Journal of Medicinal Plants Research Vol. 6(42), pp. 5469-5479, 3 November, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR12.036
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Cloning and characterization of chalcone synthase gene from *Pueraria candollei* var. *mirifica*

Piyachat Wiriyaampaiwong[1], Sudarat Thanonkeo[2] and Pornthap Thanonkeo[1,3]*

[1]Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand.
[2]Walai Rukhavej Botanical Research Institute, Mahasarakham University, Mahasarakham 44150, Thailand.
[3]Fermentation Research Center for Value Added Agricultural Products, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand.

Accepted 22 June, 2012

The full-length open reading frame of chalcone synthase (CHS) gene, designated as *PcCHS*, was obtained from *Pueraria candollei* var. *mirifica* using reverse transcription polymerase chain reaction (RT-PCR). It was 1170 bp in length, encoded for 389 amino acid residues with the relative molecular mass of 42.6 kDa. The predicted isoelectric point of the gene product was 5.10. Southern blot analysis indicated that the *P. candollei* var. *mirifica CHS* gene belonged to a multigene family. The deduced amino acid sequence of the *PcCHS*-encoded protein showed a high degree of identity to those of the CHS from *Pueraria montana*, *Glycine max*, *Phaseolus vulgaris*, *Vigna unguiculata*, *Medicago sativa* and *Pisum sativum*. The expression of *PcCHS* gene was detected in all tissues (leaf, stem and root) examined, and its expression was induced by UV-B irradiation and wounding treatment.

Key words: *Pueraria candollei* var. *mirifica*, chalcone synthase, flavonoids biosynthesis, gene expression.

INTRODUCTION

Pueraria candollei var. *mirifica*, commonly known as “White Kwao Krua”, is an indigenous herb found in the North, West and Northeastern part of Thailand especially at the high altitude of 300 to 800 m above sea level (Muangman and Cherdshewasart, 2001; Trisomboon et al., 2006). It belongs to the family Leguminosae and subfamily Papilionoideae. This plant accumulates high level of isoflavonoids that act as estrogenic hormone in its tuberous roots. These compounds have been called “phytoestrogen” consisting of miroestrol, deoxymiroestrol, daidzin, puerarin, genistin, mirificin, kwakhurin, puemiricarpene, coumestrol, daidzein and genistein (Ingham et al., 1986a, 1986b; Tahara et al., 1987; Ingham et al., 1989c; Chansakaow et al., 2000; Boonsnongcheep et al., 2010). They offer protective effect against a number of complex diseases such as osteoporosis (Setchell and Lydeking-Olsen, 2003; Urasopon et al., 2007), menopausal ailments (Krebs et al., 2004), lung cancer (Schabath et al., 2005), and breast cancer (Nebe et al., 2006). Thai women prefer to consume these compounds for promoting youthfulness and hormone replacement therapy (Cherdshewasart et al., 2007).

In higher plants, isoflavonoids are synthesized via the phenylpropanoid pathway. The first committed step in this pathway is carried out by chalcone synthase (CHS; EC 2.3.1.74). It is a member of the plant polyketide synthase superfamily, which also includes other proteins such as stilbene synthase (STS), acridone synthase, benzalacetone synthase, bibenzyl synthase, biphenyl synthase, benzophenone synthase, pyrone synthase (2-PS), pentaketide chromone synthase and *p*-coumaroyltriacetic acid synthase (Sanchez, 2008; Dao et al., 2011). CHS catalyzes the stepwise condensation of three acetate moieties derived from malonyl-CoA with 4-coumaroyl-CoA derived from the phenylpropanoid pathway to form the C15 flavonoid skeleton, naringenin

*Corresponding author. E-mail: portha@kku.ac.th. Tel/Fax: +66-43-362121.

chalcone (Kreuzaler et al., 1979; Heller and Hahlbrock, 1980). Isomerization and further substitution of this central intermediate lead to the production of flavones, isoflavonoids, anthocyanins and others (Hahlbrock and Scheel, 1989; Jiang et al., 2006; Dao et al., 2011). These compounds possess certain positive roles in different tissues of plants such as flower and fruit pigmentation, insect repellents, pollen fertility, ultraviolet (UV) protection and phytoalexins (Dixon, 1986; Clegg et al., 1997). In accordance with the specialized function of the different types of flavonoids, the activity of CHS is specifically regulated by various external stimuli, such as low temperature (Leyva et al., 1995), UV-B, UV-A and blue light (Hartmann et al., 1998; Wade et al., 2001; Zhou et al., 2007), elicitor treatments (salicylic acid, methyl jasmonate) (Schenk et al., 2000), wounding (Richard et al., 2000), and pathogen attack (Soylu, 2006) or by endogenous mechanisms in plant development and tissue differentiation such as gametophyte development and flower development (Van der Meer et al., 1992; Junghans et al., 1993; Liew et al., 1998; Claudot et al., 1999; Pang et al., 2005).

As a result of its important role in initiating flavonoid biosynthesis, chalcone synthase (*CHS*) genes have been cloned and characterized in many plant species. The first *CHS* gene was isolated from UV-irradiated parsley suspension cultures as a cDNA related to flavonol accumulation (Reimold et al., 1983). Until now, many *CHS* genes have been cloned from monocot, dicot and some gymnosperm species such as *Zea mays* (Franken et al., 1991), *Petunia hybrida* (Holton et al., 1993), *Medicago sativa* (Mckhann and Hirsch, 1994), *Trifolium subterraneum* (Arioli et al., 1994), *Bromheadia finlaysoniana* (Liew et al., 1998), *Arabidopsis thaliana* (Saslowsky et al., 2000), *Sorghum bicolor* (Lo et al., 2002), *Ginkgo biloba* (Pang et al., 2005), *Physcomitrella patens* (Jiang et al., 2006), *Centaurea jacea* (Francini et al., 2008), and *Paeonia suffruticosa* (Zhou et al., 2011).

