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Full Length Research Paper

Molecular characterization of isoflavone synthase gene from *Pueraria candollei* var. *mirifica*

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Cloning, sequencing, and characterization of the isoflavone synthase gene (2-hydroxyisoflavone synthase; *IFS*) from *Pueraria candollei* var. *mirifica* were carried out in this study. The full-length open reading frame (ORF) of the *P. candollei* var. *mirifica IFS* gene or *PcmIFS* was obtained by reverse transcription polymerase chain reaction (RT-PCR). Nucleotide sequence of *PcmIFS* was 1,566 bp long, encoded for 521 amino acid residues with the relative molecular mass of 58.9 kDa. The pI value of the *PcmIFS* gene product was 8.9 and all of the main conserved motifs that are essential for IFS activities were found. The deduced amino acid sequence of the *PcmIFS*-encoded protein showed high degree of identity to those of IFS proteins from *Pueraria montana* var. *lobata, Glycine max*, *Glycine soja*, and *Pisum sativum*. Southern blot analysis indicated that the *PcmIFS* gene belonged to a multigene family. The expression of *PcmIFS* was detected in leaf, stem, and root of the plant and its expression levels was induced by both low and high temperature stresses as well as by UV-B and wounding treatments.

Key words: Isoflavone synthase, isoflavones, *Pueraria candollei* var. *mirifica*, molecular cloning.

INTRODUCTION

Over recent years, isoflavones from *Pueraria candollei* var. *mirifica* (local name as "White Kwao Krua"), a Thai indigenous herb, have been widely studied. This plant is found in the North, West, and Northeast of Thailand and it belongs to the family Leguminoceae (Malaivijitnond et al., 2004; Trisomboon et al., 2006). The plant accumulates several isoflavones in its tuberous roots, such as daidzin (0.23 mg/g dry weight (DW)), daidzein (0.16 mg/g DW), genistin (0.06 mg/g DW), genistein (0.08 mg/g DW), and puerarin (5.52 mg/g DW) (Chansakaow et al., 2000; Thanonkeo and Panichajakul, 2006; Boonsnongcheep et al., 2010), thus, it is preferred to be consume by women for promoting youthfulness and estrogen replacement therapy (Muangman and Cherdshewasart 2001). Isoflavones are the largest group of natural isoflavonoids (Dewick, 1994). They serve as phytoalexin and also act as signal molecules for symbiosis between soybean roots and soil bacteria (*Rhizobium* species) through inducing the expression of nodulation genes in bacteria (Kosslak et al., 1987; Phillips, 1992). They also have great potential benefits for human heath, e.g. reducing certain type of cancers, reducing post-menopausal symptoms, prevent coronary heart disease, antiinflammatory effects (Shirley, 2001; Trisomboon et al., 2006), positive effect on other physiological processes such as neuro-behavioral activities (Brouns, 2002), prevention of bone loss (Urasopon et al., 2007), and antioxidant activity (Cherdshewasart and Sutjit, 2008). Due to the molecular structure of isoflavones that mimic the estrogen hormone which can be bind to estrogenic receptors; therefore, they are classified as phytoestrogen (Kuiper et al., 1998; Yu and McGonigle, 2005).

In higher plants, isoflavones are produced via a branch of phenylpropanoid pathway. It begins from the amino acid phenylalanine and intermediates of the pathway,

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naringenin, and liquiritigenin, are sequentially converted into the genistein and daidzein, respectively (Albulescu and Popovici, 2007). The enzyme involved in this reaction is 2-hydroxyisoflavanone synthase (commonly termed isoflavone synthase or IFS), a cytochrome P450 oxygenase (Dixon and Ferreira, 2002; Liu et al., 2007). The isoflavone synthase converts flavanone into 2-hydroxyisoflavanone that is unstable and automatically converts to isoflavone by removing one molecule of water between C2 and C3 (Zhang and Yu, 2009). Isoflavone synthase (*IFS*) genes have been cloned and characterized from several leguminous plants including *Glycyrrhiza echinata* (Akashi et al., 1999), *Glycine max* (Steele et al., 1999), *Lotus japonicas* (Shimada et al., 2000), *Lens culinaris*, *Lupinus albus*, *Medicago sativa*, *Pisum sativum*, *Trifolium repens*, *Vigna radiate*, *Vicia villosa* (Jung et al., 2000), *Trifolium pratense* (Kim et al., 2003), *Vigna unguiculata* (Kaur and Murphy, 2010), and *Pueraria montana* (He et al., 2011). In addition, *IFS* genes from sugar beet (*Beta vulgaris*) and medicinal plant (*Psoralea corylifolia*) have also been reported (Geigert et al., 1973; Misra et al., 2010). Overexpression of *IFS* genes in non-legume plants such as *Arabidopsis thaliana*, *Nicotiana tabacum*, *Lactuca sativa*, and *Petunia hybrida* have been carried out (Jung et al., 2000; Liu et al., 2007). With respect to the *IFS* gene in *P. candollei* var. *mirifica*, there is no report available in the literatures.

The study of gene organization and expression profiles of *IFS* gene by using modern biotechnology are essential for providing useful information for genetic transformation programs aimed at changing the production capacity of isoflavonoids in *P. candollei* var. *mirifica*. In this study, molecular cloning, sequencing, and characterization of the *IFS* gene (*PcmIFS*) in this plant were carried out. The expression profiles of *PcmIFS* in different tissues (leaf, stem, and root) and under various environmental stresses such as temperature, UV-B, and wounding treatments were also investigated.

