A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Cloning of Putative *DHCR* and *CYP85* Genes in Spinach (*Spinacia oleracea* L.)

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A thesis submitted in partial fulfillment of the requirement for the degree of master of science.

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This thesis has been examined and approved.

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Chairperson of the supervising committees

Professor Jae Hoon Kim, Ph.D., College of Applied Life Sciences, Jeju National University

_____

Professor Dong Sun Lee, Ph.D., College of Applied Life Sciences, Jeju National University

_____

Professor Key Zung Riu, Ph.D., College of Applied Life Sciences, Jeju National University

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Date

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ABBREVIATIONS

BR	Brassinosteroid
CYP	Cytochrome P450
DEPC	Diethylpyrocarbonate
DHCR	7-Dehydroxycholesterol reductase
CHR	Cholesterol
E	Ecdysone
20E	20-hydroxyecdysone
EST	Expressed sequence tag
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPLC	High performance liquid chromatography
IPTG	Isopropyl-β-thiogalactopyranoside
MVA	Mevalonate
PCR	Polymerase chain reaction
PE	Phytoecdysteroid
RACE	Rapid amplification of cDNA ends
RT-PCR	Reverse transcription polymerase chain reaction
SQ-RT-PCR	Semi-Qquantitative-RT-PCR
UV	Ultraviolet
X-Gal	5-brome-4-chlro-3-indolyl-β-D-galactopyanoside

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SUMMARY

Phytoecdysteroids (PEs) are a group of ecdysteroid produced in plant kingdom compared to zooecdysteroid that are synthesized by insect or nematode. In insects, ecdysteroids function as a hormone which control molting, metamorphosis, and other physiological processes related to development at nearly every stage. In contrast in insects, ecdysteroids show no apparent hormonal activities in plants instead they are postulated to be important as a chemical defense against non-adopted insect species. To elucidate the exact function of PEs, understanding of their metabolism is prerequisite. In plants, the content and distribution of ecdysteroids were illustrated, but the biosynthetic pathway is not clear until now. Most of the previous works in ecdysteroid biosynthesis have been investigated by metabolomic approach. In this work a genomic approach was used to identify genes which are essential for molecular biological analysis of ecdysteroid biosynthesis in plants. Two enzymes, 7-dehydrocholesterol reductase (DHCR) and CYP85 were focused on, which are supposed to catalyze formation of double bond at C7 – C8 and oxidation at C6, respectively, in the ecdysteroid ring structure. Candidate genes for these two enzymes were cloned and their expressional characteristics were discussed in spinach.

The partial gene of *So-DHCR* was cloned by the degenerate RT-PCR of first strand cDNA synthesized from mRNA using specific degenerate primers which were designed based on the conservative amino acid sequences of known DHCR's in other plants. The partial gene was confirmed in NCBI and ExPASy database and it showed high homology to other plant DHCR's with a similarity of 90% on the amino acid level. RACE technique was used for amplifying the ends of cDNA for obtaining full-length gene. The obtained full length of *So-DHCR* gene was 1,691 bp encoding 434 amino acids. Its theoretical pI was 7.89, and the molecular weight was near 45 kDa. The deduced amino acid sequence of *So-DHCR* cloned from spinach showed up to 86% similarity to those in ecdysteroid-negative plants. The sequence homology of *So-DHCR* was expected to be higher among the ecdysteroid-positive plants because they supposed to be evolved from the same ancestor. As expected the homology of *DHCR* between two ecdysteroid-positive plants, spinach and

Achyranthes japonica, was much higher with 94%. Ecdysteroid biosynthetic ability of spinach leaves was known to vary with their age, the data obtained from this study, however, showed that there was no significant difference among leaf ages in *So-DHCR* expression.

In the case of *So-CYP85*, the partial gene was cloned by the degenerate RT-PCR using specific primers designed based on the conservative amino acid sequences of *CYP85* family known in other plants. When the partial gene was analyzed against NCBI and ExPASy databases, the amino acid sequence homology to known *CYP85* family in other plants was 74%. RACE technique was used for amplifying the ends of cDNA for obtaining full-length *CYP85* gene as in *DHCR*. The full length gene of the putative *So-CYP85* was 1,696 bp encoding 464 amino acids. Its theoretical pI was 9.30, and the molecular weight was near 54 kDa. There was a heme-biding domain (FGGGTRQCPG) at the site of 407-426 bp. The protein expressed in *E. coli* exhibited a maximum absorption at 409nm in UV-visible spectroscopy. The deduced amino acid sequence of the cloned *So-CYP85* from spinach showed highest homology of 79% to that in *Vitis vinifera*, an ecdysteroid-negative plant. As expected in *So-DHCR*, the homology of *So-CYP85* between two ecdysteroid-positive plants, spinach and *Achyranthes japonica,* was much higher with 92%. The expression of the putative *So-CYP85* in spinach was higher in older leaf and lower in younger one which was consistent with the ecdysteroid biosynthetic abilities of leaves at different age as described in the previous paper. The level of *So-CYP85* expression was inversely proportional to the 20E content in the leaf and this supported the idea for the phytoecdysteroid biosynthesis to be down-regulated by the end-product 20E. The above results from this study showed several positive evidences for the cloned *DHCR* and *CYP85* genes to be involved in PE metabolism. In order to obtain direct evidences for their role in PE biosynthesis, however, further transgenic and metabolomic studies are necessary.

INTRODUCTION

Ecdysteroids are a class of steroidal compounds which are divided into two groups according to their occurrence, zooecdysteroids in animals and phytoecdysteroids (PEs) in plants. In insects, ecdysteroids function as a crucial hormone that control life cycle including metamorphosis, reproduction and diapauses by regulating many biochemical and physiological processes (Baltaev *et al*., 2000; Dinan, 2001; Makka *et al*., 2002). In plant, the distribution and content of PEs has been well studied. Until now, more than 400 structurally different PEs have been isolated (www.ecdybase.org). 20E was the most commonly detected and the most abundant PEs (Dinan *et al*., 2009). The hormonal activity of PEs is not obvious in plants. In 2007, Bakrim *et al*. reported that 20E stimulated shoot elongation at early germination stages, reduced it on the fifth day and showed a weak inhibition of root elongation on the fifth day in tomato. However, other bioassays gave negative evidence against this hypothesis (Felippe *et al*., 1980). In previous works, functional studies of PEs have been rather focused on resistance to phytophagous insects. Many papers showed that PEs have killing and repelling activities on insects (Blackford *et al*., 1997; Zolotar *et al*. 2001; Chi *et al*., 2002). The defensive activity of PE's was also reported on nematodes (Soriano *et al*., 2004).

The biosynthetic pathway was relatively well characterized in insects (Warren *et al*., 2002; 2004; Petryk *et al*., 2003; Ono *et al*., 2006) using biochemical and genetic techniques (Makka *et al*., 2002; Petryk *et al*., 2003; Gilbert, 2004). Cholesterol (CHR) is known as a primary precursor in insects and converted into 7-dehydrocholesterol (7dC). The 7dC is converted first into △4-diketol, and then into the diketol and ketodiol (Ono *et al*., 2006). The final four steps of 20E synthesis are to convert ketodiol into 3β, 5β[H]-ketotriol (2, 22dE), 2-deoxyecdysone (2dE), E and 20E sequentially. However, the early steps for conversion of 7-dehydrocholesterol to 3β, 5β [H]-ketodiol are still unknown. In contrast to insects, the biosynthetic pathway of PEs in plants is not clear. Only the putative biosynthesis of PEs in Fig. 2 was proposed in some reports. It is considered that cholesterol is also the precursor of

20E in plant, and 2-deoxyecdysone is the intermediate during synthesis of 20E. This was supported by the experiments with radioactive (^{3}H or^{14}C) molecules (cholesterol and 20dE). These two labeled compounds were found to be converted into E and 20E. There is a paper reported that labeled ecdysone was converted into 20E (Dinan *et al.,* 2009). But, it is not clear which is synthesized first between E and 20E in plants.

The 7-dehydrocholesterol reductase was known as DHCR7 or DHCR catalyzing the reaction of double bond formation at C7-C8 in the biosynthesis of CHR. The double bond at C7-C8 was known to be one of the unique structures in ecdysteroids. It was formed in the upstream of ecdysteroid biosynthesis in insect, radiolabelled CHR can't be converted into 7-dehydrocholesterol in the gland of *woc* mutants in vitro, it was reported that CHR was converted into 7-dehydrocholesterol at the early steps by C7-C8 dehydrogenation in insect (Warren *et al.*, 2001). In human, the 7-dehydrocholesterol reductase catalyzes 7-dehydrocholesterol to produce cholesterol by reduction of the $\triangle^{7}$ double bond (Hans *et al.*, 2000). In plant, the isotopes of CHR by labeling of ^{14}C or ^{3}H were converted into 20E. CHR was considered to be an important precursor of ecdysteroid biosynthesis in plant (Dinan *et al.*, 2009). The enzymes contribute to the C7-C8 double bond maybe the initial step of CHR participating in ecdysteroid biosynthesis in plant. It induced the C7-C8 double bond in the process of 7-dihydrocholesterol converting to CHR which maybe the upstream of PEs biosynthesis.