Generally, the cloned *CHS* genes are found to belong to small multi-gene family and the primary structure of the *CHS* genes are highly conserved (Pang et al., 2005). With respect to the *CHS* gene in *P. candollei* var. *mirifica*, gene organization and expression profiles in different tissues of plant or under environmental stress are scarcely found. In this study, we reported on the cloning, sequencing and characterization of the *CHS* gene in *P. candollei* var. *mirifica*. The expression profiles of this gene in different tissues and under various environmental stresses such as temperature, wounding and UV-B treatments were also investigated. This study will provide useful information for further work on enhancing the production of isoflavonoids in this plant.

MATERIALS AND METHODS

Seeds of *P. candollei* var. *mirifica* were washed thoroughly with tap water. They were subsequently surface-sterilized by shaking in 20% (v/v) of commercial Clorox (The Clorox Co., USA) containing three drops of Tween 20 for 20 min, and thoroughly rinsed 3 times with sterile distilled water. The seeds were then clipped on seed coat and soaked in sterile distilled water for 24 h prior to germinate on hormone free Murashige and Skoog salts (MS) medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose and 1% (w/v) agar in 12 oz. bottles at 25 ± 2°C with a 16/8 h light/dark cycle (light intensity 20 µmole $m^{-2}s^{-1}$, cool white fluorescent Philips TLD 18W/33) and 50% humidity for four weeks. Seedlings obtained were then used for further study.

Genomic DNA and total RNA isolation

Genomic DNA was isolated from leaf of the *P. candollei* var. *mirifica* seedling using the cetyltrimethylammonium bromide (CTAB) method (Russo et al., 1992), whereas total RNA was extracted from leaf using RNeasy Plant Mini Kit (QIAGEN, Germany). The extraction of total RNA was carried out essentially as recommended by the manufacturer's instruction except that RNase-free DNase I was added to a final concentration of 0.1 mg/ml to remove any contamination of genomic DNA.

Cloning and sequencing of *CHS* gene

An internal fragment encoding the amino acid sequence of the *CHS* gene product was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using total RNA isolated from *P. candollei* var. *mirifica* as a template and degenerated oligonucleotide primers synthesized based on the conserved region of the *CHS* gene from other Leguminosae plants in the GenBank database including *Vigna unquiculata*, *Phaseolus vulgaris*, *Pueraria montana*, *Pisum sativum*, *M. sativa* and *Arachis hypogaea*. The sequences of the two degenerated oligonucleotide primers were forward PCHSF (5′-ACTGTGTTGAWCARAGCAC-3′) and reverse PCHSR (5′-ACAAVACACCCCAKTCAA G-3′). The RT-PCR reaction was carried out using OneStep RT-PCR Kit (QIAGEN, Germany), as described by the supplier. The reaction mixture (50 µL) consisted of 10 µL of 5X QIAGEN OneStep RT-PCR buffer, 400 µM dNTP, 0.6 µM of each forward and reverse primer, 2 µl of QIAGEN OneStep RT-PCR enzyme mix and 1 µg of RNA template.

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 30 cycles at 94°C for 1 min, 58°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 product was separated on 0.7% agarose gel and target DNA was excised from the gel and purified using the NucleoSpin Extract II Kit (Macherey-Nagel, Germany). The purified PCR product was ligated into pGEM T-easy vector (Promega, Madison, WI, USA) and the resulting plasmid was transformed into the competent *Escherichia coli* JM109 using the Rapid DNA Ligation and Transformation Kit (Fermentas, USA). The plasmid DNA was isolated from a positive clone and target DNA was sequenced by the dideoxy chain termination methods using the MegaBACE 1000 automated DNA sequencer (Pharmacia Biotech, Sweden).

To obtain the sequence of 5′- and 3′ -end of the *CHS* cDNA, the SMART™ RACE cDNA Amplification Kit (ClonTech, USA) was used. The reaction was carried out essentially as recommended by the manufacturer's instruction. The complete full length open reading frame (ORF) of the *CHS* gene was confirmed by RT-PCR using the forward and reverse primers synthesized based on the known sequences of the 5′- and 3′-end of the *CHS* gene. These two primers were forward PCHSF1 (5′-ATGGTGAGCGTAGCTGAGATCCG-3′) and reverse PCHSR2 (5′-TCATATAGCCACACTATGC-3′). The RT-PCR was performed using OneStep RT-PCR Kit (QIAGEN, Germany), as described by

the supplier. The thermal cycling program was as follows: reverse transcription at 50°C for 30 min, initial denaturation at 95°C for 15 min, 30 cycles of amplification (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) and final extension at 72°C for 10 min. After RT-PCR reaction, the amplified product was separated on 0.7% agarose gel, purified, cloned and sequenced as previously described. The sequence of the *CHS* gene and deduced amino acid sequence was analyzed using GENETYX (Software Development, Tokyo, Japan), while homology searching was performed using FASTA and BLAST program in the GenBank and DDBJ databases. Phylogenetic analysis was performed using MEGA 4 program by neighbor-joining method (Saitou and Nei, 1987). The nucleotide sequence reported in this study was deposited in GenBank under the accession number JQ409456.

Southern hybridization analysis

The presence of the *P. candollei* var. *mirifica CHS* gene in its genome was determined by Southern blotting. Genomic DNA (20 µg) isolated from leaf of *P. candollei* var. *mirifica* was digested with various restriction enzymes including *Hin*dIII, *Bam*HI and *Eco*RI, then subjected to electrophoresis on 0.7% agarose gel and transferred to Hybond N^+ positively charged nylon membrane (Schleicher and Schuell) by overnight capillary transfer. The DNA was cross-linked to the membrane by UV irradiation and baked at 80°C for 2 h. All procedures were performed according to the standard methods as described by Sambrook and Russell (2001).

