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Differential expression of kenaf phenylalanine ammonia-lyase (*PAL*) ortholog during developmental stages and in response to abiotic stresses

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Abstract

Phenylalanine ammonia-lyase (PAL) is a key enzyme in the phenylpropanoid pathway. A full-length of the gene putatively encoding phenylalanine ammonia-lyase (PAL) was cloned from kenaf (*Hibiscus cannabinus* L.) using degenerate primers and the RACE (rapid amplification of cDNA ends) method. The full-length *PAL* ortholog in kenaf consists of a 2,148 bp open reading frame encoding 715 amino acids (JQ779022). The deduced amino acid sequence showed high similarity to those of PAL from *Ricinus communis* (88%) and *Vitis vinifera* (86%). The expression of the *PAL* transcript was examined in different tissues, developmental stages, and after treatments with abiotic stresses (wound, NaCl, cold, H_2O_2, ABA, SA, MeJA and drought) using quantitative real-time reverse transcriptase polymerase chain reaction (QPCR). The *PAL* ortholog was differentially expressed in the different tissues and developmental stages. The highest transcript level of the *PAL* ortholog was observed in young (4-week-old) stem and mature flower tissues, with a certain level of expression in all tissues and organs tested. Three-week-old kenaf stem tissues were used to evaluate the effects of abiotic stresses on *PAL* ortholog expression. The highest transcript level of the *PAL* ortholog was observed at an early time point (1 or 6 h) after treatments with wound (1 h), H_2O_2 (6 h) and SA (6 h), while the highest transcript level was detected at the late time point (24 h) after treatments with NaCl, cold and ABA. The *PAL* ortholog was not significantly induced by MeJA, while drought repressed the *PAL* ortholog.

Keywords: gene expression; kenaf (*Hibiscus cannabinus* L.); phenylalanine ammonia-lyase (PAL); phenylpropanoid pathway; quantitative real-time PCR.
Abbreviations: Hc- *Hibiscus cannabinus*; PAL-phenylalanine ammonia-lyase; ABA- abscisic acid; MeJA-methyl jasmonate; SA-salicylic acid.

Introduction

Kenaf (*Hibiscus cannabinus* L.) is an annual dicotyledonous plant that grows in temperature and tropical regions (Dempsey, 1975). Since kenaf has wide ecological adaptability and produces large amounts of biomass within a short growing season, it has great potential for use in biomass production (Francois et al., 1992; Lam et la., 2002; Araki and Kubota, 2005). Keanf is also an important crop in the pulp and paper industries because of the high quality of its fiber (Ahmed et al., 1988; Pande and Roy, 1996). Kenaf has moderately long fibers in its outer stem (bast) and short fibers in its core, which make its fibers a promising source of raw materials for pulp, paper and other fiber products (Anterola et al., 2002; Apel and Hirt, 2004). The amount of lignins in fibers affects the efficiency of pulping process. Bast fibers in kenaf contain a high amount of the S unit of lignins, which has less resistant linkages than the lignins composed of the G unit (Gutiérrez et al., 2004). The S unit is relatively unbranched and less condensed than the G unit. The lignin content of bast fibers in kenaf is less than 11% with a high amount of cellulose (Van Dam, et al., 1994; Gutiérrez et al., 2004; Marques et al., 2010). Various secondary metabolites are produced through the phenylpropanoid pathway, including lignins, flavonoids and coumarins, most of which are important metabolites to plants (reviewed by Vogt, 2010). This pathway starts with cinnamic acid synthesis from phenylalanine by phenylalanine ammonia lyase (PAL) in the cytosol and the pathway is modulated by PAL, which is the rate-limiting enzyme (Hisano et al., 2009). PAL activity may be regulated by feedback inhibition by the pathway product, cinnamic acid, which may modify the expression of the *PAL* gene (Christensen et al., 2001; Del Río et al., 2004). PAL is a potential target for herbicide due to its central role in plant metabolism (Basson and Dubery, 2008). Phenylpropanoid compounds have

