A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Effect of Phytoecdysteroids on Insect and Construction of Biological System for Analyzing of Phytoecdysteroids Biosynthesis

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CONTENTS

ABBREVIATIONS

20E ····· 20-Hydroxyecdysone

2D-20H ····· 2-deoxy-20-Hydroxyecdysone

7-DHC ····· 7-Dehydrocholesterol

BA ····· Benzyladenine

BR ····· Brassinosteriod

CHR ····· Cholesterol

CYP ····· Cytochrome P450

DHCR ····· 7-Dehydrocholesterol reductase

DW ····· Dry weight

E ····· Ecdysone

EST ····· Expressed sequence tag

FW ····· Fresh weight

GFP ····· Green fluorescent protein

HPLC ····· High performance liquid chromatography

LC/MS ····· Liquid chromatography/mass spectrometry

MS ····· Murashige and Skoog

NAA ····· Naphthylacetic acid

NP-TLC ····· Straight-phase thin layer chromatography

PCR ····· Polymerase chain reaction

PE ····· Phytoecdysteroid

RACE ····· Rapid amplification of cDNA ends

RP-TLC ····· Reverse-phase thin layer chromatography

RT-PCR ····· Reverse transcription polymerase chain reaction

TFA ····· Trifluoroacetic acid

TLC ····· Thin layer chromatography

UV ····· Ultraviolet

δ-ALA ····· 5-Aminolevulinic acid hydrochloride

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SUMMARY

Ecdysteroids were first recognized as steroidal hormones controlling and metamorphosis in insects. Today, it is realized that these steroids are present at all stages of insect development, regulating many biochemical and physiological processes: in newly-laid eggs, during embryonic and postembryonic developments and in adult insects, regulating aspects of development, metamorphosis, reproduction and diapauses. Ecdysteroids are the steroid hormones of all classes of arthropods and probably of other invertebrates too. Analogues of ecdysteroids (phytoecdysteroids) also occur in a certain proportion of plant species.

In 1954, the first ecdysteroid (ecdysone, E) was isolated by Butenandt and Karlson from pupae of silkworm *Bombyx mori*. Since then, the terms of zooecdysteroid (ZE) and phytoecdysteroid (PE) was used on the basis of the isolation sources. Furthermore, finding of PEs promoted the screening of source plants and understanding of PEs. Over the past 50 years, PEs researches have been advanced concerning the isolation, identification, distribution, biosynthesis and functional role in plant. Until now, more than 440 structurally different PEs have been isolated. Nevertheless, biosynthesis and functional role of PEs in plant are not fully understood.

In this study, the biological activity, biosynthesis and related genes of PEs were investigated. In part Ⅰ, the biological activities of PEs for some insect pests were studied to confirm the antifeedant activity, repellent activity and insecticidal activity. In part Ⅱ, the

genes in PEs biosynthesis were isolated from *Spinach oleracea* and its codon was optimized and synthesized for expression in *Escherichia coli* or plant. In part Ⅲ several genes in PE metabolism were expressed in *E. coli*, and their genes were used to construct the transformation system in PE-positive and negative plants. And eventually, the changes of putative PE metabolites were detected in transgenic line since experimental conditions to profile metabolites by HPLC were established.

In the antifeedant activity of PE, three insect pests were studied. For *Anomala albopilosa*, the PE showed the strong antifeedant activity. PE-treated cabbage was not fed after 48 hours, while the control cabbage was almost eaten up. For beet armyworm *Spodoptera exigua*, PE also had the potent antifeedant activity. The activity ratio of PE extraction from *A. japonica* and PE standard was 18.1% to 39.1% and 20.5% to 45.5%, respectively. The highest antifeedant ratio of PE was 45.5% at 400μg/ml. For Diamondback moth *Plutella xylostella*, PE also had the high antifeedant activity. Its activity ratio was 17.7% to 45.9% for PE standard, 24.3% to 45.5% for PE extracts from *A. japonica*, and 29.1% to 64.3% for PE extracts from *S. oleracea*. The highest antifeedant ratio of PE was 64.3% at 800μg/ml.

In repellent activity test of PE for *Cryptotympana dubia*, the remarkable repellent activity was observed.