The genes related to the final four steps have been identified in Drosophila. These four genes encode ecdysteroids hydroxylase which catalyze the hydroxylation at C2, C20, C22, and C25 of zooecdysteroids. These enzymes identified belong to the cytochrome P450 (CYP) family and the related genes were cloned in the fruit fly *Drosophila melanogaster*: CYP306A1 (Phantom: Phm, C25 hydroxylase) converts 2,22,25dE into 2,22dE (Niwa *et al.*, 2004; Warren *et al.*, 2004), CYP302A1 (Disembodied: Dib, C22 hydroxylase) converts 2,22dE into 2dE (Warren *et al.*, 2002; Gilbert, 2004), CYP315A1 (Shadow: Sad, C2 hydroxylase) converts 2dE into E (Warren *et al.*, 2002; Gilbert, 2004), CYP314A1 (Shade: Shd, C20 hydroxylase) converts E into 20E (Petryk *et al.*, 2003). In plant, there is no genomic

study in PE biosynthesis until now. However, some CYPs genes have been studied in relation to the biosynthesis of steroid. *CYP90A1*, *CYP90B1* and *CYP85* were suggested to be involved in the brassinosteroids (BRs) biosynthesis in Arabidopsis and tomato (Shimada *et al.,* 2001; Bishop *et al.*, 1999; Choe *et al.,* 1998; Szekeres *et al.,* 1996). It was reported that in BR synthesis pathway *CPY90B1* catalyzed C-22 oxidation (Fujita *et al.,* 2006), *CYP85A* catalyzed the C-6 oxidation (Shimada *et al.,* 2001). *CYP85A1* also function as *ent*-kaurene oxindase in plant (Helliwell *et al.,* 1998). The information of CYP's obtained from the studies on brassinolides seemed to be also useful in the research on PE biosynthesis because PEs is very closely related with BRs.

The CYPs are a superfamily of heme-containing proteins which display a spectral absorption peak at approximately 450nm in the reduced CO-bound state (Crespi *et al.*, 1991). This unique spectrum was also used for measurement of P450 content (Ortiz *et al.*, 1997). However, an optical absorption peak at 420nm can be detected because of changing from functional structure to non-functional (Omura *et al.*, 1999). The maximal absorption at 450nm is used for the measurement of cytochrome P450 activity (Ortiz *et al.*, 1997). CYPs were found widely in bacteria, fungi, plants, insects and animals. CYPs with at least 40% similarity are identified into a same family, those with over 55% identity belong to a subfamily (Coon *et al.*, 1992). The length of CYPs amino acids sequence is about 500 AA with molecular weight of about 45-55 kDa. There is a heme binding domain (FxxGxxxCxG) which allows identification of CYP enzymes. A typical function of P450 is the monooxygenation activity which responsible for the metabolism of exogenous compounds and biosynthesis of endogenous chemicals. For example: metabolism of drugs, plant toxins, xenobiotics, biosynthesis of cholesterol, steroid hormone in plants (Takumi *et al.,* 2008). In insects, CYPs are involved in the biosynthesis of ecdysteroids (Warren *et al.*, 2002; 2004). Hydroxylation is the most frequently catalyzed reaction for CYPs.

In this study we designed experiments based on the information obtained from previous researches to isolate the candidate genes from spinach (*S. oleracea*.) which expected to be involved in PEs biosynthesis. In subsequent experiments their expressions were examined in the plants to see the possibility of their involvement in PEs biosynthesis.

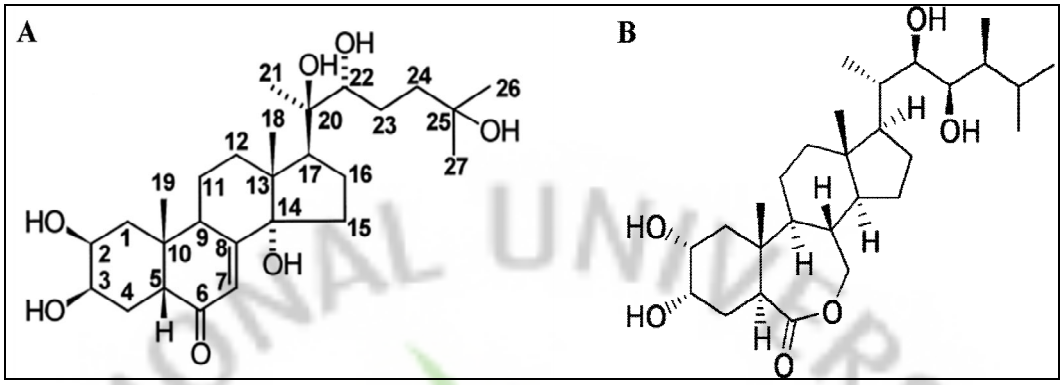

Fig. 1. Structures of 20-hydroxyecdysone (A) and brassinolide (B).

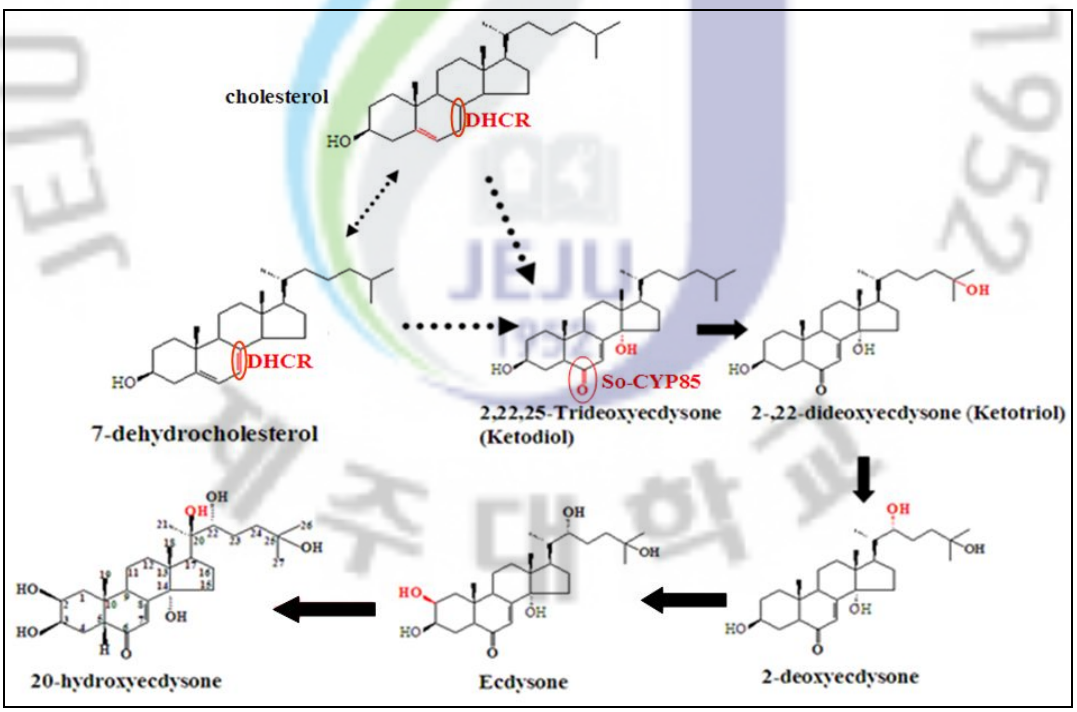

Fig. 2. Putative pathway of PE biosynthesis in plant. The steps in broken line were unknown both in insect and plant. Steps of black arrow were proved in insect. Steps of 2dE→E/20E and E→20E were proved by isotopes labeled metabolites in plant. Function sites of DHCR and CYP85 were marked in circle.

MATERIALS AND METHODS

Plant Materials

Spinach (*Spinacia oleracea* L.) used in this study was the cultivar 'GyeoWooNae' purchased from Nongwoobio company in Korean. The seeds were sowed into pots and grown in the culture room at 25℃, 18h light / 6h dark. And then the 7-day seedlings were transferred and grown under common culture conditions in the greenhouse. The whole plant was used for cDNA cloning. The single leaf pair was used for SQ-RT-PCR.

Bacterial Strains and Plasmids

Escherichia coli XL1-BLUE containing F' was used as a host strain for sub-cloning based on blue/white screening on medium containing X-gal and IPTG. The yT&A (Bio-eastern) vector was used for cloning of PCR product which is for T/A-type cloning.

Isolation of RNA from Plant

Total RNA was extracted using the Easy – BLUE kit (Intron Biotech) from the whole plant. The sample was homogenized with mortar and pestle in liquid N_2. The powder was dissolved in 1 ml of Easy – BLUE reagent per 100 mg for RNA extraction. DEPC treated water was used to dissolve RNA pellet at the last step. After that, RNase-free DNase was used to remove DNA contained in total RNA. Then half of total RNA was kept at -70℃ for further experiments. The concentration of total RNA was measured using spectrophotometer (Perkin-Elmer, Germany) at 260 nm. Three μg of total RNA was mixed with 3.75 ul of 1 × MOPS buffer and 1.25 μl of formaldehyde. The mixture was heated at 70℃ for 5 min and then immediately replaced into ice for denaturing of secondary structure. All of the mixture was loaded for electrophoresis on 1.2% agarose gel containing 0.028 g of inoacetamide and 30 μl of 1 × MOPS buffer.

mRNA was isolated from total RNA using Qiagen Oligotex Kit according to the manufacturer's instruction using 25 μl of 19.5 μg/μl total RNA. The concentration of mRNA

was measured using spectrophotometer at 260 nm.

Degenerate RT-PCR for Partial Gene Cloning

The above isolated mRNA was used to synthesis the first strand cDNA. Reverse transcription was performed using ImProm-Ⅱ™ Reverse Transcriptase (Promega) and oligo (dT) primer according to the manufacturer's instruction. RT-PCR amplification was carried out using Ex -Taq™ DNA polymerase (Takara, Japan) using the first strand cDNA as templates. GeneAMP® PCR system 9700 cycler was used for PCR reaction. Degenerate primers were designed based on the conservative amino acid of steroid hydroxylase and CYP450 family of other plants. The primers were designed using Block maker and CODEHOP program for *CYP85* and *DHCR* genes comparing the conserved amino acid sequences of previously reported genes (http://bioinformatics.weizmann.ac.il/blocks/codeho-p.html). The obtained genes were named as *So-DHCR* and *So-CYP85*. Primers used in this reaction were synthesized in Bioneer Company (Seoul, Korea).