MATERIALS AND METHODS

Preparation of plant seedlings

Mature seeds of *P. candollei* var. *mirifica* collected from 2-year-old plants grown in the Department of Biotechnology Garden, Khon Kaen University, were used in this study. Plant seedlings were prepared using the method described by Thanonkeo and Panichajakul (2006). Briefly, seeds of the plant were washed with surfactant, and then rinsed with tap water to remove the remaining surfactant. They were surface sterilized with 95% ethanol for 1 min, followed by 20 min in 30% (v/v) of commercial Clorox (The Clorox Company, USA) containing Tween 20, and then, they were rinsed with sterile distilled water. The seed coat was clipped off and immersed in sterile distilled water for 24 h prior to germinate on hormone-free MS medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose, 0.01% (w/v) inositol, and 1% (w/v) agar. The growth conditions were at 25 ± 2°C with a 16/8 h light/dark cycle (light intensity, 20 µmole m^{-2} s^{-1}) and 50% humidity. The 1-month-old plants were used for isolation of genomic DNA and RNA and expression analysis of *PcmIFS* gene.

Isolation of total RNA from *P. candollei* var. *mirifica*

The leaves, stems, and roots of plants were collected for total RNA extraction. For different temperature treatments, the seedlings were kept at 4, 25, and 37°C for 8 h and the leaf samples were collected. For UV-B treatment, the seedlings were exposed in the dark in a closed chamber with UV-B irradiation and the leaf samples were collected at 2, 4, 6, and 8 h. For wounding treatment, the leaves were cut at the edge by about 5 mm with sterile scissors and the leaf samples were collected thereafter at 0, 24, 48, and 72 h. Total RNA of all collected samples were extracted by using RNeasy Plant Mini Kit (QIAGEN, Germany), as described by the supplier. Briefly, plant samples (100 mg) were ground in liquid nitrogen using mortar and pestle. The resulting powder was transferred into 1.5-ml microtube and 450 µl of RLT buffer was added and vigorously vortexed. The mixture was transferred into QIAshredder spin column and was centrifuged at 13,000 rpm for 2 min. The supernatant was transferred into 1.5-ml microtube and 900 µl of cold ethanol (96% ethanol) were added. The mixture was transferred into RNases mini column and was centrifuged at 10,000 rpm for 15 s. After washing with RW1 and RPF buffer, total RNA in a column was eluted using RNase-free water and stored at -20°C until use.

Reverse transcription polymerase chain reaction (RT-PCR) of *PcmIFS*

The degenerated oligonucleotide primers PIFSF (5'-ATGTTG(G/C)TGGAACTTGCA-3') and PIFSR2 (5'-TATGTGCCCTTGGAAC(A/T/C)G-3') were design based on the conserved region of *IFS* genes from other leguminous plants using ClustalW. The *PcmIFS* was amplified by RT-PCR using One-Step RT-PCR Kit (QIAGEN, Germany) (Contour-Ansel et al., 2006). Thermal cycling conditions for RT-PCR reaction were initial denaturation at 95°C for 15 min and reverse transcription at 50°C for 30 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and final extension at 72°C for 10 min. After RT-PCR amplification, the amplified products were ligated into pGEM-T easy vector (Promega, USA) and transformed into the competent *Escherichia coli* JM109 using the TransformAid™ Bacterial Transformation Kit (Fermentas, USA) (Michelsen, 1995). Transformants were screened based on blue/white selection and plasmid DNA was isolated from positive clone and were purified by using NucleoSpin® Extract II Kit (MACHEREY-NAGEL, Germany) (Vogelstein and Gillespie, 1979). The purified plasmid DNA was sequenced by Dideoxy Chain Termination Method using the MegaBACE 1000 automated DNA sequencer (Pharmacia Biotech, Sweden). To obtain the sequence of 5'- and 3'-end of the *PcmIFS*, the SMART™ RACE cDNA Amplification Kit (ClonTech, USA) was used (Zhu et al., 2001). The full length ORF of the *PcmIFS* was confirmed by RT-PCR using a pair of specific primers PcmIFSF (5'-ATGTTGCTGGAACTTGCA-3') and PcmIFSR (5'-TCAAGAAGGAGGTTTAGATGC AACGC - 3') under the aforementioned conditions as previously described.

Southern blot analysis

Genomic DNA was extracted from the leaves of *P. candollei* var. *mirifica* using DNeasy Plant Mini Kit (QIAGEN, Germany) (Black and Foarde, 2007). After being digested with *Eco*RI, *Bam*HI, and *Hin*dIII, it was separated on 0.8% agarose gel and then transferred to nylon membrane (Schleicher & Schuell, Germany) by capillary action according to the standard method (Sambrook and Russell, 2001). An approximately 700 bp internal fragment of *PcmIFS* was used as DNA probe for hybridization. It was labeled with alkaline phosphatase using a chemiluminescence system (AlkPhos

Direct™, Amersham, USA) (Thanonkeo et al., 2010). All procedures for DNA labeling were carried out as recommended by the manufacturer. The membrane with cross-linked DNA was hybridized with labeled DNA probe in hybridization buffer containing 500 mM NaCl and 4% (w/v) blocking reagent for overnight at 55°C. Then, it was washed twice with the primary buffer at 55°C for 10 min and twice with the secondary buffer for 5 min at room temperature. After washing, the CDP-star detection reagent (AlkPhos Direct™, Amersham, USA) was added onto the membrane, and leaved at room temperature for 5 min. The membrane was then exposed to X-ray film (hyper film, Amersham, USA) for 1 h, thereafter, the film was developed and fixed with developer and fixer solution (Kodak, Thailand).