important roles in plant defense mechanisms, reproduction and development (reviewed by Vogt, 2010). More than one PAL genes are found in Arabidopsis (Raes et al., 2003). For example, two activities of PAL were induced by various stimuli, such as red light, UV irradiation, chilling, mechanical wounding, ozone, pathogen attacks and various plant hormones (Brodenfeldt and Mohr, 1988; Reddy et al., 1994; Singh et al., 1999; Campos-Vargas and Saltveit, 2002; Jiang and Joyce, 2003; Lafuente et al., 2003; Campos-Vargas et al., 2005; Chen et al., 2006). In higher plants, PAL genes exist as a family of genes (Fukasawa-Akada et al., 1996; Butland et al., 1998; Kumar and Ellis, 2001; Cochrane et al., 2004), and each gene may have distinct metabolic functions, such as flavonoids, lignin biosynthesis, etc. In this study, a putative *PAL* ortholog was cloned for the first time, and the expression of the ortholog was characterized in different tissues, developmental stages and under stress conditions in 3-week-old stem tissues of kenaf after treatments with various stresses [wound, NaCl, cold, H_2O_2, abscisic acid (ABA), salicylic acid (SA), methyl jasmonate (MeJA) and drought]. Understanding the expression pattern of *PAL* is important for identifying targets for biotechnological modification that could improve product synthesis.

Results and discussion

Kenaf PAL ortholog shares sequence characteristics with other PALs

In order to clone the full-length of *PAL* ortholog in kenaf, we used degenerate primers and the RACE (rapid amplification of cDNA ends) system. *PAL* ortholog (GenBank Accession No. JQ779022) consists of a 2,148 bp open reading frame (ORF) encoding 715 amino acids with 176.92 kDa and 4.92 pI, as calculated by the ExPASy Proteomics Server (Fig 1). Blast search showed that the deduced kenaf PAL ortholog shared 88, 86, 85, 85, 85, 85, 85, 83, 81, 80 and 73% similarities with the amino acid sequences of PALs from *Ricinus communis* (XP002519521), *Vitis vinifera* (ABM67591), *Populus trichocarpa* (ACC63887), *Populus trichocarpa* x *Populus deltoids* (AAA33805), *Morus alba* var. *multicaulis* (AEE81750), *Pyrus* x *bretschneideri* (ADF59061), *Catharanthus roseus* (BAA95629), *Arabidopsis thaliana* PAL1 (At2g37040), *A. thaliana* PAL2 (At3g53260), *A. thaliana* PAL4 (At3g10340) and *A. thaliana* PAL3 (At5g04230), respectively (Fig 2). The first 23 amino acids are different among other PAL sequences, while the rest of the sequences are highly conserved. The PAL ortholog grouped in a sub-cluster of 4 proteins: *Populus trichocarpa* x *Populus deltoids*, *Populus trichocarpa*, *Ricinus communis* and *Morus alba* var. *multicaulis* based on a phylogenetic tree (Fig 3). Amino acid ammonia-lyases catalyze the addition of ammonia to achiral olefinic acids to form chiral L-amino acids, and include histidine ammonia-lyases (HAL), aspartate ammonia-lyases (AAL), 3-methylaspartate ammonia-lyases (MAL), and phenylalanine ammonia-lyase (PAL), etc (Hanson and Havir, 1973). The C-terminal region shares a high sequence identity, and contains a multi-helix region that plays an important role in the regulation of the enzyme activity by destabilizing the active conformation of the Tyr110-loop (Lee et al., 2003; Pilák et al. 2006). According to X-ray structures, HAL contains the cofactor 3,5-dihydro-5-methylidene-4*H*-imidazol-4-one (MIO) by cyclization and dehydration of residues within the Ala-Ser-Gly sequences (Schwede et al., 1999; Langer et al., 2001; Baedeker and Schulz, 2002). The Ala-Ser-Gly signature was also observed in the putative PAL ortholog in kenaf (resides 201-203; Fig 1 and 2). Three central α helices form a triple coiled coil creating an electropositive platform for cofactor MIO. Tyr110 is of the most conserved