To prepare a probe for Southern hybridization analysis, an internal fragment encoding the amino acid sequence of the *CHS* gene product was amplified by RT-PCR using total RNA isolated from *P. candollei* var. *mirifica* as a template and forward PCHSF and reverse PCHSR primers. The *CHS* PCR fragment was alkaline phosphatase labeled using Gene Images AlkPhos Direct Labeling and Detection System (AlkPhos DirectTM, Amersham). All procedures for DNA labeling were carried out as recommended by the manufacturer. The Hybond N^+ positively charged nylon membrane with cross-linked DNA was pre-hybridized at 50°C for 2 h in hybridization oven (Hybrid limited equipment class I) with gentle rotation. After incubation, the labeled-DNA probe was added and subsequently incubated at 55°C an overnight. The membrane was washed according to the standard procedure for Southern blot analysis. After washing, the CDP-*Star* detection reagent (AlkPhos Direct™, Amersham) was added onto the membrane and left at room temperature for 5 min. The membrane was then exposed to X-ray film (Hyper film, Amersham) for 1 h, thereafter it was developed and fixed with developer and fixer solution (Kodak), respectively.

Expression analysis of the *P. candollei* var. *mirifica CHS* gene

The expression level of *P. candollei* var. *mirifica CHS* gene in different tissues and under various environmental stresses was determined by RT-PCR. The leaf, stem and root of 4-week-old plant seedlings were collected for total RNA extraction. For temperature stress, 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 1500 µJ/m^2 UV-B irradiation treatment (Pang et al., 2005) and the leaf samples were collected at 0, 10, 20 and 30 min. 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, 12, 24, 48 and 72 h, and immediately frozen in liquid nitrogen and stored at -80°C until use (Pang et al., 2005). Total RNA of all collected samples were extracted by using RNeasy Plant Mini Kit (QIAGEN, Germany), as described by the supplier, and used as template in RT-PCR analysis.

RT-PCR was carried out using OneStep RT-PCR Kit (QIAGEN, Germany) with the specific primers (PCHSF1 and PCHSR2) synthesized based on the 5′- and 3′-region of the *P. candollei* var. *mirifica CHS* gene. The reaction mixture (50 µL) consisted of 10 µL of 5X QIAGEN OneStep RT-PCR buffer, 400 µM dNTP, 0.6 µM of each forward and reverse primer, 2 µL of QIAGEN OneStep RT-PCR enzyme mix and 1 µg of RNA template. As a control, 10 µg samples of total RNA were subjected to agarose gel electrophoresis (0.9% agarose) and 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 30 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min. The amplified products at the 15^{th}, 18^{th}, 21^{st}, 24^{th}, 27^{th} and 30^{th} cycle were subjected to electrophoresis on a 1.0% agarose gel. After staining with ethidium bromide, the relative amounts measured as the intensity of the signal in the gel of the PCR products were compared using the Gel Image Master (Gel Doc XR^+) (Bio-Rad, USA). The experiment was repeated at least twice (as biological duplication). Under these conditions, the OneStep RT-PCR was able to specifically detect mRNA because no band was observed when reverse transcriptase was omitted.

RESULTS AND DISCUSSION

Sequence analysis of the *P. candollei* var. *mirifica CHS* gene

RT-PCR, using degenerate *CHS* primers synthesized based on the conserved region of the *CHS* genes from other Leguminosae plants, was performed to amplify products from *P. candollei* var. *mirifica* RNA. A major amplification product of the expected length (approximately 1.0 kb) was obtained (data not shown) and subsequently cloned into the pGEM T-easy vector and sequenced. Homology analysis of nucleotide sequence of this amplified fragment revealed high degree of identity with the corresponding region of *CHS* sequences from other plant species, suggesting that this fragment is a part of *CHS* gene in *P. candollei* var. *mirifica*. The sequences of the 5′- and 3′-end of the *P. candollei* var. *mirifica CHS* cDNA was amplified by RT-PCR using the SMART™ RACE cDNA Amplification Kit (ClonTech, USA) as earlier described. After sequences assembling, the full length ORF of the *CHS* gene was confirmed by RT-PCR using specific primers synthesized based on the known sequences of the 5'- and 3'-end of the *CHS* gene. After DNA sequencing, a complete full length ORF encoding the entire amino acid sequence of the *CHS* gene product was obtained (GenBank accession number JQ409456). The nucleotide sequence of this ORF, designated as *PcCHS*, its flanking region and the deduced 389-residue amino acid sequence were shown in Figure 1. The length of the *P. candollei* var. *mirifica CHS* cDNA coding region (1170 bp) is in good agreement with the size of the known *CHS* genes in other plant species (Nakajima et al., 1991; Liew et al., 1998; Pang et al., 2005; Francini et al., 2008; Zhou et al., 2011). The coding region ends with a TGA stop codon, and it encodes a polypeptide with a calculated relative molecular mass of 42.6 kDa. The predicted isoelectric