Expression analysis of *PcmIFS* in different tissues and under stress conditions

Total RNA isolated from different tissues (leaf, stem, and root) and from leaves of stressed plants was used as template for expression analysis of *PcmIFS* by RT-PCR. RT-PCR was carried out using OneStep RT-PCR Kit (QIAGEN, Germany) (Contour-Ansel et al., 2006), with a pair of specific primers PcmIFSF and PcmIFSR. The reaction mixture (50 µl) consisting of 10 µl of 5x QIAGEN OneStep RT-PCR buffer, 400 µM dNTP, 1 µl of each primer (forward and reverse primers), 2 µl of QIAGEN One-Step RT-PCR Enzyme mix (reverse transcriptase and *Taq* DNA polymerase) and 1 µg of RNA template. As a control, 10 µg samples of the total RNA were subjected to agarose gel electrophoresis (0.9% agarose) and were stained with ethidium bromide. Actin gene was used as an internal control. Thermal cycling conditions for RT-PCR reaction were initial denaturation at 95°C for 15 min and reverse transcription at 50°C for 30 min followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The samples of amplified products were collected at 20th, 23rd, 26th, 29th, 32nd and 35th cycles, and were separated on 1% agarose gel. After staining with ethidium bromide, the relative amounts of the products were compared using the Gel Image Master (Pharmacia Biotech). The experiment was repeated at least twice.

RESULTS AND DISCUSSION

Analysis of the full length *IFS* cDNA of *P. candollei* var. *mirifica*

RT-PCR was carried out to amplify an internal fragment of *IFS* gene from *P. candollei* var. *mirifica* using degenerate primers synthesized based on the nucleotide sequences of *IFS* genes from other leguminous plants. A PCR product of an approximately 700 bp was achieved (data not shown) and it was cloned into pGEM T-easy vector, and then, it was sequenced. Homology analysis of the nucleotide sequence of this amplified fragment showed high degree of identity with the corresponding region of *IFS* sequences from other leguminous plants, suggesting that this DNA fragment is a part of the *IFS* gene in *P. candollei* var. *mirifica*. To isolate full length *IFS* cDNA, RT-PCR amplification using the SMART™ RACE cDNA Amplification Kit (ClonTech, USA) (Zhu et al., 2001) was performed and the resulting 1.6-kb PCR product was obtained. Nucleotide sequencing analysis revealed that this amplified fragment was a full length *IFS* cDNA of *P. candollei* var. *mirifica*.

The full length *P. candollei* var. *mirifica IFS* cDNA, designated as *PcmIFS*, was 1,566 nucleotides in length and contained a typical translation initiation codon (ATG) and termination codon (TGA), and was encoded a polypeptide of 521 amino acid residues (Figure 1). The deduced polypeptide had a relative molecular mass of 58.9 kDa and the predicted isoelectric point of the IFS protein was 8.90. The full *PcmIFS* cDNA sequence has been submitted to the GenBank under the accession number JQ746654. The P450 conserved motifs of the IFS protein from *P. candollei* var. *mirifica* are as shown in Figure 2. At the N-terminal sequence, an endomembrane (ER) targeting sequence (LLELAIGLVVLALFLHLR) (Sakaguchi et al., 1984, 1987) and a region rich in basic amino acid residues at position 25 (lysine; K), 27 (lysine; K), 30 (arginine; R), and 31 (histidine; H) required for proper orientation in the ER membrane (Sakaguchi et al., 1987; Yamazaki et al., 1993) were found. The proline rich region which is important for correct folding of the protein in the ER membrane was also located at the N-terminal sequence next to the basic amino acids rich region. The highly conserved "I" helix, FSAGTDST, proposed to be involved in oxygen binding by P450 proteins (Steele et al., 1999) and the highly conserved motif, PERF, an unknown function among P450 sequences and the heme-iron ligand signature, FGSGRRMCPG, were also exhibited at the position near the C-terminal sequence of the IFS protein (Udvardi et al., 1994; Steele et al., 1999). The amino acid, lysine "K^{375}", which is essential for aryl migration of flavanone molecule to produce isoflavone skeleton (Sawada et al., 2002) was also presented.

Homology and phylogenetic analysis of *PcmIFS*

Homology analysis was performed to estimate the degree of amino acid identity between the *PcmIFS* gene product with IFS proteins from other plant species. As shown in Figure 2, the *P. candollei* var. *mirifica* IFS protein showed high sequence identity to the known IFS sequences from leguminous plants, such as *P. montana* var. *lobata*. (AAM12530) (94%), *G. max* (ACA81460) (92%), *Glycine soja* (ACA81487) (92%), and *P. sativum* (AAF34533) (91%). The IFS sequence of *P. candollei* var. *mirifica* also showed high degree of similarity to IFS protein of non-leguminous plants (data not shown), suggesting that this polypeptide is well conserved (Sawada et al., 2002). All the main conserved motifs of P450 were found in these putative proteins, however, one amino acid change (F/V/L) in the ER targeting sequence was observed between the species. A phylogenetic analysis based on the *IFS* nucleotide sequences of legume- and non-legume plants was performed using the neighbor-joining method (Saitou and Nei, 1987) (Figure 3). The results showed that *PcmIFS* was closely related to all known *IFS* genes, especially that of leguminous plant, *P. montana* var. *lobata*, suggesting that these plants share a relatively recent common ancestor. Studies by Kaur and Murphy