The above isolated mRNA was used to synthesis the first strand cDNA. mRNA was reverse transcribed using ImProm-Ⅱ TM Reverse Transcriptase (Promega) and oligo (dT) primer according to the manufacturer's instruction. PCR amplification was carried out using Ex-Taq™ DNA polymerase (Takara, Japan) with the template of first strand cDNA. Thermal cycling in a total of 50 µl of PCR reaction, 5 units of Ex-Taq polymerase (Takara Korea, Korea), 5 µl of 10X Ex-Taq buffer, 5 µl of 2.5 mM dNTP, 2 µl of template, 20 pmol of each degenerate forward and reverse primers were used. The primers sequences used for degenerate RT-PCR were listed in Table 1. After initial denaturation at 95 ℃ for 5 min, thermal cycling was carried out following program below: 94 ℃ for 45 sec, annealing temperature were 60 ℃ for 45 sec and 65 ℃ for 60 sec for the gene of *So DHCR* and *So-CYP85* respectively, 72 ℃ for 1 min. Followed by a final extension at 72 ℃ for 10 min. The PCR product was analyzed in 1.2% agarose gel (TAE buffer) and staining with ethidium bromide. The target band of each PCR product was eluted and ligated into the yT & A vector and transformed into *XL1-BLUE* competent cell according to the manufacturer's instruction.

Table 1. Degenerate primer sequences for gene cloning in spinach.

Target gene	Primer sequence
So-CYP85	F: 5`- GGAACTATGGGATGGCCTATTTTYGGNGARAC - 3`
	R: 5`- TCTTCCAGAGCCTTAGGATGATCRTGNARRTA - 3`
So-DHCR	F: 5`-GAATTTTGATATTAAGGTTTTTACTAATTGTAGATTYGGNATGATG- 3`
	R 5`- :TTTTTCACAATACAGCTTCCAATACTTNCCRTAYTT -3`

Y, C/T, N, A/G/C/T, R, A/C

Table 2. Primer sequences for cloning full length cDNA of *So-CYP85* by RACE

Target gene	Primer sequence
So-CYP85 - 3'RACE	1st: 5'-GGTTAGTACCAGGGTACCCACAGTC-3'
	2nd: 5'-GGGGTGTCCAACAATAGTGTC-3'
So-CYP85 - 5'RACE	1st: 5'-AGAAGGGCACCCCTCATGTACT-3'
	2nd: 5'-GTTGGGCAGTTGAGCTTGATT-3'
cDNA - 3' linker	1st: 5'-GCGAGCACAGAATTAATACGACT-3'
	2nd: 5'-CGCGGATCCGAATTAATACGACTCACTATAGG-3'
cDNA - 5' linker	1st: 5'-CGACTGGAGCACGAGGACACTGA-3'
	2nd: 5'-GGACACTGACATGGACTGAAGGAGTA-3'

The positive recombinant vector (confirmed by electrophoresis) was digested by *Hind* Ⅲ. Empty yT & A vector was used as a control. Four ul of recombinant vector was digested by 1 μl of *Hind*Ⅲ. Then, it was incubated at 37℃ for overnight, and analyzed by 1.2% agarose gel electrophoresis.

The positive recombinant vector (confirmed by *Hind*Ⅲ) was confirmed by PCR. The reaction system and program were same with degenerated PCR for target gene. The PCR product was analyzed in 1.2% agarose gel. The positive recombinant vector was send for sequencing in the central laboratory, Jeju National University.

Each partial nucleotide and amino acid sequences was analyzed using Blast in NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). ExPASY (http://us.expasy.org/tools) was used for analysis of reading frame and stop code. The hypothesized function of partial gene was assumed comparing the gene in Genebank. Primers for RACE were designed based on the sequence of the partial genes.

Cloning of Full Length cDNA

Full-length cDNAs were generated by RACE technique using GeneRacer™ Kit (Invitrogen™) for 5' end, FirstChoice RLM-RACE Kit (Ambion) for 3' end according to the supplier's instruction. Degenerated forward and reverse primers of *So-DHCR* were used for the first time PCR of 5' and 3' end RACEs. New specific primers for *So-CYP85* were designed for the first time PCR based on the partial sequences produced by degenerated PCR. Nested primers for the second time PCR were designed from the partial sequences produced by degenerated PCR. The PCR reaction was performed using GeneAMP® PCR system 9700 cycler.

Thermal cycling in a total of 50 μl of PCR reaction, 5 units of Ex-Taq polymerase (Takara Korea, Korea), 5 μl of 10X Ex-Taq buffer, 5 μl of 2.5 mM dNTP, 2 μl of template, 20 pmol of each degenerate forward and reverse primers were used. The primers sequences used for RACE PCR were listed in Table 2. After initial denaturation at 95 ℃ for 5 min, thermal cycling was carried out following program: 94 ℃ for 45 sec, annealing temperature

were 60 ℃ for 40 sec and 65 ℃ for 40 sec for the gene of *So-DHCR* and *So-CYP85* respectively, 72 ℃ for 1 min. Followed by a final extension at 72 ℃ for 10 min. The PCR product was analyzed in 1.2% agarose gel and staining with ethidium bromide. The target band of each PCR product was eluted and ligated into yT & A vector which will be transformed into *XL1-BLUE* competent cell. The plasmid was extracted and checked by electrophoresis, and then confirmed by *Hind*III restriction endonuclease digestion and PCR. The correct insertion of the plasmids was sequenced from the central laboratory in Jeju National University.

Each end fragment sequence of nucleotide and amino acid were analyzed using NCBI (http://www.ncbi. nlm.nih.gov/BLAST/), EnCyclon (http://www.encyclon.net/), ExPASY (http://us.expasy.org/tools), and CAP3 sequence assembly program (http://pbil.univ-lyon.fr/ cap3. php).

Protein Expression of *So-CYP85* in *E. coli*

The ORF of *So-CYP85* was amplified using PCR with forward primer (5`- GGT AAT CAT ATG GCT AAG AAA ACG AGC TCT TCT GCT TTG TTA AGA TGG – 3`) and reverse primer (5` - GGT AAT GTC GAC TCA GTG GTG GTG GTG GTG ATA ACT CGA AAC TCG AAT -3`).

The PCR fragment was purified and digested with *Nde* I and *Sal* I restriction enzyme. Digested *So-CYP85* was cloned into the pCW vector containing *Nde* I and *Sal* I restriction enzyme sites. The ligation mixture was transformed into *E. coli* strains DH5α competent cells (Invitrogen, USA), and then, the plasmid DNA was purified using a miniprep kit (Promega, Madison, WI). The recovered plasmid DNA was verified by sequencing for the construction of *So-CYP85* system.

The constructed *So*-CYP85 DNA plasmid was transformed into *E. coli* BL21 (Rosetta3) and then plated on LB medium/ampicillin (100 μg/ml). A single colony was picked and transferred into single 5 ml of LB liquid medium/ampicillin. After overnight incubation at 37°C, the starter culture was diluted into 1:l of LB expression medium/ampicillin (100 μg/ml)

containing 1 mM IPTG, 0.5 mM δ-ALA, and 1 mM thiamine. The expression culture was grown at 37°C for 3 h and then at 28 °C with shaking at 200 rpm for 2 days. After the being harvested, the cells was centrifuged at 15，000 g (30 min, 4 °C) and examined via sodium dodecyl sulfate polyacrylamide gel electrophoresis. Spectra (300–500 nm) were then recorded using a SHIMADZU UV-1800 spectrophotometer.

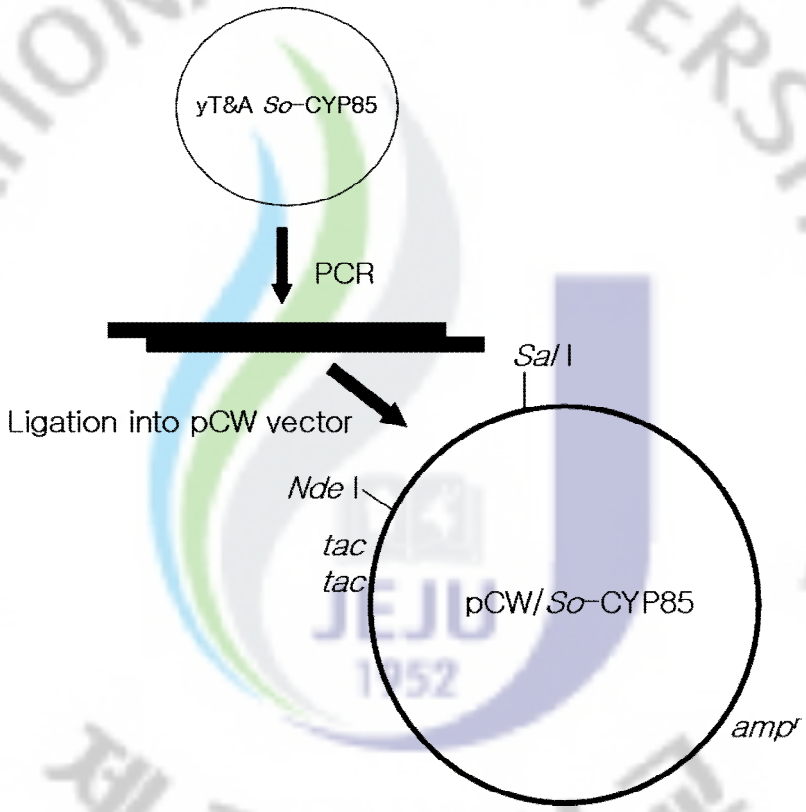

Fig. 3. Construction scheme for *So-CYP85* expression vector.