In the insecticidal activity of PE for *Culex pipiens pallens*, there was a strong insecticidal activity. The mortality of mosquito of PE standard was 27.1% to 60.4%, and the value of LC_{50} was 1.9μg/ml.

At the conclusion of part Ⅰ, the PE showed a remarkable antifeedant, repellent and insecticidal activity on tested insect pests. These results showed that the PE might operate on the plant defense against non-adapted phytophagous insect.

Spinach, *S. oleracea*, was chosen for cloning of genes related to PE biosynthesis, due to a potent biological activity of its PE extracts against herbivorous insect.

Four genes related to PE biosynthesis, *DHCR, CYP85, CYP90B* and *CYP92A* were isolated from *S. oleracea*. All of these genes were homologous to other plant genes identified with same function and shared more than 60% identities at the amino acid level.

To enhance the expression and solubility of plant P450s (CYP85, CYP90B, CYP92A) and insect CYP314A1 in *E. coli*, the peptide segment, MAKKTSS residues, replaced the hydrophobic region in the amino-terminal region of the enzyme. And 5 histidine-tags were attached to C-terminal of protein for Ni^{2+}-chelate affinity column for purification.

At the conclusion of part Ⅱ, four candidate genes were isolated from spinach. And then, all codons in each gene were optimized and synthesized for over expression in *E. coli* or plant. Based on the above results, four candidate genes were obtained the over-expression in *E. coli* in part III.

Transformation systems in PE-positive (spinach) and negative (tobacco and Arabidopsis) plants were constructed for stable expression and transient expression of candidate genes of PE biosynthesis, respectively. In the former case, transgenic callus of spinach obtained by transformation of *Aj-CYP85* gene to get a constant expression. Transgenic plants of Arabidopsis also acquired by floral dipping method for *DHCR, CYP85, CYP90B* and

CYP92A, respectively. In the latter case, three genes, *CYP85*, *CYP90B* and *CYP92A,* were infiltrated into a leave of spinach to get over-expression or knock-down, and obtained a temporarily expression in plant leaves.

Metabolite profiling was established by HPLC. This condition could separate several metabolites between 7-dehydrocholesterol and 20-hydroxyecdysone in downstream of putative PE metabolic pathway. The over expressed plant of *CYP85* gene and the knockdown plants of *CYP90B* and *CYP92A* genes were analyzed in metabolites changes.

At the conclusion of part Ⅲ, four genes were expressed in *E. coli* for the enzymatic study. Several transgenic lines against those genes were constructed through the transformation system in PE-positive and negative plants. And finally, metabolite profiling was established by HPLC, and changes of putative PE metabolites were detected in transgenic lines.

Consequently, defensive roles of PE against insects were elucidated, and basic information on a molecular level was established for insight into regulation of PE pathway in this study.

INTRODUCTION

Phytoecdysteroids

Ecdysteroids were first recognized as steroidal hormones controlling and metamorphosis in insects. Today, it is realized that these steroids are present at all stages of insect development, regulating many biochemical and physiological processes: in newly-laid eggs, during embryonic and postembryonic developments and in adult insects, regulating aspects of development, metamorphosis, reproduction and diapauses. Ecdysteroids are the steroid hormones of all classes of arthropods and probably of other invertebrates too. Analogues of ecdysteroids (phytoecdysteroids) also occur in a certain proportion of plant species (Dinan 2001).

In 1954, the first ecdysteroid (ecdysone, E) was isolated by Butenandt and Karlson from pupae of silkworm *Bombyx mori* (Butenandt and Karlson 1954). And then, in 1965, its structure was unambiguously identified by Huber and Hoppe (Huber and Hoppe 1965). Afterward β-ecdysone (20-hydroxyecdysone, 20E) containing additional hydroxyl group at C20 of E was also isolated from *Bombyx mori* (Lafont, Koolman *et al*. 1993).