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ATGTTGCTGGAACTTGCAATTGGTTTAGTTGTGTTAGCTTTGTTTCTGCACTTGCGTCCC      60
M  L  L  E  L  A  I  G  L  V  V  L  A  L  F  L  H  L  R  P
ACACCGAGTGCAAAATCCAAAGCCCTTCGCCACCTTCCAAACCCTCCTAGTCCAAAGCCT     120
T  P  S  A  K  S  K  A  L  R  H  L  P  N  P  P  S  P  K  P
TGTCTTCCCTTCATTGGTCACCTTCACCTTTTGAAAGATAAACTTCTCCACTACGCCCTC     180
C  L  P  F  I  G  H  L  H  L  L  K  D  K  L  L  H  Y  A  L
ATCGATCTCTCCAAACGATATGGGCCCTTATACTCTCTCTACTTTGGGTCCATGCCCACT     240
I  D  L  S  K  R  Y  G  P  L  Y  S  L  Y  F  G  S  M  P  T
GTTGTTGCCTCCACCCCTGAGTTGTTCAAACTCTTTCTTCAAACCCACGAGGCTTCTTCC     300
V  V  A  S  T  P  E  L  F  K  L  F  L  Q  T  H  E  A  S  S
TTCAACACAAGGTTCCAAACCTCAGCCATAAGGCGCCTCACTTACGACAACTCTGTGGCC     360
F  N  T  R  F  Q  T  S  A  I  R  R  L  T  Y  D  N  S  V  A
ATGGTTCCCTTCGGACCTTACTGGAAGTTCATCAGGAAGCTCATCATGAACGACCTCCTC     420
M  V  P  F  G  P  Y  W  K  F  I  R  K  L  I  M  N  D  L  L
AACGCCACCACCGTTAACAAGTTGAGGCCCTTGAGGACCCAACAAATCCGCAAGGTCCTG     480
N  A  T  T  V  N  K  L  R  P  L  R  T  Q  Q  I  R  K  V  L
AGGGTGATGGCCCAAAGTGCAGAGGCCCAACAACCCCTTAATGTGACCGAGGAGCTTCTA     540
R  V  M  A  Q  S  A  E  A  Q  Q  P  L  N  V  T  E  E  L  L
AAGTGGACAAACAGCACCATCTCGATGATGATGTTGGGTGAGGCTGAAGAGGTCAGAGAC     600
K  W  T  N  S  T  I  S  M  M  M  L  G  E  A  E  E  V  R  D
ATGGCTCGTGAGGCGGTTAAGATTTTTGGGGAATACAGTCTCACTGACTTCATCTGGCCA     660
M  A  R  E  A  V  K  I  F  G  E  Y  S  L  T  D  F  I  W  P
TTGAAGAATCTCAAGGTTGGACAGTATGAGAAGAGGATTGAAGATATATTCAACAAATTC     720
L  K  N  L  K  V  G  Q  Y  E  K  R  I  E  D  I  F  N  K  F
GACCCTGTCATTGAAAGGGTTATCAAGAAGCGCCGAGAGATCGTCAGAAGGAGAAAGAAC     780
D  P  V  I  E  R  V  I  K  K  R  R  E  I  V  R  R  R  K  N
GGAGAAGCTGTTGAGGATGAGGCCAGCGGGGTTTTCCTCGACACTTTGCTTGAATTCGCT     840
G  E  A  V  E  D  E  A  S  G  V  F  L  D  T  L  L  E  F  A
GAGGATGAGACCATGGAGATCAAAATTACCAAGGAGCAAATCAAGGGTCTTGTTGTCGAC     900
E  D  E  T  M  E  I  K  I  T  K  E  Q  I  K  G  L  V  V  D
TTCTTCTCAGCAGGGACAGATTCAACAGCTGTGGCAACAGAGTGGGCTTTGGCAGAGCTC     960
F  F  S  A  G  T  D  S  T  A  V  A  T  E  W  A  L  A  E  L
ATCAACAATCCTAGGGTGTTGCAAAAGGCTCGGGATGAGGTCTACAGTGTTGTGGGAAAA    1020
I  N  N  P  R  V  L  Q  K  A  R  D  E  V  Y  S  V  V  G  K
GATAGACTTGTTGACGAAGTTGATACTCAAAACCTTCCTTACATTAGAGCCATTGTGAAG    1080
D  R  L  V  D  E  V  D  T  Q  N  L  P  Y  I  R  A  I  V  K
GAGACATTCCGTATGCACCCACCACTCCCTGTGGTCAAAAGAAAGTGTGTGGAAGAGTGT    1140
E  T  F  R  M  H  P  P  L  P  V  V  K  R  K  C  V  E  E  C
GAGATTGACGGCTATGTGATCCCTGAGGGAGCTTTGATTCTTTTCAATGTTTGGGCTGTA    1200
E  I  D  G  Y  V  I  P  E  G  A  L  I  L  F  N  V  W  A  V
GGAAGAGACCCAAAATATTGGGACAGACCATTGGAATTTCGTCCTGAGAGGTTCTTAGAA    1260
G  R  D  P  K  Y  W  D  R  P  L  E  F  R  P  E  R  F  L  E
AGTGGTGCTGAAGGGGAAGTAGGACCTATTGATCTTAGGGGCCAACATTTCCAACTTCTC    1320
S  G  A  E  G  E  V  G  P  I  D  L  R  G  Q  H  F  Q  L  L
CCGTTTGGATCTGGAAGGAGAATGTGCCCTGGAGTTAATTTGGCCACTTCAGGAATGGCA    1380
P  F  G  S  G  R  R  M  C  P  G  V  N  L  A  T  S  G  M  A
ACACTGCTTGCAACTCTTATCCAGTGCTTTGACCTGCAAGTAGTGGGCCCAAAAGGAGAA    1440
T  L  L  A  T  L  I  Q  C  F  D  L  Q  V  V  G  P  K  G  E
ATATTGAAAGGTAAGGACGCCAAAGTTAGCATGGAAGAGAGAGCTGGCCTCACTGTTCCA    1500
I  L  K  G  K  D  A  K  V  S  M  E  E  R  A  G  L  T  V  P
AGGGCACATAATCTCGTGTGTGTTCCACTTGCAAGGACAAGCGTTGCATCTAAACCTCCT    1560
R  A  H  N  L  V  C  V  P  L  A  R  T  S  V  A  S  K  P  P
TCTTGA                                                          1566
S  *
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Figure 1. Nucleotide and deduced amino acid sequences of the *P. candollei* var. *mirifica PcmIFS* gene. Translation stop codon is indicated by an asterisk. The ER targeting sequence is highlighted in grey color and the region rich in basic amino acid residues is indicated by boxes around the amino acids. The proline rich region is in bold. The highly conserved "I" helix and the conserved motif of unknown function among P450 sequences are indicated by underline and double-underline, respectively. The heme binding motif and amino acid lysine "K" required for aryl migration of flavanone molecule are in bold italic and shaded box, respectively.