Analysis of Gene Expression in Spinach

Gene expression in different leaves of spinach was examined using RT-PCR. Total RNA was prepared at the '3rd leaf pair' stage when the '4th leaf pair' emerge. The 1st, 2nd and 3rd leave pairs including petioles were separated individually. Total RNA was extracted from about 100 mg of leaf powder after grinding in liquid nitrogen with 1 ml of Easy-Blue regent. At the last step, 60 µl of DEPC-treated water was used for RNA elution. Then, the concentration of total RNA was adjusted to 100 ng/µl. First-strand cDNA was synthesized by RT-PCR according to the protocol of Maxime RT Premix Kit using 1µg of total RNA (removed DNA). The cDNA material was stored at -20℃. The *Actin* or *GAPDH* house keeping gene was used as control.

For repetition, 12 plants of *S. oleracea* were prepared and divided into 3 groups as 3 repetition containing 4 plants in each single group. The leaf pair at the same position (1st, 2nd and 3rd) of the four plants in each group was ground together. The powder of 100 mg was used for total-RNA extraction and SQ-RT-PCR.

Analysis of Phytoecdysteroid by HPLC

The leaves of plant were separated as in Fig. 4 (C). The leaf samples were dried 2 days at 55℃ in dry chamber. Dried samples were ground into powder in 1.5 ml tubes with a round metal stick. Methanol and hexane were used to extract and purify PEs respectively. Dry crude PEs of aqueous methanol phase was dissolved in 2 ml of 10% 2-propanol (Fig. 5).

The concentration of 20E in different leaves of *S. oleracea* was analyzed by HPLC (Table 4). The standard 20E compound was purchased from ZERUN PHARMACEUTICAL CO., LTD, Shanghai, China.

Fig. 4. The plant and the individual leaves of *S. oleracea* used for total RNA extraction and 20E analysis. A: The plant of spinach at '3rd leaf pair' stage, B: The plant of spinach at '4th leaf pair' stage, C: The individual leaf of spinach used in experiment at '3rd leaf pair' stage. The leaves were numbered from the basal set near root to the youngest apical ones: COT: cotyledon, L1: the 1st leaf pair of true leaf, L2: the 2nd leaf pair of true leaf, L3: the 3rd leaf pair of true leaf, L4: the 4th leaf pair of true leaf.

Table 3. Operation condition of HPLC for 20-hydroxyecdysone analysis

Parameter	Condition
Instruments	Vacuum degasser, P4000 pump, As1000 auto sampler with column oven UV6000LP Photo Diode Array detector
Column	ODS column (4.6×250mm column ID, 5 u)
Mobile phase	10% 2-propanol (0.1% TFA)
Flow rate	1.2 ml/min
Detection wavelength	242 nm
Scan wavelength	200 - 360 nm
Column temperature	40℃
Sample injection	20 µl
Run time	45 min

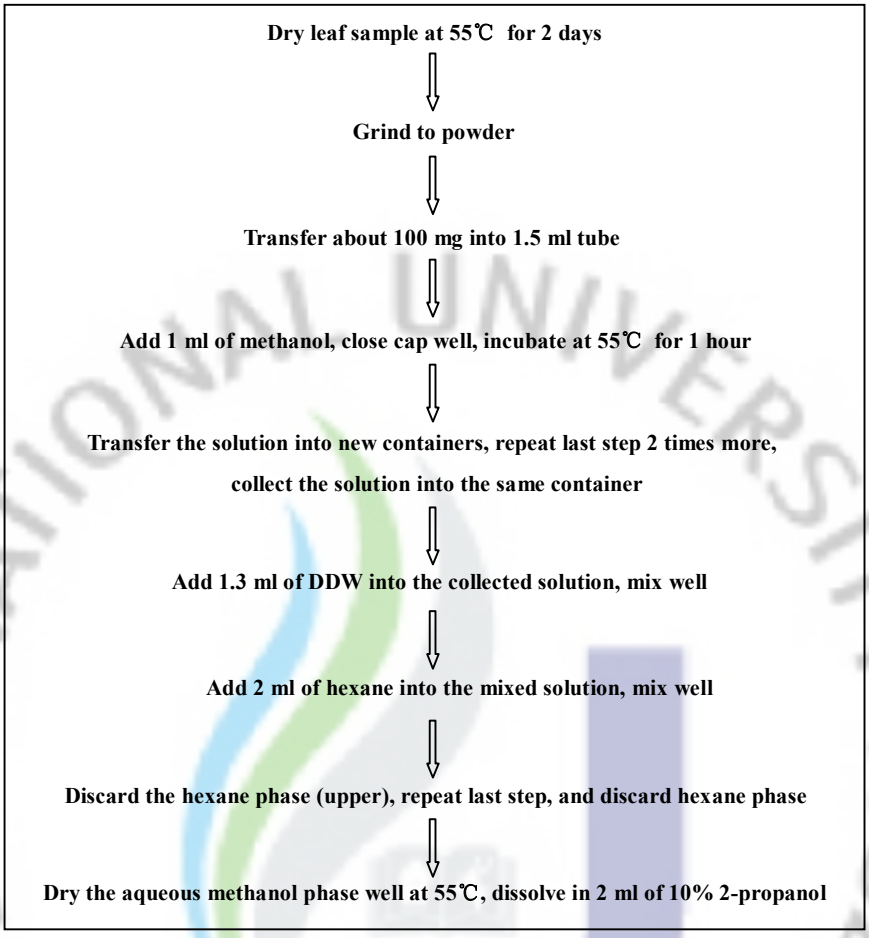

Fig. 5: Experimental procedures for extraction and analysis scheme of PEs from root and leaves of spinach.

RESULTS

Isolation of RNA

Total RNA was extracted from about 100 mg of powder ground from whole plant. One ml of Easy-BLUE reagent was used for total RNA extraction. RNA pellet was dissolved in 60 µl of DEPC treated water at the last step. The concentration of total RNA was 19.58 ng / µl. The value of OD260/280 was 1.69 showing that the purity of RNA was good enough for further experiments (Fig.6). The mRNA was isolated from 500 ng of total RNA.

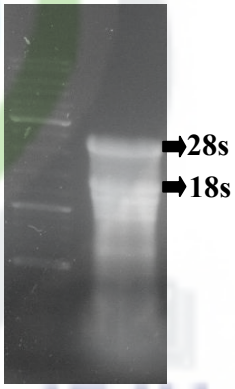

Fig. 6. Agarose gel electrophoresis showing the total RNA isolated from *S. oleracea.*

Table 4. Putative genes isolated from first strand cDNA by degenerate RT-PCR

clone	Nucleotide (bp)	% Amino acid identity with other organism / putative function
So-CYP85	788	74% with *Nicotiana tabacum* (ABG36709.1) / C6-oxidase
So-DHCR	692	90% with *Gossypium hirsuteum* (ABA01480.1) / sterol delta-7 reductase

Cloning and Expression of putative *DHCR*

Cloning of candidate gene For cloning of *DHCR* candidates, degenerate RT-PCR was performed and the nucleotide sequence of the PCR products were determined. More than five sequences of the partial gene were analyzed using NCBI and ExPaSy databases. Some were not the expected sequences. Then, one target sequence was selected for amplifying the 3' and 5' end of cDNA by RACE.

In degenerate RT-PCR, there was no band at the first time PCR using cDNA as templates. Then the second time PCR was carried out using the first time PCR product as template. The band was eluted and purified for TA cloning. Sequencing result proved that the band was the target band. One partial sequence of *So-DHCR* with a 692 bp nucleotide acid in length was cloned. This fragment showed a 90% identity of that in *Gossypium hirsuteum* at amino acid level. Two pairs of specific primers were designed based on the partial sequence for 5' and 3' end RACE of cDNA. A length of 640 bp nucleotide acid of 5' end, and a length of 1,100 bp nucleotide acid of 3' end fragment were hypothesized.

The full length of *So-DHCR* was obtained by overlapping of 5' and 3' end fragments. The cloning of *So-DHCR* was confirmed by *Hind*III digestion. One band of approximately 2,800 bp which was the main part of yT & A vector was observed on the agarose gel for 5' and 3' end RACE. Two bands with 500 bp of 5' and one band with 1,000 bp of 3' end RACE were observed on the agarose gel. The result indicated that the target DNA had been successfully inserted into the vector. Then, the positive recombinant plasmid was confirmed by PCR. The plasmid of positive clone was extracted for bidirectional sequencing.

Nucleotide sequence analysis revealed that full length of *So-DHCR* was 1,696 bp nucleotide acids. The coding region (ORF) of this cDNA was 1,302 bp nucleotides which encoded a deduced protein of 434 amino acids. The nucleotides from 1 to 41 and 1,344 to 1,691 are 5' UTR and 3' UTR, respectively. The theoretical pI was 7.89, and the molecular weight was near 45 kDa that was similar with the Arabidopsis DHCR with a molecular weight of 49 kDa.

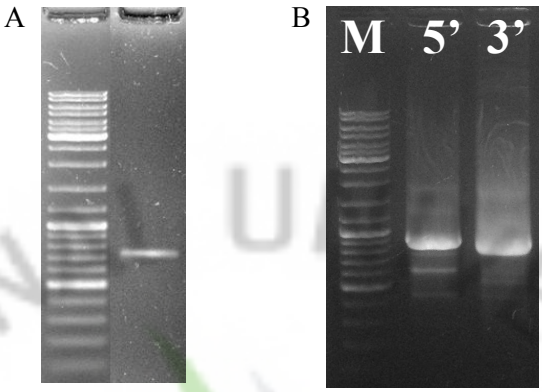

Fig. 7. Electrophoresis of *So-DHCR* gene. A: Products of degenerate RT-PCR of *So-DHCR*, B: 5' and 3' end RACE products of *So-DHCR* using nested primers.