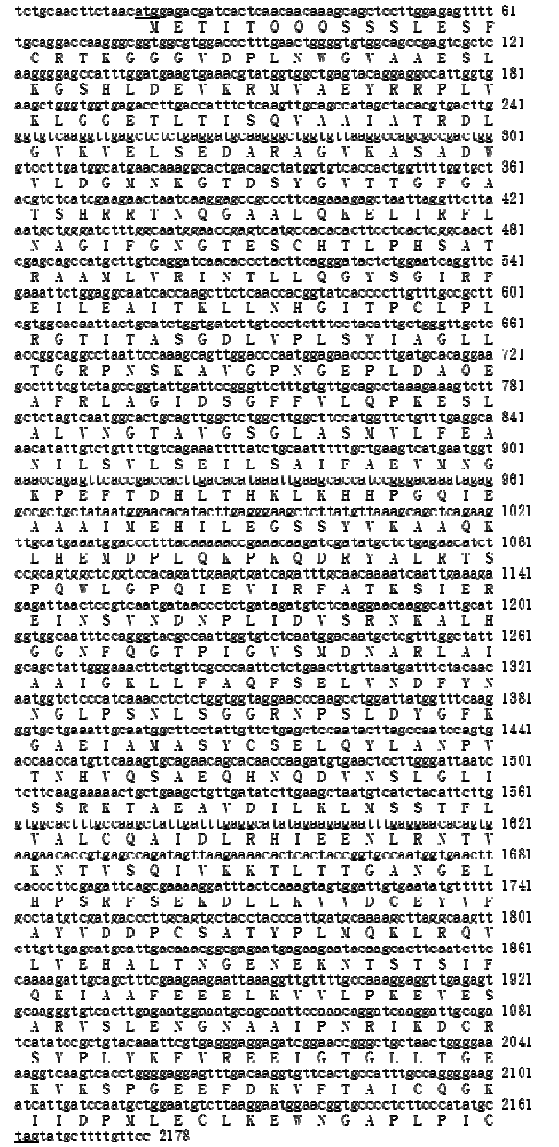

Fig 1. The full length cDNA and deduced amino acid sequence of the kenaf phenylalanine ammonia-lyase (PAL) ortholog. The start codon (ATG) and stop codon (TAA) are underlined and in bold. The conserved residues of PAL were boxed: Tyr110, Ala-Ser-Gly and Gly493.

residues within the MIO-containing ammonia-lyase family and is essential to the catalytic activity (Fig 1 and 2; Pilbák et al., 2006). Gly493 was also observed in the putative PAL ortholog in kenaf, which is the first residue of the α-helix α17 and one of the 3 central α-helices forming an electropositive platform for the cofactor MIO (Fig 1 and 2; Calabrese et al., 2004). This result suggests that the PAL ortholog of kenaf belongs to a PAL enzyme. Therefore, we designated the *PAL* ortholog of kenaf as *HcPAL*. In the Arabidopsis genome, there are 4 PAL genes (PAL1, 2, 3 and 4) (Raes et al., 2003). The deduced amino acid sequences of HcPAL have the highest similarities with AtPAL1 (83%) and AtPAL2 (81%). While the *pal1* and *pal2* single mutants showed no obvious visible phenotypes in growth and development, the *pal1 pal2* double mutants showed infertility, reduction in lignin, modification in cell wall structure, and deficiency in anthocyanin pigments (Rohde et al., 2004; Huang

et al., 2010). The double mutants were more sensitive to UV-B radiation but more tolerant to drought than wild-type Arabidopsis. These results indicate that the function of *PAL1* and *PAL2* was redundant. The *pal1 pal2 pal3 pal4* quadruple mutants were stunted and sterile and displayed reduced accumulation of SA, making the plants more susceptible to *Pseudomonas syringae* (Huang et al., 2010).

HcPAL expression in kenaf

The level of the *HcPAL* transcript was analyzed in various tissues and organs using QPCR. The *HcPAL* transcript was detected in all tissues and organs tested, including the root, stem, petiole, leaf and flower (Fig 4A). Since *PAL* plays crucial role in the pathway, *PAL* is expressed in all tissues and organs. The highest transcript levels of *HcPAL* were detected in the young stem (4-week old) and mature flower. During stem development, the transcript level of *HcPAL* gradually increased up to 4 weeks, and then subsequently decreased (Fig 4B). The transcript levels of *HcPAL* were similar during 8- to 20-week of stem development. The highest transcript level was observed in the mature flower during flower development, while the highest transcript level was detected in the young leaf during leaf development (Fig 4C and 5D). These results were in agreement with the findings of previous reports. High levels of the *PAL* transcripts were detected in the root and flower of tobacco plant, while low level of transcript was found in the mature leaf (Fukasawa-Akada et al., 1996). Four transcripts of the Arabidopsis *PAL* genes were detected in inflorescent stem (Raes et al., 2003). The highest expression was observed in *PAL1, PAL4* and *PAL2*, while the *PAL3* transcript was only detected at a very low level (Raes et al., 2003). Arabidopsis *PAL1* and *PAL2* were known to be important for lignin biosynthesis (Oh et al., 2003; Rohde et al., 2004). Arabidopsis *PAL1* was most closely related to *PAL2*, while *PAL3* clustered together with *PAL4*. Arabidopsis *PAL1* and *PAL2* also shared common structures in the promoter regions and showed similar expression patterns (Raes et al., 2003). A high level of *HcPAL* transcript was also observed during flower development in kenaf. Since a high level of phenylpropanoid compounds, such as si-napate and flavonoids, was found in Arabidopsis flower, *PAL* expression was required during flower development (Chapple et al., 1994). While *HcPAL* was highly expressed in the stem, the tomato *PAL5* transcript was not detected in the stem by northern blot analysis (Guo and Wang, 2009). This result indicates that different *PAL* genes accumulate differently in different tissues. A similar result was observed in raspberry (Kumar and Ellis, 2001). Again, this indicates that there are distinct regulatory mechanisms for the different *PAL* genes. Overall, the expression results also confirmed the putative function of *HcPAL* as *PAL*.