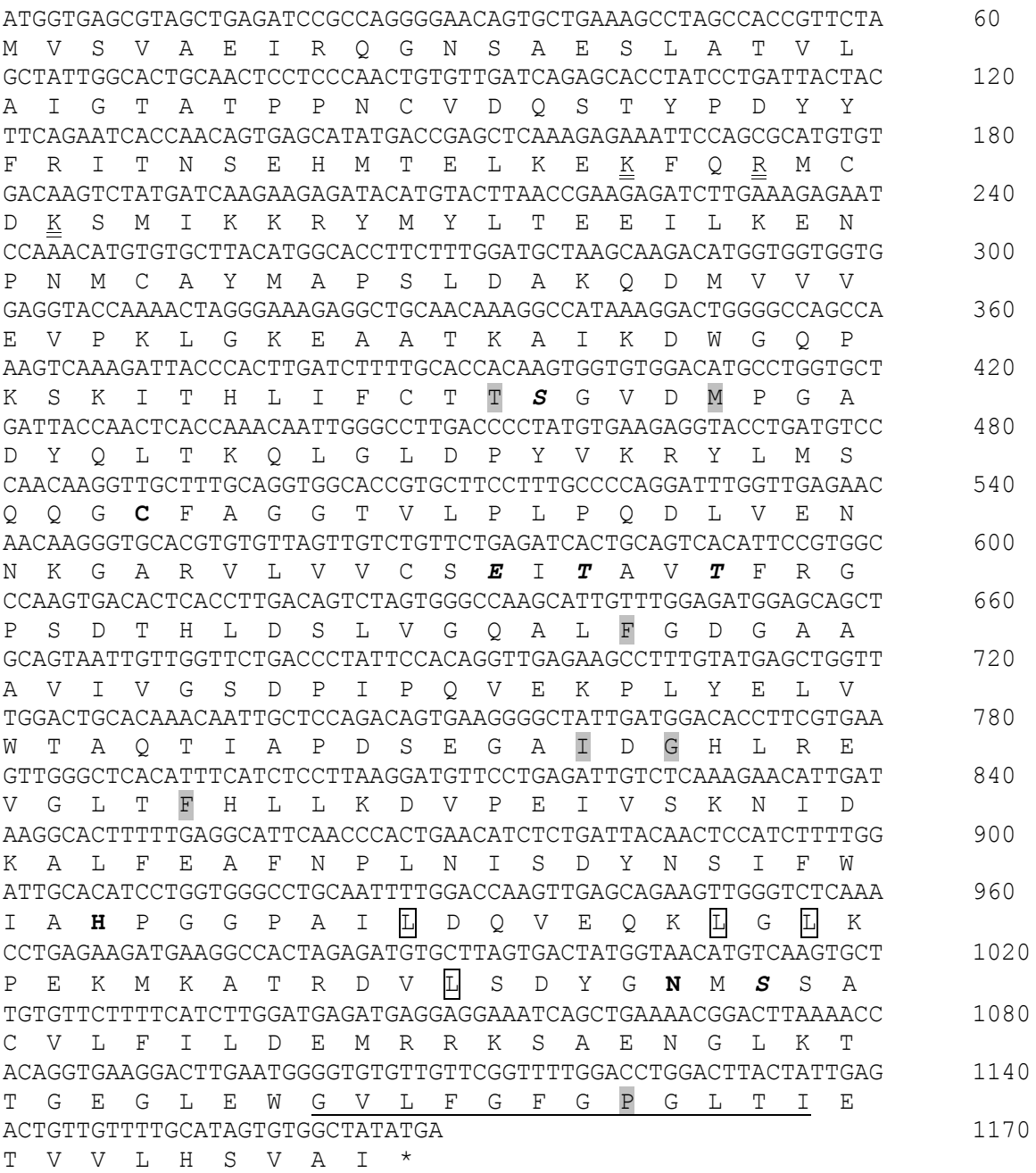

Figure 1. Nucleotide and deduced amino acid sequences of the *P. candollei* var. *mirifica CHS* gene. Nucleotides are numbered from the first nucleotide from 5' end of the sequence. Amino acids are indicated below the nucleotide sequence in single-letter codes. Translation stop codon is indicated by an asterisk. The conserved CHS active site residues are marked in bold while the family signature is indicated by underline. The leucine zipper motifs are indicated by boxes around the amino acids. The seven amino acid residues of the cyclization pocket are shaded and the residues of coumaroyl pocket, including S^{133}, E^{192}, T^{194}, T^{197} and S^{338} are italic and bold. The CoA binding active sites such as K^{55}, R^{58} and K^{62} are double-underlined.

point of this polypeptide is 5.10.

The highly conserved CHS active site residues responsible for the reaction of multiple decarboxylation and condensation, including C^{164}, H^{303} and N^{336} (Ferrer et al., 1999), and the putative CHS family signature (GVLFGFGPGLTI) (Suh et al., 2000) were found in the *PcCHS*. The leucine zipper motif, L^{310}, L^{317}, L^{319} and L^{331} (Claudot et al., 1999), as well as the functionality of the active site (Helariutta et al., 1995) were also found in the *PcCHS* gene product (Figure 1). The seven amino acid residues of the cyclization pocket, including T^{132}, M^{137}, F^{215}, I^{254}, G^{256}, F^{265} and P^{375}, the residues of coumaroyl

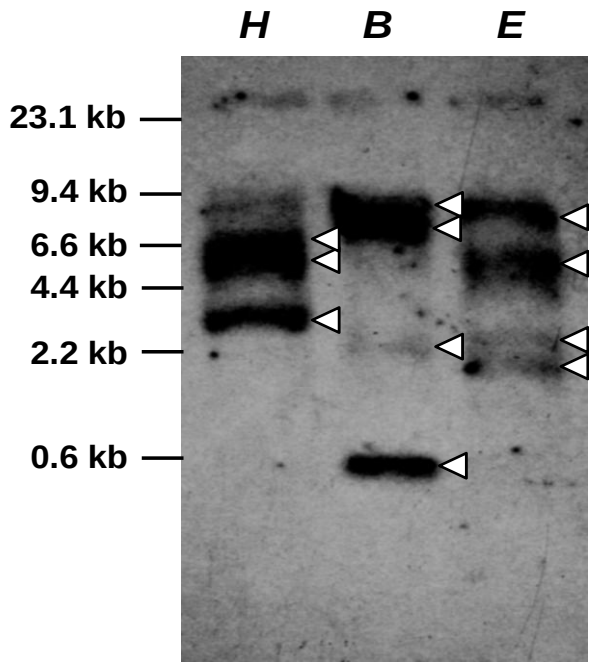

Figure 2. Southern blot analysis of the genomic DNA of *P. candollei* var. *mirifica*. Genomic DNA isolated from leaves was digested with *Hin*dIII (H), *Bam*HI (B) and *Eco*RI (E), electrophoresed on 0.7% agarose gel and transferred to nylon membrane. The membrane was hybridized with an internal fragment of the *CHS* gene amplified with the primer PCHSF and PCHSR.