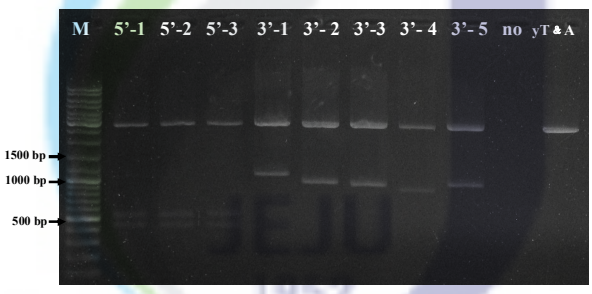

Fig. 8. Agarose gel showing: 5’ and 3’ end fragment of *So-DHCR* digested using *Hind* Ⅲ restriction endonuclease. 5’-1, 5’-2, 5’-3 are 5’ end fragment, from 3’-1 to 3’-5 are 3’ end fragment of *So-DHCR*. ‘Line no’ means no loading of sample. yT & A line means just load yT & A vector as a control.

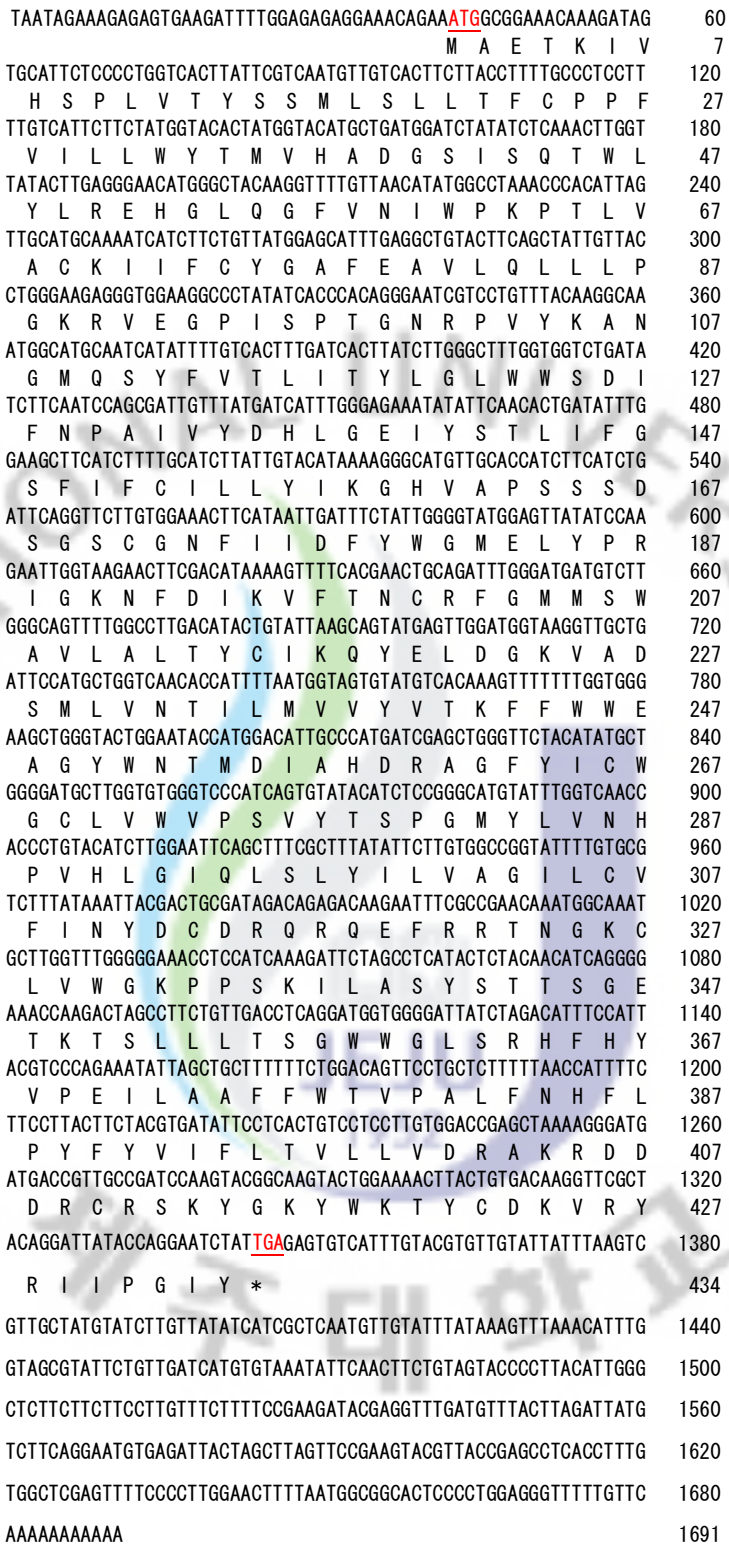

Fig. 9. Nucleotide and deduced amino acid sequence of *So-DHCR* from *S. oleracea*. The nucleotide and deduced amino acid are numbered in the right margins. The termination cordon is marked by an asterisk. The start and end codes were underlined in red.

Homology of the cloned gene to other known genes The determined 1691 bp nucleotide sequence corresponding to deduced amino acid of 434 amino acids was subjected to search against known sequences using the blastp search in NCBI database. The sequence of *So-DHCR* in spinach showed high homologous with that of known *DHCR* gene in various plant species. The deduced amino acid sequence of *So-DHCR* was compared with that in other plants using ClustalW and GeneDoc. The result showed high identities of sequence with: *Arabidopsis thaliana* (86%), *Tropaeolum majus* (85%), *Gossypium hirsutum* (85%), *Ricinus communis* (86%) and *Achyranthes japonica* (94%).

Fig. 10. Alignment of deduced amino acid sequences of *DHCR* isolated from *S. oleracea* with that from other plant in the NCBI database. The deduced amino acid of *DHCR* from *Arabidopsis thaliana* (NP_175460.1), *Ricinus communis* (EEF48723.1), *Tropaeolum majus* (AAR29980.1), *Gossypium hirsutum* (ABA01480.1) and *Achyranthes japonica* are shown. Numbers indicate the position of amino acid residues. Conserved residues are high lighted (in black when present in all sequences). The introduced gaps (dash) were produced by alignment program ClustalW and GeneDoc program automatically to maximize similarity among the sequences. Numbers of nucleotides are indicated on the right margin.

Expression of the endogenous gene within spinach Total RNA was used for RT-PCR under the same PCR reaction system and program used for gene cloning. The annealing temperature was different for the gene *So-DHCR*, *GAPDH* and *Actin* which own a temperature of 50℃, 56℃, and 50℃, individually.

The gene expression level of *So-DHCR* in different leaf pairs of spinach showed no apparent difference. The expression level was little higher in L2 at the '4th leaf pair' stage. This may due to the concentration of the total RNA.

The concentration of 20E was analyzed by HPLC (see portion of *So-CYP85*). The highest concentration of 20E was in the young leaf L3 or L4 at '3rd or 4th leaf pair' stage. The lowest concentration of 20E was in the elder leaf L1 or L2 at '3rd or 4th leaf pair' stage. It was nearly 10 times of the 20E concentration in the highest site as that in the lowest site. The transcription level of *So-DHCR* didn't accord with the change of 20E in plant of spinach.

Primer sequences for semi-quantitative RT-PCR (530 bp product):

F1: ATGGCGGAAACAAAGATAGTGCA

R1: TCGCTGGATTGAAGATATCAGAC

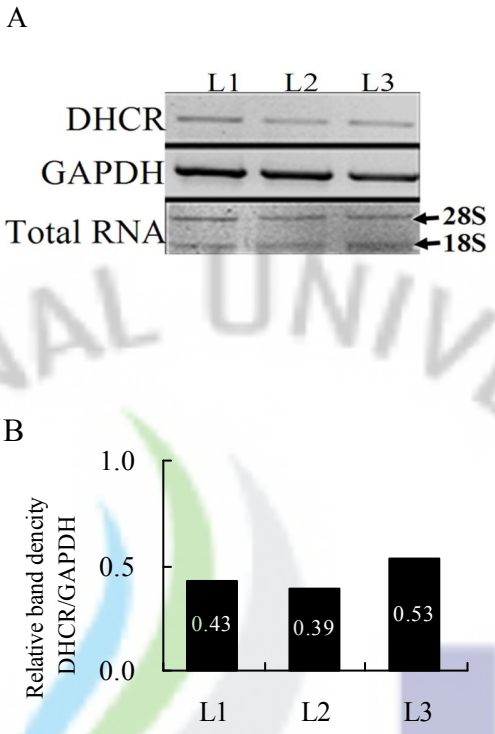

Fig. 11. Agarose gel electrophoresis of SQ-RT-PCR and total RNA for *So-DHCR* at '3rd leaf pair' stage. L1, L2, L3: 1st, 2nd, 3rd leaf pair of plant. A: SQ-RT-PCR, B: Relative band density of *So-DHCR*.

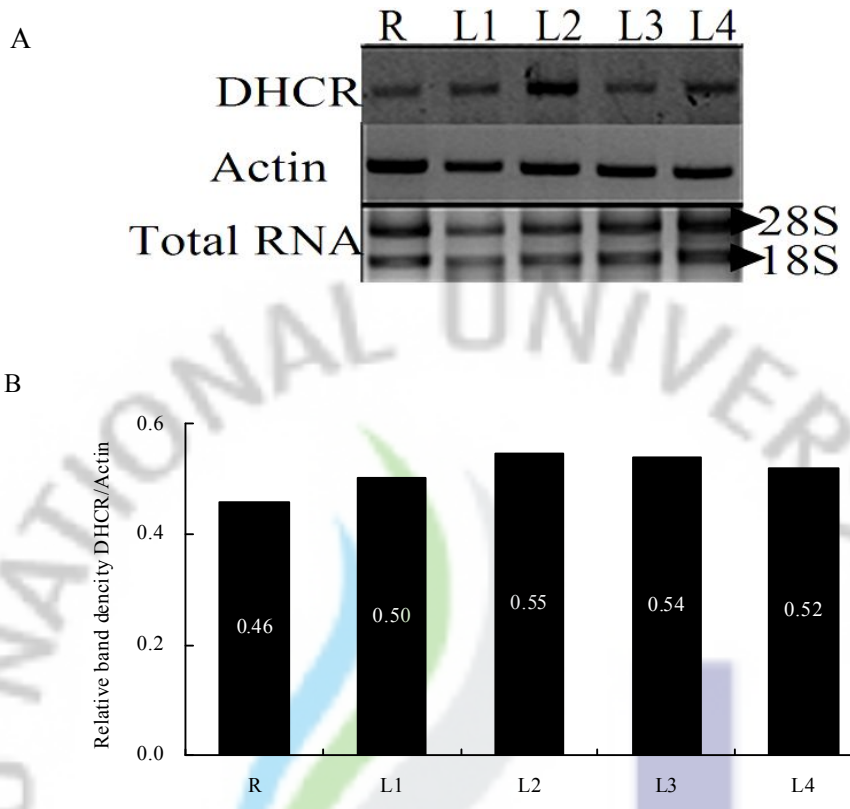

Fig. 12. Agarose gel electrophoresis of SQ-RT-PCR and total RNA for *So-DHCR* at '4th leaf pair' stage. R: root, L1, L2, L3: 1st, 2nd, 3rd leaf pair of plant, A: SQ-RT-PCR, B: Relative band density of *So-DHCR*.