HcPAL expression in response to various abiotic stresses

Defense mechanisms are activated by stresses, which lead to the induction of defense enzymes and cell wall reinforcement, including lignin deposition (Hano et al., 2006; Desender et al., 2007; Hamann et al., 2009). Phytohormones including ABA, SA, JA and ethylene may be produced by stresses, which may control the expression of various genes. The phytohormones produced can amplify the initial signals to generate a second round signaling pathway (Mahajan et al., 2005; Shao et al., 2007). In this study, various abiotic stresses and signal molecules, such as wound, NaCl, cold, H_2O_2, ABA, SA, MeJA and drought, were applied to kenaf plants to examine the expression patterns of *HcPAL* using QPCR (Fig 5). Stem tissues of 3-week-old plants were harvested after treatments for

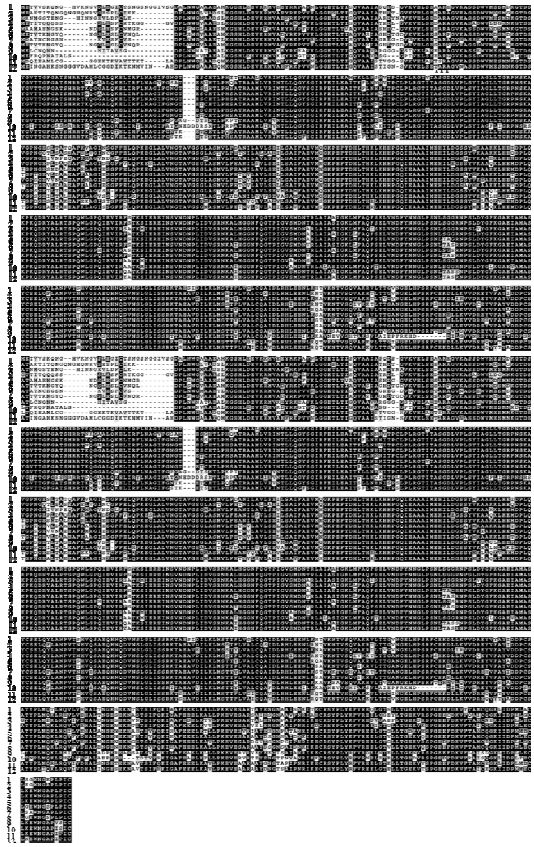

Fig 2. Multiple alignment of deduced amino acid sequences of the phenylalanine ammonia-lyase (PAL) ortholog with other PAL sequences. Alignment was conducted using ClustalW and BOXSHADE sequence alignment program in Biology WorkBench. Residues shaded in black were identical amino acids with other PAL sequences. The conserved residues (Ala-Ser-Gly) were marked with asterisk. The PAL sequences used were as follows: (1) *Morus alba* var. multicaulis (AEE81750), (2) *Pyrus* x *bretschneideri* (ADF59061), (3) *Catharanthus roseus* (BAA95629), (4) *Hibiscus cannabinus* (JQ779022), (5) *Ricinus communis* (XP002519521), (6) *Populus trichocarpa* (ACC63887), (7) *Vitis vinifera* (ABM67591),
(8) *Populus trichocarpa* x *Populus deltoids* (AAA33805), (9) *Arabidopsis thaliana* PAL4 (At3g10340), (10) *A. thaliana* PAL3 (At5g04230), (11) *A. thaliana* PAL2 (At3g53260), (12) *A. thaliana* PAL1 (At2g37040).