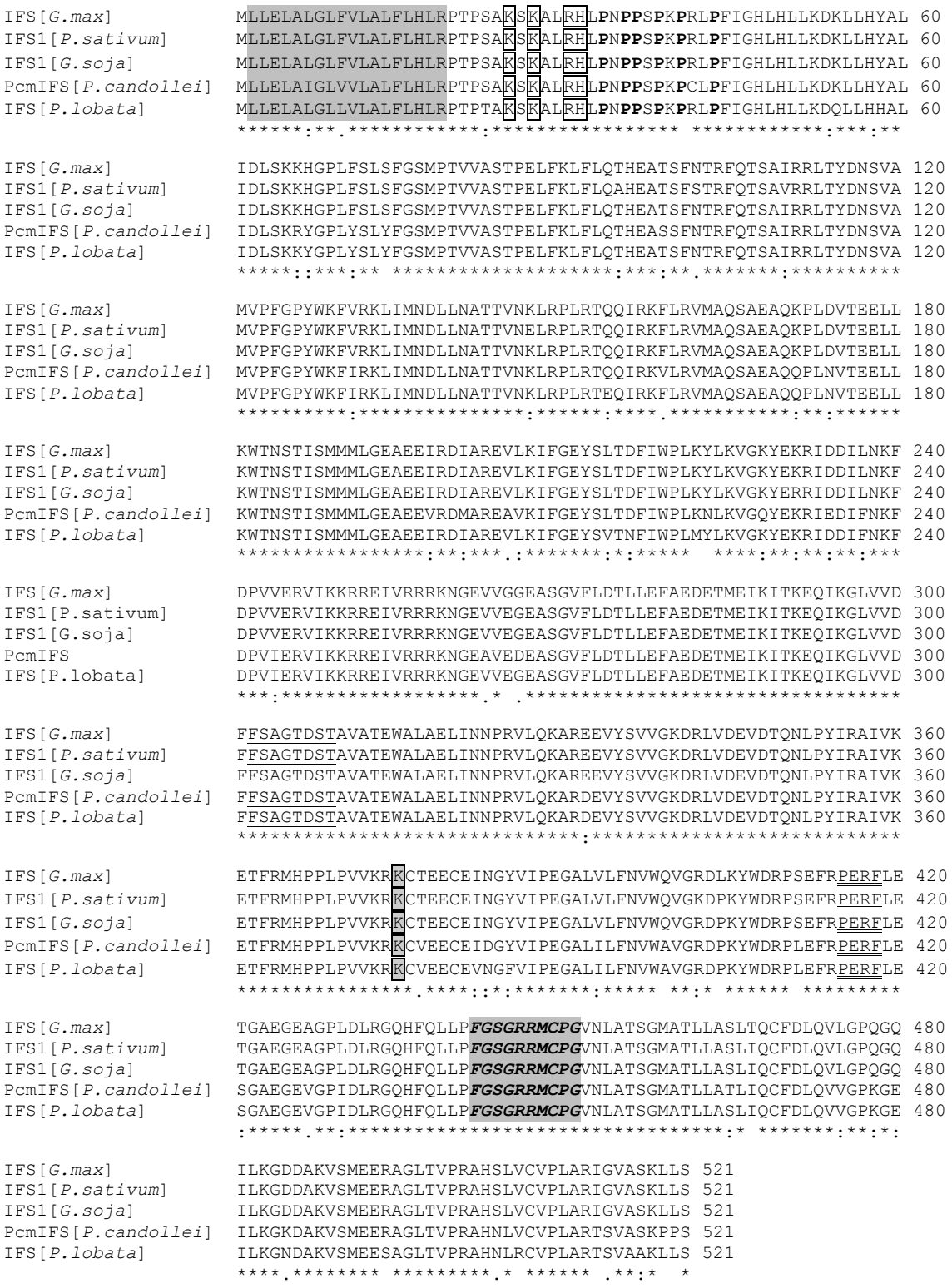

Figure 2. Multiple alignment of the deduced amino acid sequence of PcmIFS and IFS proteins from *G. max* (ACA81460), *P. sativum* (AAF34533), *G. soja* (ACA81487) and *P. montana* var. *lobata*. (AAM12530). All of the conserved motifs are indicated as described in Figure 1.