pocket, including S^{133}, E^{192}, T^{194}, T^{197} and S^{338}, and the CoA binding active sites such as K^{55}, R^{58} and K^{62} were also conserved in the *PcCHS* (Zhou et al., 2011). The *CHS* genes studied so far contain a single intron and two exons, with the only exception of *CHS* gene from *Antirrhinum majus* and *P. patens* which contains two introns (Sommer and Saedler, 1986; Jiang et al., 2006). The exon 1 of *CHS* genes ranged from 110 - 190 bp, while exon 2 was about 1 kb in most plant species, such as in *Pinus sylvestris* (Fliegmann et al., 1992), *A. thaliana* (Saslowsky et al., 2000) and *G. biloba* (Pang et al., 2005). Usually, the position of the single intron in the *CHS* genes is conserved, almost at the first cysteine codon, but the length of the intron varies in different plant species, ranging from less than 100 bp to more than 1 kb (Yang et al., 2002). It has been reported in the *CHS* gene from *A. thaliana* that the length of intron was 85 bp (Feinbaum and Ausubel, 1988), while that for the *G. biloba CHS* gene was 119 bp (Pang et al., 2005). The large intron has been reported for the *CHS* genes from soybean (645 bp) (Akada et al., 1993) and walnut (669 bp) (Claudot et al., 1999). There are few reports on divergence of intron in the *CHS* genes, e.g., in sorghum and petunia, the length of intron for each member was different ranging from 150 bp to 2.0 kb and 700 bp to almost 4.0 kb, respectively. These findings indicated that each member was specific at intron level rather than at exon level (Koes et al., 1989; Lo et al., 2002). With respect to the position and length of intron in the *P. candollei* var. *mirifica CHS* gene, further studies on cloning, sequencing and characterization of the genomic DNA are needed and these are now under our investigation.

Southern blot analysis

Southern blot analysis was carried out in order to estimate the number of genes coding for CHS in the genome of *P. candollei* var. *mirifica*. Genomic DNA isolated from leaves was digested separately with *Hin*dIII, *Bam*HI and *Eco*RI, transferred to Hybond N^+ positively charged nylon membrane and hybridized with the alkaline-labeled coding sequence of *PcCHS* as probe. The result showed that 3 – 4 hybridization bands were observed in each of the restriction enzyme-digested lanes, suggesting that the *PcCHS* gene belonged to a multigene family (Figure 2). This is in agreement with that of *P. hybrida* (Koes et al., 1989), *Z. mays* (Franken et al., 1991), *P. lobata* (Nakajima et al., 1996), *Pisum sativum* (Ito et al., 1997), *B. finlaysoniana* (Liew et al., 1998), *A. thaliana* (Saslowsky et al., 2000), *S. bicolor* (Lo et al., 2002), *G. biloba* (Pang et al., 2005), *Glycine max* (Akada et al., 1993; Matsumura et al., 2005) and *P. patens* (Jiang et al., 2006), in which a multigene family of the *CHS* gene has been reported. In the *P. sativum* genome, at least eight *CHS* genes have been characterized (Ito et al., 1997). A total of seven *CHS* genes have been isolated from a genomic library of *S. bicolor* (Lo et al., 2002) and nine *CHS* genes were found in the genome of *G. max* (Matsumura et al., 2005).

The *CHS* multigene family in higher plant is directly related to the functional diversity of flavonoids. Some of these genes are constitutively expressed, while others are transcriptionally induced by environmental factors (Pang et al., 2005). In *P. sativum*, for example, the eight members of *CHS* gene family showed different expression patterns under elicitor treatment and UV irradiation (Ito et al., 1997). In *P. patens*, there were eight *CHS* gene classes that are expressed at different developmental stages or under different growth conditions (Jiang et al., 2006).

Comparison of the amino acid sequence of the *P. candollei* var. *mirifica CHS* gene with *CHS* genes of other plants

The deduced amino acid sequence of the *P. candollei* var. *mirifica PcCHS* showed high sequence identity to the known CHS proteins from other plants such as *P. montana* var. *lobata* (D10223) (95%), *P. vulgaris* (X06411) (92%), *V. unguiculata* (X74821) (89%), *G. max* (FJ770471) (87%), *M. sativa* (L02902) (86%) and *P.*

sativum (X63335) (86%), respectively (Figure 3). The main conserved amino acid residues, (C^{164}, H^{303} and N^{336}), which functions as the active site of the enzyme (Ferrer et al., 1999) and the putative CHS family signature (GVLFGFGPGLTI) (Suh et al., 2000) were presented in these putative proteins. However, amino acid residue of the putative CHS family signature was changed in *V. unguiculata* to serine (S) instead of threonine (T). This might be due to a difference in the plant genotype. The leucine zipper motif (L^{310}, L^{317}, L^{319} and L^{331}) also matched the consensus sequence that is conserved in the plant CHS proteins (Claudot et al., 1999).

A phylogenetic tree was constructed based on multiple alignments of the various plant CHS amino acid sequences using the neighbor-joining method (Saitou and Nei, 1987). The result revealed a close relationship between the CHS from *P. candollei* var. *mirifica* and other Leguminosae plants (Figure 4), suggesting that these CHS proteins share a common ancestor.

Expression analysis of the *P. candollei* var. *mirifica* *CHS* gene

To determine the expression patterns of the *PcCHS* gene, we compared the transcript level of this gene in different tissues of *P. candollei* var. *mirifica*, including leaf, stem and root. As shown in Figure 5, the *PcCHS* transcripts were detected in all tissues examined. The highest accumulation of the *PcCHS* transcript was found in leaf, followed by root and stem. Based on the intensity of the signal in the gel measured by Gel Image Master, the accumulation level of the *PcCHS* transcript in leaf was approximately 1.3 and 6.9 times higher than those in root and stem, respectively. This is not surprising because this plant has been shown to accumulate high amount of isoflavonoids such as daidzein and genistein in tuberous roots, which are proposed to be synthesized from leaves of the plant (Thanonkeo and Panichajakul, 2006). The expression profiles of *PcCHS* gene in the present study are consistent with that of Liew et al. (1998) who determined the expression pattern of *CHS* gene in the orchid *B. finlaysoniana* by RT-PCR analysis and reported that the *CHS* transcripts were visualized in all parts of the plant (leaf, stem, root and flower) and all floral organs (column, lip, petal, sepal and stalk) examined. In white spruce, levels of *CHS* mRNA were also observed in all tissues (needle, stem and root) examined (Richard et al., 2000). Our results are also in accordance with other investigations that have been examined the accumulation level of *CHS* transcripts in other plants, but with some differences. In alfalfa, the *CHS* transcript levels are high in root tips and entire young roots, but very low in aerial portions of the plant (stems, leaves and flowers) (McKhann and Hirsch, 1994). The *CHS* transcript levels have also been shown to be relatively high in roots of pea (Harker et al., 1990) and soybean (Estabrook and Sengupta-Gopalan, 1991), indicating that high transcript levels of *CHS* in roots may be a common feature in Leguminosae plants (McKhann and Hirsch, 1994).