QPCR analysis. While all treatments induced the expression of *HcPAL*, ABA (at early time points) and drought repressed expression. The changes in *HcPAL* expression by MeJA treatment were not significant.

Wound, In wound treatment, the level of the *HcPAL* transcript was maximal 1 h after treatment, and then gradually decreased to the level of the control. Wounding induced genes related to lignin biosynthesis, resulting in the accumulation of lignin surrounding the wound sites, such as *PAL*, *C4H* (cinnamate 4-hydroxylase), *F5H* (ferulate 5-hydroxylase), *CAD* (cinnamyl alcohol dehydrogenase), *CCR* (cinnamoyl-CoA reductase) and *4CL* (4-coumarate:CoA ligase) (Delessert et al., 2004; Soltani et al., 2006; Moura et al., 2010). A similar expression pattern of *PAL* was observed in the suspension cells obtained from *Scutellaria baicalensis* (*SbPAL*; Xu et al., 2010). The *SbPAL1* transcript accumulated transiently within 1-3 h after wounding,

and then decreased to the control level. The other two *PAL* transcripts (*SbPAL2* and *SbPAL3*) increased to maximum levels within 24 h after wounding, and then returned to control levels.

NaCl, The *HcPAL* transcript was significantly induced by 200 mM NaCl treatment and was maximal after 24 h. Induction by NaCl was also observed in the tomato *PAL* gene, *SlPAL5* (Guo and Wang, 2009). NaCl treatment induced up to 90% accumulation in lignin contents in soybean roots (Neves et al., 2010). NaCl caused an increase in lignification or altered the monomeric composition of the lignin, which is one of the mechanisms used to overcome high salt conditions (Neves et al., 2010).

Cold, *HcPAL* transcripts were induced within 6 h and reached a maximum level by 24 h after cold (10°C) treatment. Low temperature increased the activity of PAL, which led to the accumulation of ρ-coumaric, ferulic, synaptic acids and the esterified soluble forms of these acids in the leaf mesophyll cells of oilseed rape leaf (Solecka and Kacperska, 1995; Solecka et al., 1999). The accumulation of the esterified forms may be important to protect plants from free phenols (Whetten and Sederoff, 1995). There are four *PAL* genes in Arabidopsis (*AtPAL1, 2, 3* and *4*) that expressed in inflorescent stem (Raes et al., 2003). Both *AtPAL1* and *AtPAL2* increased by low temperatures (Olsen et al., 2008). These results indicated that *AtPAL1* and *AtPAL2* involved in abiotic environmental-triggered flavonoid synthesis.

H_2O_2, The *HcPAL* transcript level was maximal after 6 h of treatment with 10 mM H_2O_2 and then gradually returned to control levels. In Arabidopsis cell cultures, treatment with 5 mM H_2O_2 induced *AtPAL1* (Desikan et al., 1998). Gayoso et al. (2010) suggested that there may be a possible relationship between the H_2O_2 content and PAL activity in the roots tomatoes resistant to *Verticillium dahliae*. In susceptible tomato plants, a delay was observed in the expression of *PAL* genes in response to the production of H_2O_2 compared to resistant plants. The induction of *PAL* genes by H_2O_2 was also detected in Arabidopsis cell cultures (Desikan et al., 1998). Treatment with H_2O_2 induced the accumulation of ρ-coumaric acid and the activity of peroxidase (Gayoso et al., 2010). The induced ρ-coumaric acid may be used to maintain cell walls in plants by cross-linking the lignins to the polysaccharides in cell walls (Pan et al., 1998).

ABA, The level of the *HcPAL* transcript increased significantly after various treatments but not by 100 µM ABA. The level of the *HcPAL* transcript decreased relative to the control up to 6 h after treatment, and was recovered at 12 h and reached a maximum 24 h after treatment. ABA is known to be involved in the response to various environmental stresses, such as drought and salt (reviewed in Zhu, 2002). ABA treatment reduced the transcript level of tomato *SlPAL5* and the transcript level was not recovered to the control level after 24 h of treatment, while *SlPAL5* was induced significantly by various abiotic stresses, such as NaCl, mannitol and cold (Guo and Wang, 2009). Therefore, tomato *SlPAL5* was categorized into the ABA-independent cascade. Many genes were induced by ABA treatment and the genes were also induced by drought, salt, osmotic stress and cold treatments (Shinozaki and Yamaguchi-Shinozaki, 1996). Some genes induced by water stress were not induced by exogenous ABA application, which indicates the existence of both ABA-independent and ABA-dependent signal transduction pathways (Bray, 1997).