(2010) also illustrated high relationship between *IFS* genes of leguminous plant species. The conservation of activity-specific motifs as well as phylogenetic analysis suggests that this *PcmIFS* cDNA may encode functional

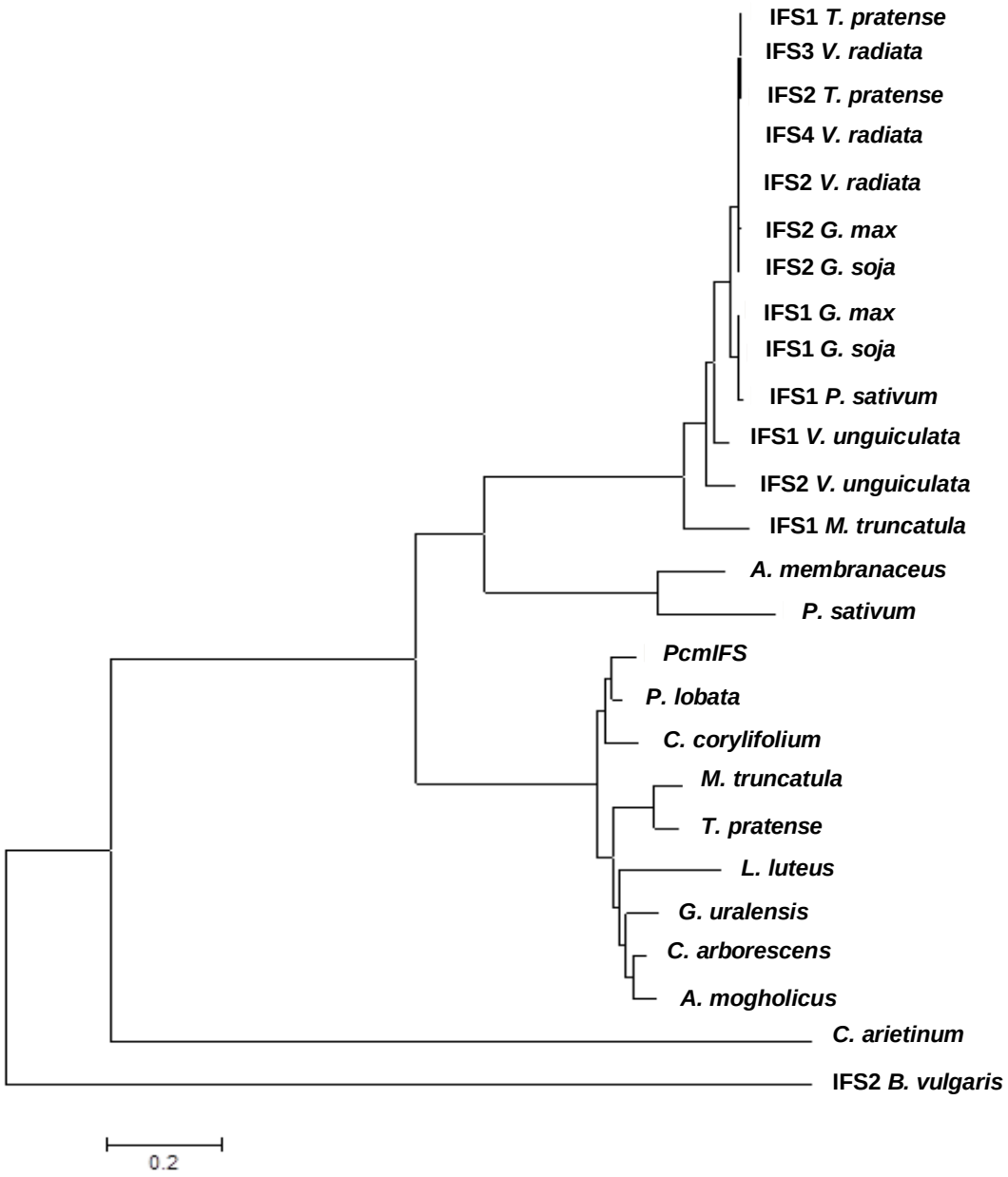

Figure 3. An unrooted phylogenetic tree showing the relationships between *PcmIFS* and *IFS* genes from other plant species using MEGA 4 program by neighbor-joining method.

IFS from *P. candollei* var. *mirifica* (Misra et al., 2010).

Southern blot analysis

A copy number of the *PcmIFS* in genome of *P. candollei* var. *mirifica* was determined by southern blot analysis. Genomic DNA isolated from *P. candollei* var. *mirifica* was digested with *Eco*RI, *Bam*HI, and *Hin*dIII and was hybridized with *PcmIFS* cDNA labeled probe. The result showed that two to three hybridization bands were observed (Figure 4), indicating that *PcmIFS* belonged to a multigene family like *IFS* genes in other plant species (Steele et al., 1999; Akashi et al., 1999; Jung et al. 2000). In *L. japonicas*, two to three copies of *IFS* gene have been noticed (Shimada et al., 2000), and in *V. unguiculata*, two copies of *IFS* gene have been reported. Furthermore, two copies of *IFS* gene in non-legume plants such as sugar beet have been detected (Jung et al., 2000). On the contrary, a single copy of the *IFS* gene in the genome of *T. pratense* has been found (Kim et al., 2003).

Most of the *IFS* genes in legume plant contained one intron, e.g. the *IFS* gene in *T. pratense* possessed one

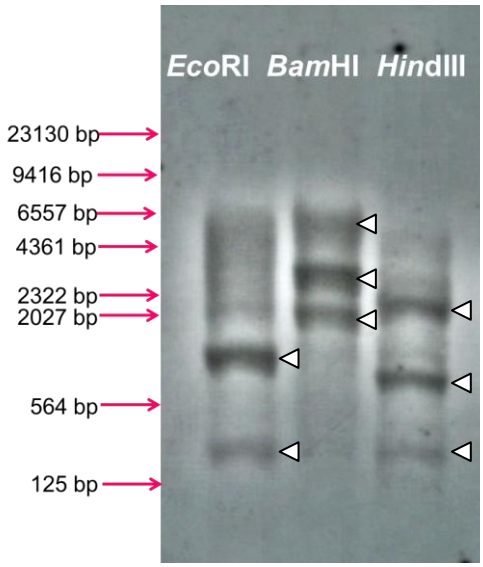

Figure 4. Southern blot analysis of the genomic DNA of *P. candollei* var. *mirifica*. Genomic DNA isolated from leaves was digested with *Eco*RI, *Bam*HI, and *Hin*dIII, electrophoresed on 1% agarose gel and transferred to nylon membrane. The membrane was hybridized with an internal fragment of the *IFS* gene amplified with the primer PcmIFSF and PcmIFSR.

intron of 140 bp (Kim et al., 2003). In *V. unguiculata*, the *IFS1* and *IFS2* also contained one intron of 290 and 82 bp, respectively (Kaur and Murphy, 2010). Therefore, genomic DNA cloning of the *PcmIFS* should be done in the future in order to determine a number of intron in *P. candollei* var. *mirifica*.

