ services/TargetP/), and Mega5 (http://www/ megasoftware. net/). The statistical significance was determined based on Duncan's multiple range test at a significance of $P \leq 0.05$ using SASS (SASS Inc., Cary, NC, USA).

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