SA and MeJA, Treatments with 5 mM SA and 100 µM MeJA induced the accumulation of *HcPAL*. The accumulation of the *HcPAL*

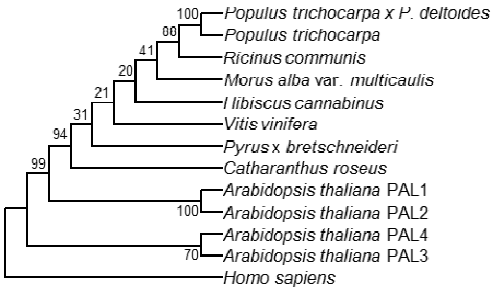

Fig 3. Phylogenetic tree of kenaf phenylalanine ammonia-lyase (PAL) ortholog. The tree was generated using the neighbor-joining method of ClustalW and Mega5 with amino acid sequences of the kenaf PAL ortholog and other plants. The bootstrap values from 1000 replcations are in percent at the nodes. The PAL sequences used were as follows: (1) *Arabidopsis thaliana* PAL4 (At3g10340), (2) *A. thaliana* PAL3 (At5g04230), (3) *A. thaliana* PAL1 (At2g37040), (4) *A. thaliana* PAL2 (At3g53260), (5) *Catharanthus roseus* (BAA95629), (6) *Pyrus* x *bretschneideri* (ADF59061), (7) *Morus alba* var. multicaulis (AEE81750), (8) *Populus trichocarpa* x *Populus deltoids* (AAA33805), (9) *Vitis vinifera* (ABM67591), (10) *Populus trichocarpa* (ACC63887), (11) *Ricinus communis* (XP002519521), (12) *Hibiscus cannabinus* (JQ779022). Human (ACJ38232) sequence was used as an outgroup.

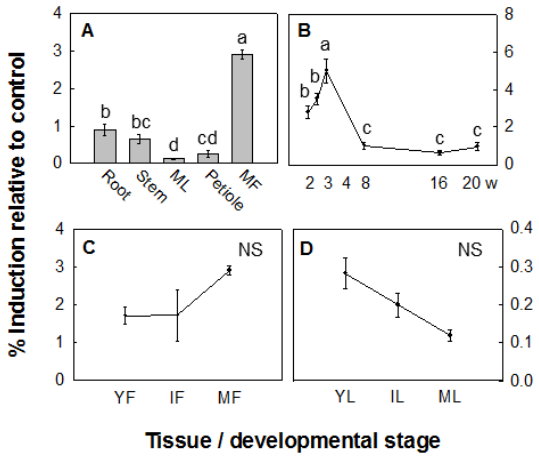

Fig 4. Expression of the kenaf phenylalanine ammonia-lyase (*PAL*) ortholog during developmental stages. Quantitation of the relative transcript levels were analyzed using QPCR with respect to *ACTIN* transcripts. The percent induction relative to the control was calculated after deduction of the control transcript level. (A) expression of *HcPAL* ortholog in various tissues and organs from 16-week-old kenaf plants. (B) expression of *HcPAL* ortholog during stem development (2, 3, 4, 16, 20 weeks after sowing), (C) expression of *HcPAL* ortholog during flower development (YF, young flower; IF, immature flower; MF, mature flower), and (D) expression of *HcPAL* ortholog during leaf development (YL, young leaf; IL, immature leaf; ML, mature leaf). Vertical bars represent the means ± SE (n = 3). Significant differences at a 5% level between the mean values are indicated by different letters above each point. NS, not significant.