Cloning and Expression of putative *C*YP85

Cloning of candidate gene First strand cDNA was synthesized from 288 ng of mRNA by RT-PCR. Two clear bands were got for *So-CYP85* during electrophoresis. These two bands were eluted and purified together for TA cloning. The sequencing result proved that the short sequence band was not the target band for *So-CYP85*. However, it was difficult to divide the two bands individually when cut the bands. So, it was also a method for TA cloning by eluting the two bands together.

*So-C*YP85 with a 788 bp nucleoside acids was screened from the partial sequences of degenerate RT-PCR. The sequence of this partial gene was similar with a brassinosteroid C-6 oxidase gene's (*CYP85A*). Homologue of the partial sequences of cloned *So-CYP85* was compared with that in other plants. This partial sequence showed a 74% identity of amino acid sequence of *Nicotiana tabacum*. A length of 574 bp for 5' and 818 bp for 3' end fragment were hypothesized.

Primer sequences for semi-quantitative RT-PCR (437 bp product) :

F:5`- TGCTGTGATTTTCAGCTTGTTTTG -3`

R: 5`- ATTTTGCCAATTGGAGAGATGGG -3`

The cDNA for RACE was synthesized from 288 ng of mRNA. The linkers of 5' and 3' end were used as primers in a nested PCR together with the gene specific primers. A contig of full length cDNA was obtained by linking the 5' and 3' end fragments. Nucleotide sequence analysis revealed that the full length of *So-CYP85* was 1,696 bp in nucleotide sequence. The coding region (ORF) of this cDNA was 1,392 bp nucleotides which encoded a deduced protein of 464 amino acids. There was a heme-biding domain (FGGGTRQCPG) at site of 407-426 bp. The isoionic point was 9.30. The molecular weight of amino acid encoded by this gene was 54 kDa. This was very similar with the CYP85A in Arabidopsis (54 kDa) and *Solanum lycopersicum* (54 kDa).

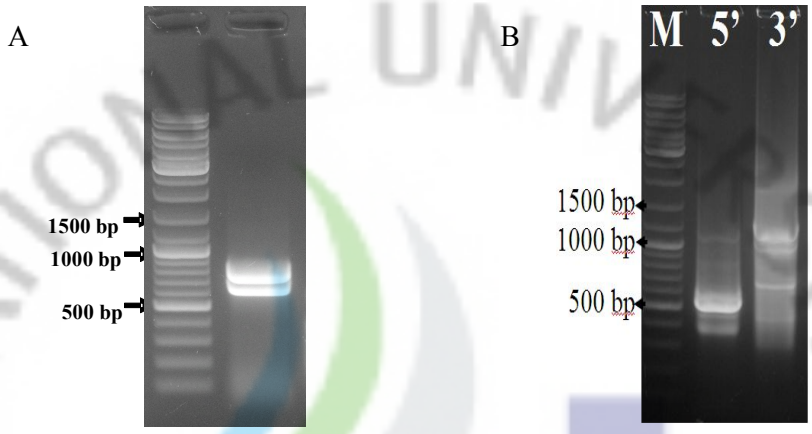

Fig. 13. Electrophoresis for *So-CYP85*. A: Products of degenerate PCR of *CYP85*, B: 5' and 3' RACE products of gene *CYP85* using nested primer.

Fig. 14. Nucleotide and deduced amino acid sequence of *So-CYP85* in *S. oleracea*. The nucleotide and deduced amino acid were numbered in the right margins. The terminator cordon is marked by an asterisk. The start and end code were marked in red. The heme-binding signature sequence was underlined.

Homology of the cloned gene to other known genes The determined 1,696 bp nucleotide sequence encoding a deduced 464 amino acids was subjected to a search against known sequences using the blastp search in NCBI database. The result showed high homologous with known *CYP85A* sequence in various plant species. The deduced amino acid sequence of *So-CYP85* was compared to that in other plants using ClustalW and GeneDoc. The result showed high identities with those of *Achyranthes japonica* (92%), *Nicotiana tabacum* (78%), *Solanum lycopersicum* (76%), *Vitis vinifera* (79%), *Phaseolus vulgaris* (77%), and *Arabidopsis thaliana* (67%).

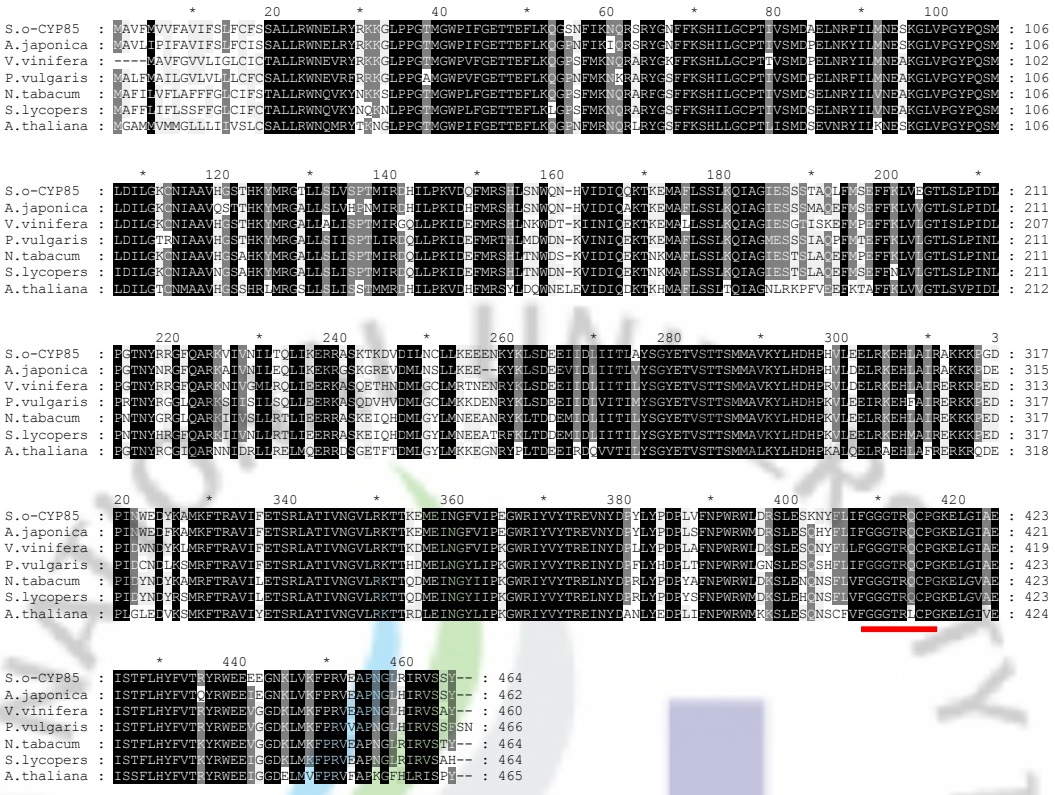

Fig. 15. Alignment of deduced amino acid sequences of *So-CYP85* isolated from *S. oleracea* with those of other plant *CYP85* genes in the NCBI database. The deduced amino acid of *CYP85* from *Achyranthes japonica*, *Nicotiana tabacum* (ABG36709.1), *Solanum lycopersicum* (Q43147.1), *Vitis vinifera* (ABB60086.1), *Phaseolus vulgaris* (Q69F95.2), and *Arabidopsis thaliana* (Q9FMA5.1) were showed. Numbers indicate the position of amino acid residues. Conserved residues were high lighted (in black when present in all sequences). The alignment was created with GeneDoc program. Gaps in the sequences indicated by dashes were produced by alignment program ClustalW and GeneDoc program automatically. Numbers of nucleotides were indicated on the right margin. Heme-binding site was underlined in red.

UV absorption spectrum of the gene product The expressed products were analyzed and a target band was observed in supernatant at about 54 kDa. This indicated that the recombinant protein *So-CYP85* expressed in *E. coli* was soluble. The protein was exhibited a maximum at 409 nm by UV-visible spectroscopy.