The *CHS* multigene family in higher plants has been shown to induced by various environmental factors such as pathogen attack (Zhu et al., 1996), light and UV radiation (Ito et al., 1997; Gläßgen et al., 1998; Pang et al., 2005), elicitor treatment (Richard et al., 2000), wounding (Pang et al., 2005) and ozone exposure (Francini et al., 2008). Thus, in this study, we examined the changes in expression levels of *PcCHS* gene in *P. candollei* var. *mirifica* in response to low or high temperature, UV-B radiation and wounding and the results are summarized in Figure 6. The accumulation level of *PcCHS* transcripts was decreased when plants were exposed to low (4°C) and high temperature (37°C), as compared to normal growth temperature (25°C) (Figure 6a). These results indicated that the expression of *PcCHS* was suppressed by low or high temperature. Under UV-B radiation, the transcript accumulation of *PcCHS* was raised to 10 min after UV-B exposure and slightly reduced thereafter (Figure 6b). Based on the intensity of the signal in the gel, the accumulation level of *PcCHS* transcript at 10 min after UV-B exposure was approximately 2.6 and 2.8 times higher than those at 20 and 30 min after exposure. Overexposure to UV-B radiation might cause serious damage of the plant, meaning that the self defense system of the plant was destroyed by the overexposure to UV-B (Lee et al., 2008).

Under wounding treatment, the transcription level of *PcCHS* slightly increased and reached the highest level at 24 h (Figure 6c). Based on the intensity of the signal in the gel, the accumulation level of *PcCHS* transcript at 24 h after wounding treatment was approximately 1.1 times higher than those at 12 and control treatment (without wounding). It was also about 1.2 and 1.5 times higher than those found at 48 and 72 h after wounding. These results suggested that wounding treatment enhanced the expression level of *PcCHS* in *P. candollei* var. *mirifica*, as was reported in other plants. In white spruce, for example, wounding induced maximal *CHS* mRNA accumulation approximately 8 h after treatment (Richard et al., 2000). In soybean and alfalfa, wounding by cutting hypocotyls tissue (soybean) or leaves (alfalfa) induced maximal *CHS* mRNA accumulation approximately 4 - 8 h after treatments (Creelman et al., 1992; Junghans et al., 1993).

Some elicitors such as jasmonic acid and methyl jasmonate have been shown to have great impact on the expression of *CHS* in several plant species including *Petroselinum crispum* (Dittrich et al., 1992), parsley (Ellard-Ivey and Douglas, 1996) and white spruce (Richard et al., 2000). Whether the expression of *PcCHS* in *P. candollei* var. *mirifica* is regulated by these compounds remains to be investigated.