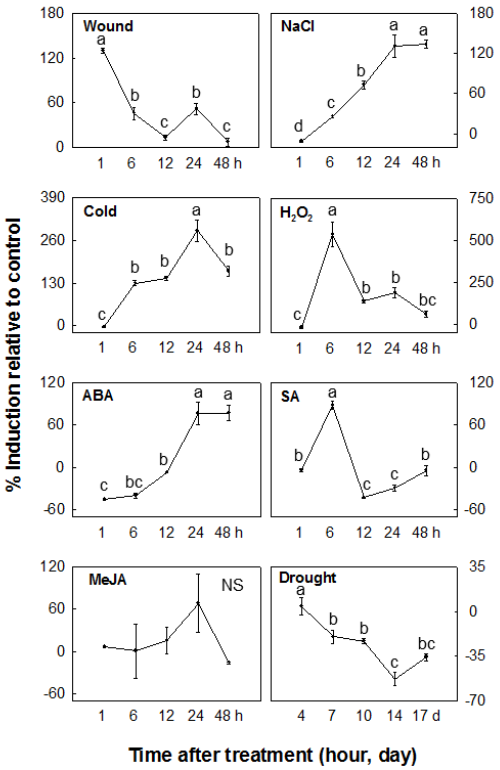

Fig 5. Expression of kenaf phenylalanine ammonia-lyase (*PAL*) ortholog after treatments with abiotic stresses and signal molecules. 3-week old stem tissues were subjected to various abiotic stresses and signal molecules : wound, NaCl, cold, H_2O_2, ABA, SA, MeJA and drought. Quantitation of the relative transcript levels were analyzed using QPCR with respect to *ACTIN* transcripts. The percent induction relative to the control was calculated after deduction of the control transcript level. Vertical bars represent the means ± SE (n = 3). Significant differences at a 5% level between the mean values are indicated by different letters above each point. NS, not significant.

transcript was maximal 6 h after treatment with SA, and then gradually returned to control level. A similar expression pattern of *HcPAL* was observed after treatment with SA and H_2O_2. MeJA treatment also induced *HcPAL*; however, the induction was not statistically significant. Treatment with elicitor molecules, such as SA and MeJA, resulted in the activation of defense mechanisms, including induction of cell wall strengthening and defense enzymes (Desender et al., 2007). *PAL* transcripts from *Lycoris radiata* (*LrPAL*) were significantly induced by MeJA, and moderately increased by SA (Jiang et al., 2011). The addition of exogenous SA also induced PAL activity in pear and *Saussurea medusa* cell cultures (Cao et al., 2006; Yu et al., 2006). Addition of MeJA induced expression of enzymes that were important to the PAL pathway, including the *PAL* gene (Lois et al., 1989; Gundlach et al., 1992; Walters et al., 2002)

Drought, Application of drought to kenaf repressed the expression of *HcPAL*. The expression of *HcPAL* was significantly down-regulated after 14 days of treatment. A similar down-regulation of expression was reported in *Camellia sinensis PAL (CsPAL)* (Singh et al., 2009). *HcPAL* transcripts decreased in response to both drought and ABA treatments. *CsPAL* was also down-regulated by both drought and ABA treatments. It was also reported that the activity of PAL was decreased by ABA treatment (Ward et al., 1989; Graham and Graham, 1996). The down-regulation of *PAL* may be due to enhanced cellular injury, increased membrane permeability, and the reduction in the rate of net photosynthesis during drought and ABA treatment. In conclusion, a full-length *PAL* gene putatively encoding phenylalanine ammonia-lyase was cloned from *Hibiscus cannabinus* L., which is an enzyme that is involved in the initial step of the phenylproanoid pathway. The expression of *PAL* is controlled by developmental stages and environmental stresses. Therefore, it is essential to understand how *PAL* expression is regulated during developmental stages and in response to various abiotic stresses.

Materials and methods

Plant materials

Kenaf seeds (*Hibiscus cannabinus* L., C-9) were obtained from Advanced Radiation Technology Institute (Korea Atomic Energy Research Institute, Jeongeup 580-185, Korea). The seeds are originally from Russia (GenBank of Korea Rural Development Administration IT No. 202789). Non-soil mixture (TOBIETEC, Chungbuk, Korea) was used to germinate seeds in pots, and the germinated seedlings were grown for up to 4 weeks in a controlled environment condition with 16-h light /8-h dark, 100 µmol m^{-2} s^{-1} at 22°C with watering twice a week. After 4-week growth in a controlled environment condition, seedlings were transplanted into 20-cm pots with a non-soil mixture and grown in a greenhouse with natural sunlight for up to 20 weeks, watering twice a week. Tissue samples (root, stem, petiole, leaf and flower) were harvested from 16-week-old kenaf plants. Harvested tissue samples were frozen in liquid nitrogen and stored at -80°C. Leaf development was separated into three stages: 1) young leaf (YL), < 2 cm long; 2) immature leaf (IL), 3-5 cm long; and 3) mature leaf (ML), > 9 cm long. Flower samples were also separated into three developmental stages: 1) young flower (YF), unopened green flower, < 2 cm long with green sepal; 2) immature flower (IF), unopened white flower, > 3 cm long with green sepal; and 3) mature flower, open white flower.