Expression of *PcmIFS* in different tissues and under stress conditions

To study the expression profiles of *PcmIFS* in different tissues and under stress conditions, total RNA prepared from leaves, stems, roots, and leaves of 1-month-old plants after treatment with various temperatures, UV-B irradiation and wounding was subjected to RT-PCR. As shown in Figure 5a, *PcmIFS* expression was detected in all tissues investigated, including leaves, stems, and roots. The transcription levels of *PcmIFS* in leaves and roots were the highest. This could be explained by the fact that this plant synthesized isoflavones such as daidzein, genistein mainly in the leaves and the compounds were stored in its tuberous roots (Thanonkeo and Panichajakul, 2006). Another possibility is that the high transcription level of *PcmIFS* in roots may be related to symbiotic interaction between the isoflavones and nitrogen-fixing bacteria, as described by Shimada et al. (2000). Kim et al. (2003) investigated the expression of *IFS-Tp* in *T. pratense* and found that *IFS-Tp* was detected at almost the same level in all tissues tested, including leaf, stem, and root. Dhaubhadel et al. (2003) investigated the expression profiles of the *IFS1* and *IFS2* genes in soybean seeds and found that the *IFS1* was present in all tissues and all developmental stages including embryo, seed, pod, flower, seed coat, leaf, stem, and root, but the highest expression was observed in seed coat and root tissues. On the contrary, the expression of *IFS2* was detected only in embryo and pod tissue during late stage of development. In *P. corylifolia*, most tissues including leaf, stem, root, and fruit accumulated more or less similar amount of *IFS* transcript, though flower showed least expression (Misra et al., 2010).

The expression profiles of *PcmIFS* in response to environmental stimuli including different temperatures, UV-B irradiation, and wounding were examined and the results are shown in Figure 5b to d. Under different temperature treatments, transcription level of *PcmIFS* was the highest at 4°C, as compared to that at 25 and 37°C (Figure 5b). Our results are in agreement with the finding of Tsukamoto et al. (1995) and Carrão-Panizzi et al. (1999) who reported the high level of isoflavones accumulation in soybean at low temperatures. UV-B has been reported to enhance the expression of *IFS* gene as well as the biosynthesis of isoflavones in several plant species (Jansen et al., 1998; Kim et al., 2003). Therefore, we examined the effect of UV-B treatment on the expression of *PcmIFS* in this study. The expression of *PcmIFS* was increased and it reached its maximum at 4 and 6 h after treatment, and then slightly declined at 8 h (Figure 5c). In other legume plants such as *T. pratense* and *Astragalus mongholicus*, the expression of *IFS* genes was increased and it reached its maximum at 6 and 10 h after UV-B treatment, respectively (Kim et al., 2003; Xu et al., 2011). The reduction of *PcmIFS* expression at 8 h after treatment might be due to the over exposure of plant to UV-B which in turn causes serious damage or destruction of self defense system of plant cells (Lee et al., 2008). In previous studies, we observed that wounding treatment enhanced the expression of *CHS* gene in *P. candollei* var. *mirifica* (Wiriyaampaiwong, 2012). Thus, the effect of wounding treatment on the expression of *PcmIFS* was analyzed and the results are summarized in Figure 5d. In response to wounding treatment, the transcription level of *PcmIFS* was increased and it reached the highest level at 48 h. This result is in agreement with the finding of Reymond and Farmer (1998) and Bailey et al. (2005) who reported that wounding treatment induced the expression of many genes in *Arabidopsis* and *Theobroma cacao*, respectively.

In this study, we emphasized on the expression profiles of *PcmIFS* upon physical stresses like temperature, UV-B irradiation, and wounding treatment. However, there are some elicitors, such as yeast extract, methyl jasmonate and ethylene, which have been shown to function as