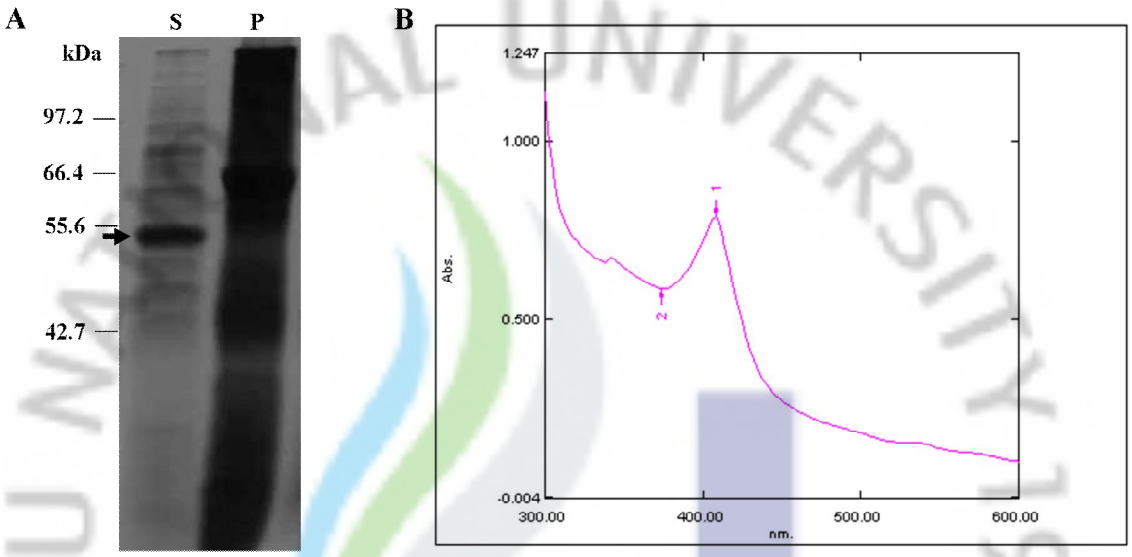

Fig. 16. Expressed protein of *So*-CYP85 in *E. coli*.
A: SDS-PAGE analysis of *So*-CYP85 in *E. coli* expressing recombinant using pCW vector**.** A molecular weight is shown on the left. Arrow indicated *So*-CYP85. S: supernatant, P: pellet. B: Spectrum analysis of *So*-CYP85.

Expression of the endogenous gene within spinach For gene expression analysis, same weight of 1μg of total RNA was used for RT-PCR under the same PCR reaction system and program in gene cloning. The annealing temperature for the gene *So-CYP85*, *GAPDH* and *Actin* was 56℃, 56℃, and 50℃. RP-HPLC was used to analysis the content of 20E in different parts of spinach.

The gene expression level of *So-CYP85* in the three leaf pairs of spinach at '3rd leaf pair' stage decreased following the age of leaves. The highest expression level of *So-CYP85* was in the elder leaf L1. And the lowest expression level was in the young leaf L3.

Table 5. Compositions of cDNA synthesis and SQ-RT-PCR reaction for *So-CYP85*

Template for cDNA synthesis	1μg of total RNA
Template for PCR	2 ul of the cDNA
Each Primer	20 pmol
10× Ex-Taq Buffer	5 μl
Mix dNTP	5 μl
Ex-Taq	0.3 μl (1.5 unit)
DDW	33.7 μl
Total volume	50 μl
Denature temperature / time	94℃ / 5 min
Cycles (35)	94℃ / 40 sec, 56 ℃/ 40 sec, 72℃/ 40 sec
Last extension	72℃/ 7 min

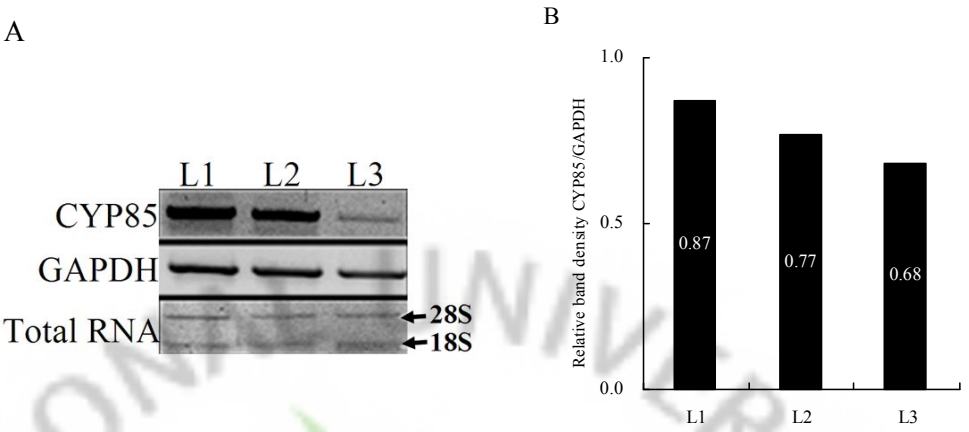

Fig. 17. Agarose gel electrophoresis of semi-quantitative RT-PCR and total RNA of *So-CYP85* at '3rd leaf pair' stage. L1 L2 L3: 1st, 2nd, 3rd leaf pair of plant. A: SQ-RT-PCR, B: Relative band density of *So-CYP85*.

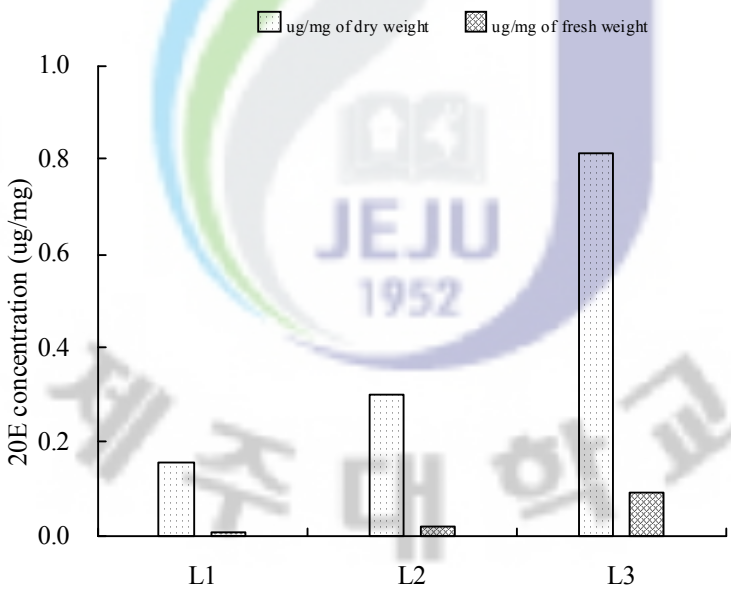

Fig. 18. Distribution of 20E within the leaves of spinach. L1-L3: the leaf pairs of spinach from bottom to top. The decrease of gene expression level from old leaves to young leaves and the highest content of 20E in the young leaves illuminate that 20E maybe biosynthesized in the old organs in spinach, and transfer to young leaves.

The leaves grown on different site were used for 20E analysis. Concentration of 20E in different aerial organs of plant at '3rd leaf pair' stage was analyzed using HPLC. Total, 12 plants divided into 3 groups containing 4 individual single plants were used for HPLC analysis. Concentration of 20E was analyzed in dry or fresh samples (showed in Fig.18). The concentration of 20E was highest in young leaf L3, and lower in the elder leaf L1, L2. The concentration of 20E in youngest shoot was 8 times as higher as that in oldest leaves at '3rd leaf pair' growth stage. The increase of 20E from 'leaf 2' to 'leaf 3' was quicker than that from 'L1' to 'L2'. And the concentration increased obviously in dry samples compared to fresh ones.

The different expression level of gene *So-CYP85* in root and each leaf pair of aerial parts was also studied in spinach at '4th leaf pair' stage. Expression level of gene *So-CYP85* was highest in the leaf L2 during root and 4 leaf pairs, and then decreased. In the elder leaf L1, the level of *So-CYP85* expression was little lower than that in young leaf, this may because that the expression of gene decrease during the growth of the same leaf. The level of *So-CYP85* expression was low in root compare to that in leaves. Overall, there was no enormous distinction during the gene expression in the different parts of spinach at '4th leaf pair' stage.

The concentration of 20E was differential in the root and each aerial part. The highest concentration of 20E emerged in the youngest leaf L4. On the contrast, the lowest concentration of 20E was in the leaf L2. It was nearly 10 times higher of 20E concentration in L4 than that in L2. This result was similar with that got by Bakrim (2008). In their study, they found that 20E transfer from old leaves to young leaves. The level of *So-CYP85* expression and the distribution of 20E in different part of spinach maybe give an evidence that 20E was synthesized in old leaves and transfer into new leaves. However, these evidences are not hard to support it. More studies will be needed. This identified with the hypothesis function of 20E — an antifeedant to some herbivore insect. It is propitious for young and important apical bud to survive.

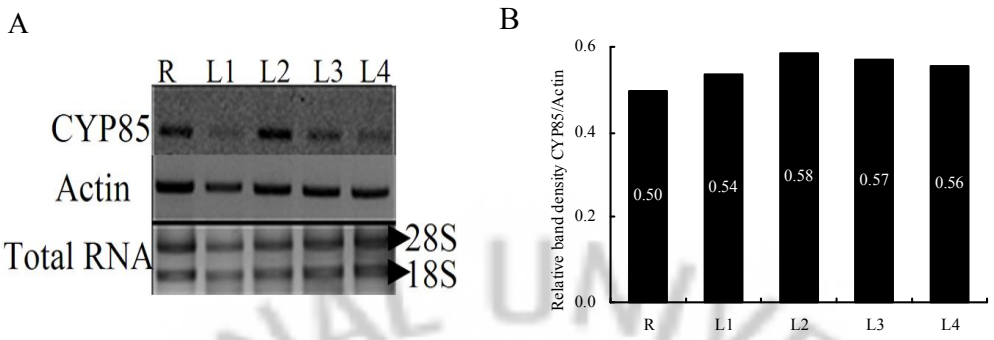

Fig. 19. Agarose gel electrophoresis of semi-quantitative RT-PCR and total RNA of *So-CYP85* at '4th leaf pair' stage. R: root, L1, L2,L3: 1st, 2nd, 3rd leaf pair of plant. A: SQ-RT-PCR, B: Relative band density of *So-CYP85*.