Stress treatments

Three-week-old seedlings grown in a growth room were subjected to stresses. Stem tissues were harvested for QPCR analysis. Seedlings were watered with ABA (100 µM), H_2O_2 (10 mM), SA (5 mM), NaCl (200 mM), or cold (10°C). Plants were watered with distilled water and used as the control. Stems were cut longitudinally with scissors in opposite sides (less than 1-mm deep) for wound treatment. Stems were sprayed with 100 µM MeJA , which was dissolved in 0.004% ethanol. The treated seedlings were covered with a vinyl bag until harvest. Stem tissues were harvested 1, 6, 12, 24 and 48 h after treatments. For control plants, seedling were sprayed with 0.004% ethanol and covered with a vinyl bag. For cold treatment, seedlings were incubated in cold room (10°C) with the same light condition for 1, 6, 12, 24 and 48 h. Harvested tissues were frozen in liquid nitrogen and stored at -80°C until use.

RNA extraction

Total RNA was extracted from various kenaf tissues and treated with DNase I as previously described (Ghosh et al., 2012). The RNA integrity and quantity were verified with an agarose gel and spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA).

Cloning of a full-length gene

First-strand cDNA was synthesized from 2 μg RNA, which consisted of a mixture of stem and leaf RNA, using Superscript® III first-strand synthesis supermix (Invitrogen, Carlsbad, CA, USA) with a gene specific primer (PALr2, 5'-TG(A/G)TC(A/C/G)GT(A/G)AACTC-3'). The degenerate primers were designed to amplify the fragment of a transcriptionally active *PAL* gene based on the consensus sequences of the *PAL* orthologs of *Populus trichocarpa* (ACC63887), *Populus trichocarpa* x *Populus deltoids* (AAA33805), *Arabidopsis thaliana* PAL1 (At2g37040), *A. thaliana* PAL2 (At3g53260), *A. thaliana* PAL3 (At5g04230), and *A. thaliana* PAL4 (At3g10340). The forward primer sequence is as follows: 5'-GC(C/G/T)AG (T/C)AGTGA(T/C)TGGGT-3'. The PCR product was purified from the agarose gel using a Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI, USA). The purified PCR product was cloned into pGEM® -T easy Vector (Promega) and DNA sequences were analyzed by Cosmogenetech Co. (Seoul, Korea). Both 5' and 3' RACE (rapid amplification of cDNA ends, Invitrogen) were applied to clone a full length of PAL ortholog in keanf.

QPCR analysis

QPCR was performed to examine the expression pattern of *PAL* ortholog as described by Bae et al. (2008). Mx3000P QPCR System (Agilent, Santa Clara, CA, USA) with SYBR Green QPCR Master Mix (Agilent) were used for QPCR. Kenaf *ACTIN* gene (DQ866836) was used as an expression control with the primer sequences: forward primer, 5'-ATGGACAAGTCAT TACTATTGGAGC-3'; reverse primer, 5'-AGTGATTTCCTTGCTCATACGGT-3'. The forward (5'-GGTGTCACTTGAGAATGGAAATG-3') and reverse (5'-AAAGCATACTAGC ATATGGGAAGAG-3') primers of *PAL* ortholog were as designed using the Primer 3 software of Biology Workbench (expected size, 246 bp; http://workbench.sdsc.edu/).

Data analyses

DNA and protein sequences were analyzed using NCBI Blast (http://blast. ncbi.nlm.nih.gov/), Biology WorkBench, ExPASy Proteomics Server (http://expasy.org/tools/ pi_tool.html). Mega5 (http://www.megasoftware.net/) was used to construct a phylogenetic tree with amino acid sequences by the neighbor joining method. Data for gene expression levels were statistical analyzed for significance using SASS (SASS Inc., Cary, NC, USA). The statistical significance of the mean differences was analyzed using Duncan's multiple range test at a significance level of $P \leq 0.05$.

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