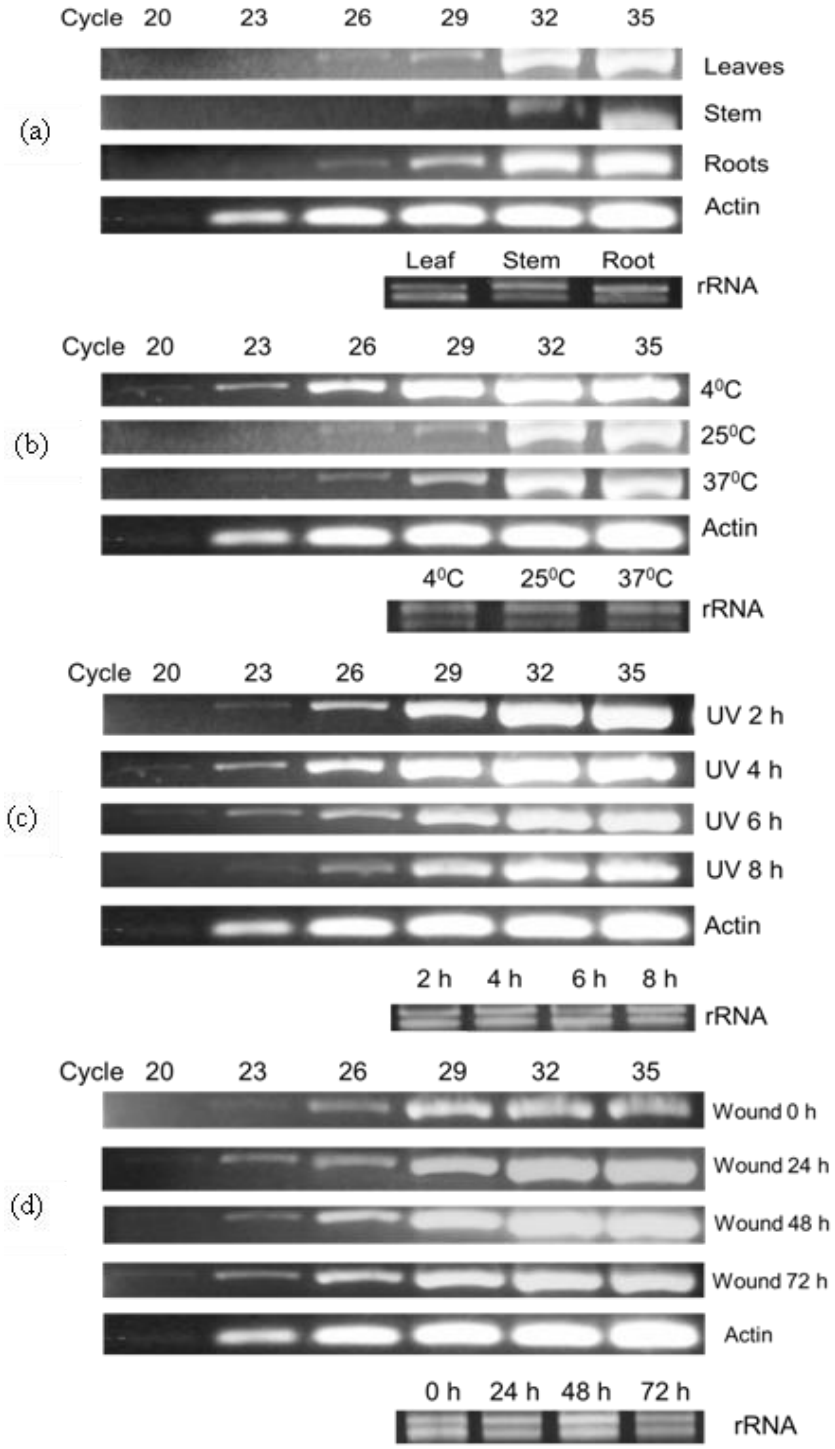

Figure 5. Expression profiles of *PcmIFS* in different parts of plant (a), under different temperatures (b), UV-B irradiation (c), and wounding treatment (d). Actin gene was used as an internal control for RT-PCR system. The numbers above the lanes represent the number of PCR cycles. Ethidium bromide-stained rRNA was used as a control to normalize the amount of total RNAs (10 µg).

regulators of many genes involved in isoflavones biosynthesis pathway as well as many different plant defense genes (Naoumkina et al., 2007; 2008; Mejia-Teniente et al., 2010). In *M. truncatula*, L-phenylalanine ammonia-lyase (*PAL*), cinnamate 4- hydroxylase (*C4H*), 4-coumarate CoA ligase, chalcone synthase (*CHS*),

chalcone reductase (*CHR*), and chalcone isomerase (*CHI*) genes were up-regulated in response to yeast extract. Likewise, the CHI-like gene (*TC107065*) and *WRKY* gene families were highly induced upon methyl jasmonate treatment (Naoumkina et al., 2007; 2008). In *T. cacao*, a type III peroxidase (*TcPer-1*) and endo-1,4-β-glucanase (*TcGlu-1*) genes were up-regulated after ethylene treatment (Bailey et al., 2005). Whether the expression of *PcmIFS* is regulated by these compounds remains to be investigated.

Conclusion

P. candollei var. *mirifica* has long been used for promoting youthfulness and estrogen replacement therapy due to its active compounds in the tuberous roots (Muangman and Cherdshewasart, 2001). Recently, extracts and purified compounds from this herb have been shown to contain antiinflammatory (Shirley, 2001; Trisomboon et al., 2006), neurobehavioral (Brouns, 2002), prevention of bone loss (Urasopon et al., 2007), and antioxidant activities (Cherdshewasart and Sutjit, 2008). These activities have been associated with isoflavones synthesized in this plant. Although, numerous studies have been carried out for the use of this plant in several other purposes, no study has been carried out in relation to molecular characterization of genes involved in the synthesis of isoflavones from this plant. This is the first report to characterize the *IFS* gene, *PcmIFS*, from this medicinal plant. The full *IFS* cDNA, designated as *PcmIFS*, was successfully cloned from *P. candollei* var. *mirifica* and it belonged to a multigene family. The *PcmIFS* gene product shared common features with known IFS proteins from leguminous plant species. *PcmIFS* was constitutively expressed in all tissues of plant, including leaf, stem, and root and its expression was induced by abiotic stresses, including low and high temperature stresses, UV-B irradiation, and wounding treatments. Since the formation of isoflavones is complex and several candidate genes, such as chalcone synthase (*CHS*), chalcone isomerase (*CHI*), and 2-hydroxyisoflavanone dehydratase (*IFD*) have been proposed to be involved in this process. Thus, further study on the characterization of these genes and their regulation related to the biosynthesis pathway of isoflavones should be carried out. The knowledge gained concerning such studies would not be only useful in understanding the isoflavone biosynthesis in *P. candollei* var. *mirifica*, but also provide molecular wealth for the improvement of this medicinal plant as well as other non-legumes plants as well.

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