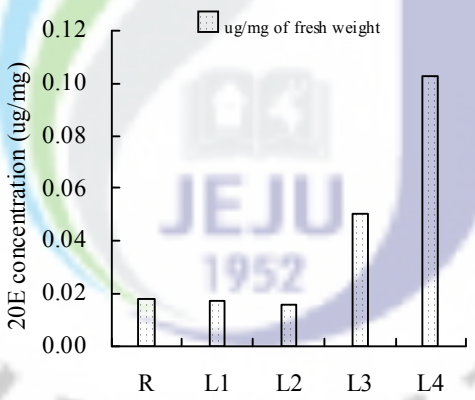

Fig. 20. Distribution of 20E in root and leaf of *S. oleracea*.

R: root, L1-L4: the leaf pairs of spinach from bottom to top.

DISCUSSION

Ecdysteroids have a carbonyl oxygen at C6 and a double bond at C7-C8 in the B-ring and these structures are very distinctive compared to other steroidal compounds. Both of these structures are essential for biological activity of ecdysteroids. Studies on ecdysteroids biosynthesis started ca. 30 years ago (Dinan *et al.,* 2009). However, the biosynthetic pathways for the formation of these structures still remained known in plants (Ono *et al.*, 2006). Molecular biological approach is alternative way to reveal the detailed processes in the formations of these unique structures. The first step for molecular biological study is to isolate corresponding genes. In this study we tried to isolate two candidate genes from an ecdysteroids producing plant spinach, DHCR and CYP85 which are expected to be involved in double bond formation at C7-C8 and oxidation at C6, respectively.

Characteristics of the Putative *DHCR* Gene

Previous papers have revealed that cholesterol is a direct precursor of 7-dehydrocholesterol in the early steps of ecdysteroid biosynthesis (Fujimoto *et al*., 1997, Ohyama *et al*., 1999). For the production of C7-C8 double bond, an enzyme functioning as dehydrogenase is necessary. In this study, a gene was isolated as a candidate for the dehydrogenase from spinach. A full length cDNA for DHCR was cloned and sequenced using degenerate RT-PCR and RACE technique. The identity of *DHCR* was approximately 85% with those in other plants on amino acid level: *Arabidopsis thaliana*, *Tropaeolum majus*, *Gossypium hirsutum* and *Ricinus communis* in which the enzyme has been already suggested as a putative 7-dehydrocholesterol reductase. So, the cloned gene from spinach was supposed to encode 7-dehydrocholesterol reductase (sterol-7-reductase) or cholesterol synthesase. The molecular weight of deduced protein of spinach DHCR was 45 kDa and it was similar to that of Arabidopsis DHCR with a molecular weight of 49 kDa. In particular, the homology of this gene in amino acid sequence was especially high, 94%, to that of *A.*

japonica synthesizing PEs. The high sequence similarity in ORF between DHCRs in different species of ecdysteroid producing plant suggests that they probably take part in the same catalytic process of the C7-C8 double bond formation in ecdysteroids biosynthesis.

Recently, an informative paper on PEs biosynthesis in spinach was reported by Ahmed *et al*. (2008). The authors observed that 20E was synthesized only in the source organ (old leaves) and not in the sink organ (new apical leaves). In the experiment ecdysone was strongly reduced in leaves (4% versus 19% to control). They also revealed that the conversion of MVA into ecdysone and 20E was down-regulated by feed back control of 20E. In the experiment 20E was completely abolished (0% versus 2% in control leaves). Therefore we examined the expression of the putative DHCR gene in spinach plant to see the possibilities of its involvement in PE synthesis. The gene expression was analyzed in the leaf pair L1, L2 and L3 at "3rd leaf pair stage", and L1, L2, L3 and L4 at "4th leaf pair" leaf pair stage". The transcription level of *DHCR* was slightly variable in different leaf pair of spinach. However, the difference in the level of gene expression among the tested leaves was not obvious. On the other hand the concentration of 20E was significantly different among the leaves at different age which was consistent with the previous report. Theses results showed that the expression of *DHCR* was not affected by 20E level. The precise function and target metabolic step of the isolated *DHCR* gene remained for further transgenic studies.

Characteristics of the Putative *So-CYP85* Gene

CYP450 family is a group of compound representing a super family of heme-thiolate enzymes. They involved in carbon hydroxylation or oxidation reactions in steroid biosynthesis. The oxidative reaction can be described as $SH+O_2+NADPH+H^+ \rightarrow SOH+H_2O+NADPH^+$. 'S' represents a compound with hydroxyl or oxygen group. It has been studied that CYPs take part in the last 4 steps of 20E biosynthesis in *Drosophila* ((Warren *et al*., 2002; Petryk *et al*., 2003; Warren *et al*., 2004; Gilbert, 2004; Niwa *et al*., 2004). They catalyze the four steps of hydroxylation during 20E biosynthesis. In plant, the PEs contains many hydroxyl groups in the structure contrast to the putative precursor CHR.

The other plant steroid—BR, the biosynthesis of it has been studied well. Some enzymes were identified catalyzing the production of BR by certain carbon site hydroxylation. These enzymes was classified belong to CYP familys, containing CYP85 and CYP90 subfamilies (Nomura *et al.,* 2006). The CYPs were also found in mammal including CYP11, CYP17 and CYP19 involving in the biosynthesis of steroid (David, F. V. Lewis and Peter Lee-Robichaud, 1998). In the biosynthesis pathway of BR, C-6 was thought likely to be the rate-limiting step for the production of castasterone (CS) which was converted from 6-deoxo CS via 6-hydroxy intermediate catalyzed by the CYP85A subfamily (Takahito *et al.*, 2005). In *Arabidopsis CYP85A* catalyzed the production of castasterone from 6-deoxocastasterone via the oxidation at C6 atom (Nomura *et al.,* 2001). Castasterone was converted into brassinolide catalyzed by the enzyme *CYP85A3* in yeast (Nomura *et al*., 2005). Nomura *et al*. also studied the heterologous expression of the CYP85A in yeast. They found that CYP85A3 (in tomato) and CYP85A2 (in Arabidopsis) catalyzed oxidation reaction at the C-6 of 6-deoxoCS. These results showed that the expression of *CYP85A* catalyzed the oxidation at C-6 in the biosynthesis of BRs.

The structure of 20E is similar to brassinolide containing many hydroxyl groups and a C-6 oxygen sit. Therefore, *CYP85A* was hypothesized to be C-6 oxydase in the biosynthesis of ecdysteroid. The study of *CYP85* is benefit to understand the biosynthesis of 20E in plant. In this study, a full length of gene *So-CYP85* was cloned in spinach. The deduce amino acid showed a 67% and 76% identity with that in *Arabidopsis thaliana* and *Solanum lycopersicum*, particularly, a high identity of 92% with *A. japonica.* And also, a heme-binding site was contained in the amino acid sequence encoded by spinach *So-CYP85*. Therefore, this gene cloned in spinach was identified belong to CYP85 subfamily in CYP450. And the molecular weight of amino acid encoded by this gene was about 54 kDa. This was very similar with the CYP85A in Arabidopsis (54 kDa) and *Solanum lycopersicum* (54 kDa). The high identity on amino acid and the similar characteristics on enzyme structure give us a clue that spinach *So*-CYP85 gene maybe function as a C-6 oxidation in the 20E biosynthesis process in spinach.

In order to understand the expression type of *CYP85* in spinach, the transcription was confirmed in the root and different leaf pairs. The highest transcription level of *CYP85* was found in the old leaves of spinach, and lowest in the young leaves. This phenomenon was not only found in the plant at "3rd leaf pair" but also at "4th leaf pair" stage. The high transcription of gene implies that the related product should be also high. Nevertheless, the concentration of 20E was unexpected in different leaf pair of spinach as describing above. Transcription of *CYP85* was opposite to the concentration or accumulates of 20E which was lowest in old and highest in young leaves. Therefore, 20E may be redistributed after being synthesized in spinach. This was confirmed in the result of Bakrim's (2008). They found that 20E was synthesized in the old leaves, and then translocated to the young leaves in spinach. The transcription level of *CYP85* accorded to this very well. They found that the conversion of MVA into 20E was inhibited by exogenous 20E in the leaves *in vitro*. On the other side, the expression of *CYP85A* was regulated by the negative feedback of BR (Bancos *et al.*, 2002). CYP85A1 catalyzes the production of CS from 6-deoxoCS in the tomato Dwarf. The CYP85A1 in tomato and Arabidopsis was subject to negative feedback regulation of BR (Takahito *et al.*, 2005). So, the diversity of transcription level and 20E concentration may be due to the redistribution of 20E after synthesis. The expression of *CYP85* may be suppressed by 20E in the young leaves of spinach where the concentration of 20E is high.

Conclusion

Most of the previous works in ecdysteroid biosynthesis have been investigated by metabolomic approach. In this work a genomic approach was used to identify genes which are essential for molecular biological analysis of ecdysteroid biosynthesis in plants. We focused on two enzymes, 7-dehydrocholesterol reductase (DHCR) and CYP85 which are supposed to catalyze formation of double bond at C7 – C8 and oxidation at C6, respectively, in the ecdysteroid ring structure. The full length cDNAs for putative DHCR and CYP85 were successfully cloned from spinach by using degenerate RT-PCR and RACE technique. The high homology in amino acid sequence and close similarity in molecular weight of

deduced protein with other known plant DHCR's provided positive evidences for the candidate as a DHCR. In the case of CYP85, the deduced protein of the isolated gene also showed high homology in amino acid sequence and close similarity in molecular weight with CYP85 family known in other plants. In addition the CYP85 candidate contained heme-binding domain and showed the typical absorption spectrum of cytochrome P450. Reverse correlation between the gene expression and 20E concentration in plant organs provided another evidence for the CYP85 candidate to be involved in PE biosynthesis. However, identification of target steps in ecdysteroid biosynthesis and elucidation of exact functions of the above two putative genes remained for further studies.

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