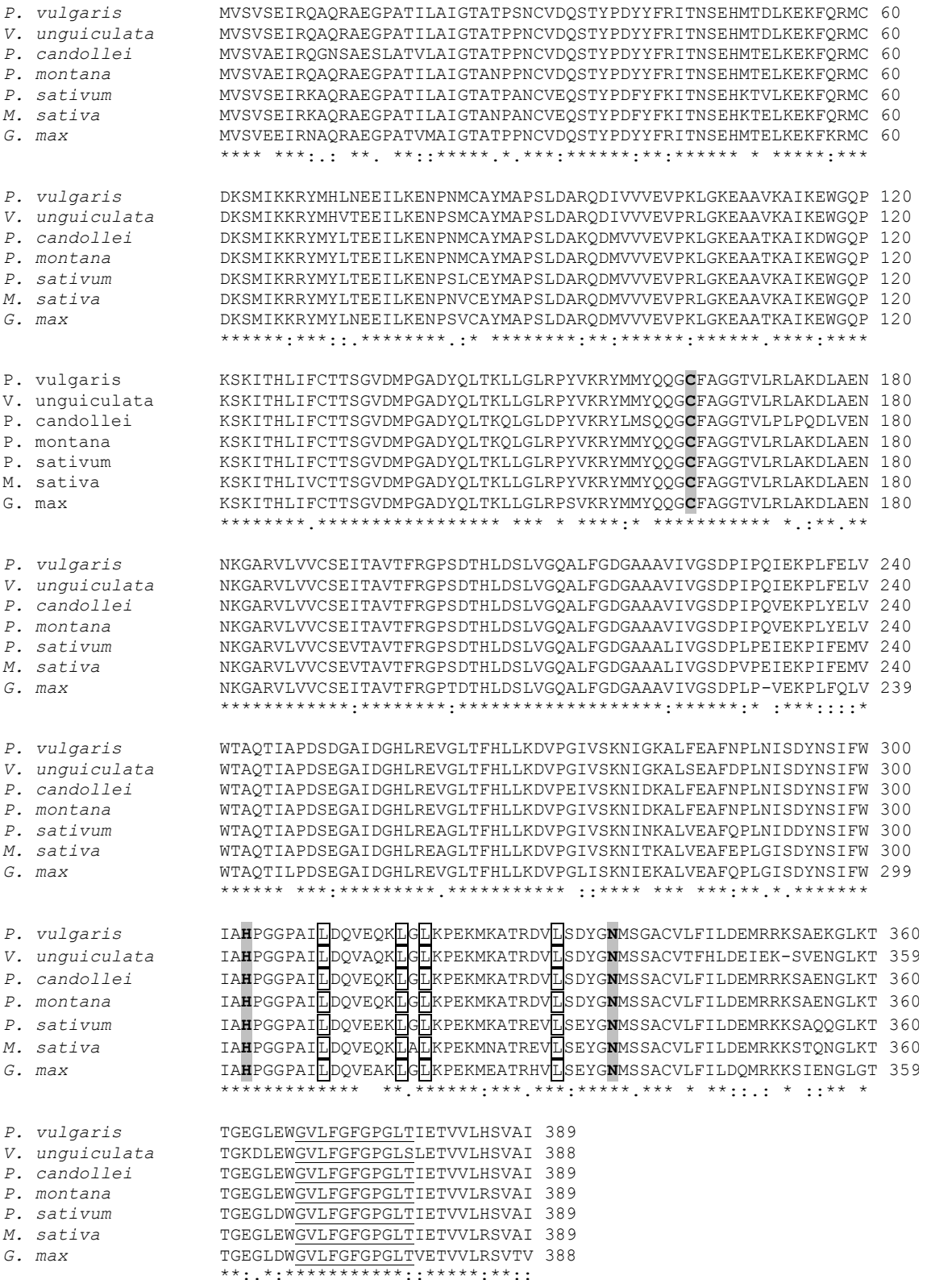

Figure 3. Multiple alignment of the deduced amino acid sequence of the *P. candollei* var. *mirifica* CHS and CHS protein from *P. vulgaris* (X06411), *V. unguiculata* (X74821), *P. montana* (D10223), *G. max* (FJ770471), *P. sativum* (X63335) and *M. sativa* (L02902). Amino acid residues identical and similar between each other are shown by asterisks and dots, respectively. Gaps introduced to maximize the alignment are indicated by a horizontal dash. The conserved CHS active site residues and the family signature are marked in shaded bold letter and underline, respectively. The leucine zipper motifs are indicated by boxes around the amino acids.

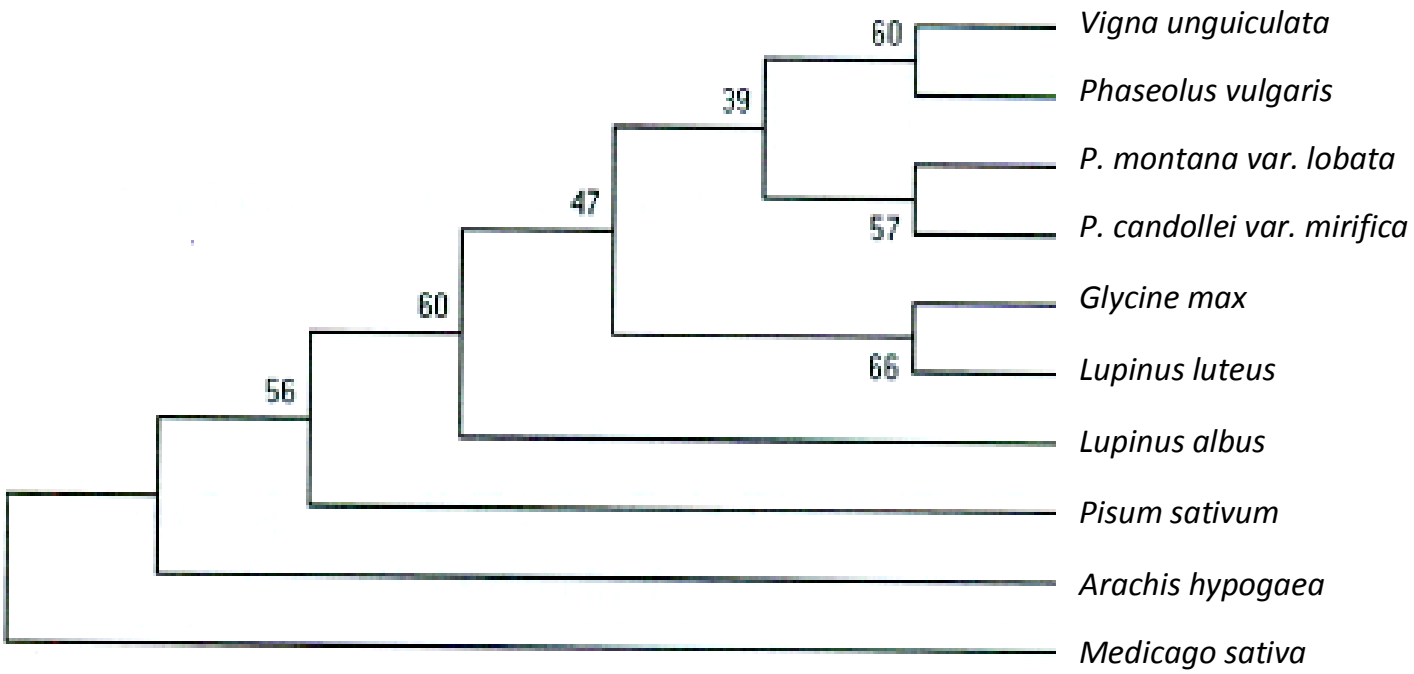

Figure 4. An unrooted phylogenetic tree showing the relationships between CHS protein from *P. candollei* var. *mirifica* and other plant species. The tree was built using MEGA 4 program by neighbor-joining method. Numbers represents bootstrap values.

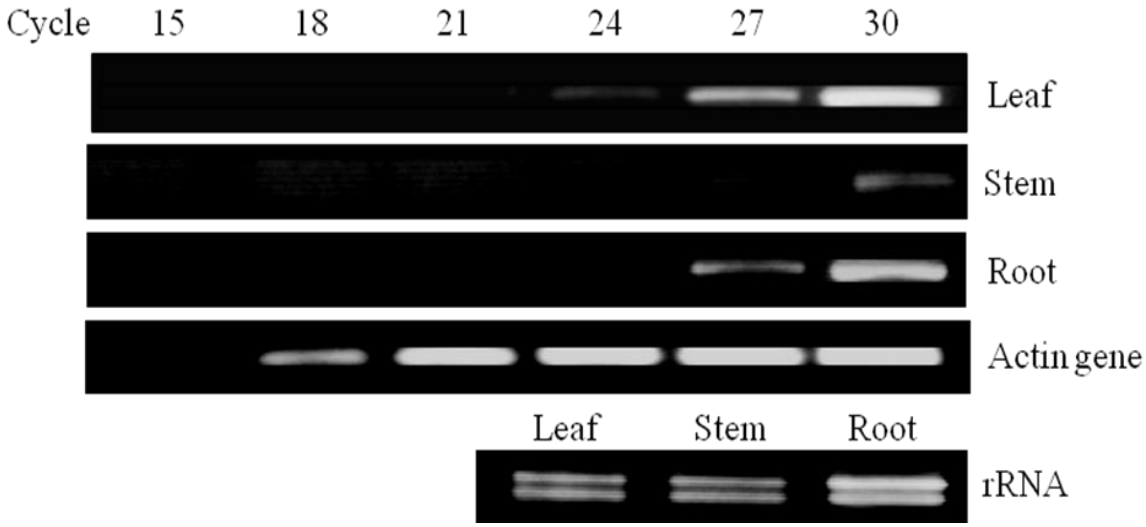

Figure 5. RT-PCR analysis of the *P. candollei* var. *mirifica CHS* gene expression in different tissues of plant. Total RNAs were isolated from leaf, stem, root and then subjected to RT-PCR analysis with primers specific for the *CHS* gene as described in materials and methods. Actin gene was used as 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).

Conclusion

The *CHS* gene, designated as *PcCHS*, which is involved in the biosynthesis of isoflavonoids, was cloned from *P. candollei* var. *mirifica*. This gene shared common features in term of amino acid sequence such as the highly conserved CHS active site, the putative CHS family signature, as well as the leucine zipper motif with known *CHS* gene products from other Leguminosae plants. The *PcCHS* belonged to a multigene family in *P. candollei* var. *mirifica* and its expression was enhanced by environmental stresses, including UV-B and

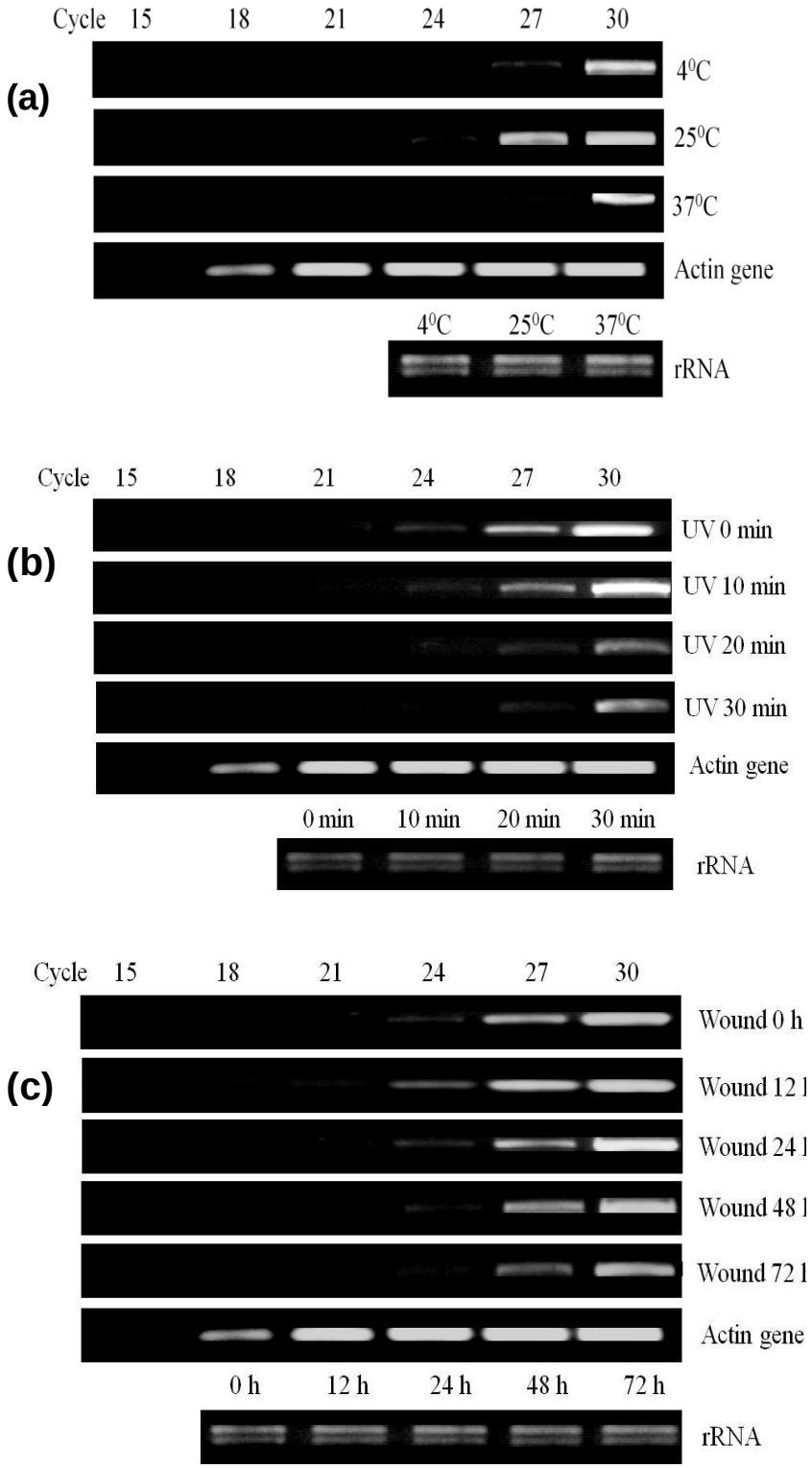

Figure 6. RT-PCR analysis of the *P. candollei* var. *mirifica CHS* gene expression under various environmental stresses. Total RNAs were isolated from plant exposed to different temperatures (a), UV-B (b) and wounding (c), and then subjected to RT-PCR analysis with primers specific for the *CHS* gene as described in materials and methods. 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).

wounding. The results obtained herein will assist in further studies on enhancing the expression of the *PcCHS* gene, which may lead to the increase in the accumulation of useful bioactive compounds in isoflavonoids biosynthetic pathway in this plant.

ACKNOWLEDGEMENTS

This work was financially supported by the Rajamangala's University of Technology Isan, Kalasin Campus and the Fermentation Research Center for Value Added Agricultural Products (FerVAAP), Khon Kaen University.

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