In 1966, the first ecdysteroids in plant were isolated by Nakanishi *et al*. from the leaves *Podocarpus nakaii* (Nakanishi, Koreeda *et al*. 1966). These first four compounds having molting hormone activity were named as Ponasterone A, B, C and D. At almost same time, β-ecdysone was isolated from the wood of *Podocarpus elatus*, the rhizomes of *Polypodium vulgare* and the root of *Achyranthes fauriei* (Galbraith and Horn 1966; Heinrich and

Hoffmeister 1967; Takamoto, Ogawa *et al*. 1967).

Since then, the terms of zooecdysteroid (ZE) and phytoecdysteroid (PE) was used on the basis of the isolation sources. And, finding of PEs promoted the screening of source plants and understanding of PEs. Over the past 50 years, PEs researches have been advanced concerning the isolation, identification, distribution, biosynthesis and functional role in plant. Until now, more than 440 structurally different PEs have been isolated. Nevertheless, biosynthesis and functional role of PEs in plant is not fully understood (Baltaev 2000; Dinan, Savchenko *et al*. 2001; Lafont, Harmatha *et al*. 2002).

Structure of PEs

PEs have a varied chemical structure and appears to be derived from C_{27}, C_{28} or C_{29} sterols. Several modifications of the structure are required to produce PEs. The major transformations are conversion of the trans A/B ring juncture in sterols to a *cis* A/B ring juncture in the ecdysteroid, the introduction of a 7-en-6-one chromophore and the introduction of 14α-hydroxy group (Rees 1985). Additional hydroxylations at C-2, C-20, C-22, and C-25 are required to produce the most commonly identified phytoecdysteroid, 20E, **Figure 1**, with the addition of a C-5 β-hydroxy group producing the often reported Polypodine B (Adler and Grebenok 1999).

The diversity of PEs is produced from the number, position and orientation of hydroxyl group and modification of the basic structure (Lafont and Horn 1989; Bathori and Pongracz 2005). The commonly hydroxylated sites are 2β-, 3β-, 14α-, 20R- and 22R- positions, which

together bring on the highly biologically active Ponasterone A. PEs containing eight hydroxyl group, nusilsterone, 5-hydroxy abutasterone, integristerone, have been also isolated. The C28 and C29 PEs may exist in either free form or polar conjugates of glucosides, sulfates, and phosphates or non-polar conjugates of acetates and benzoates (Adler and Grebenok 1999). These results suggest that over 1000 possible structures might occur in nature (Dinan 2001). However, in many cases, PEs is isolated in a free state. Also, structurally modified PEs is found only in minor ecdysteroids and the biological activity of these molecules reveal less than 20E.

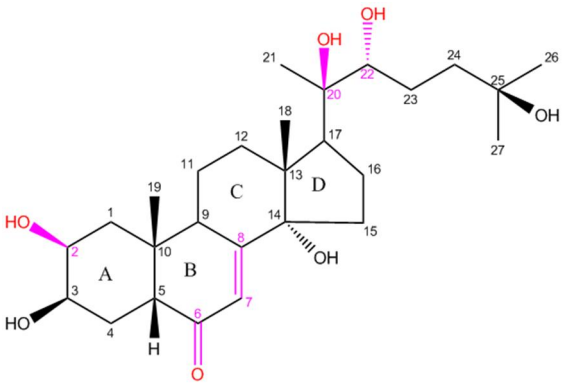
Screening of PE-containing plant in plant kingdom have been realized by development of radioimmunoassay (RIA, detection of structural similarity to ecdysone) and BII bioassay (detection of biological activity) method (Dinan 1995; Dinan, Bourne *et al*. 2001). Subsequently, much information about the distribution of ecdysteroids in the plant kingdom and also within the different parts of the plant has been reported. However, this information is fragmentary for understanding of possible roles of PEs in plant. Metabolic modifications of the basic structure have led to the identification of over 440 phytoecdysteroid compounds (Lafont, Harmatha *et al*. 2002). In addition to these true ecdysteroids, plants often make ecdysteroid-related compounds which are loosely defined based upon their structure and biological activity (Lafont and Horn 1989).

The characteristic of PEs biosynthesis in plant have been studied concerning fluctuation and distribution within plant part, and biosynthesis pathway. The fluctuation of PEs within plant at different growth stages was demonstrated in *T. cuspidate*, *R. carthamoides*, *L.*

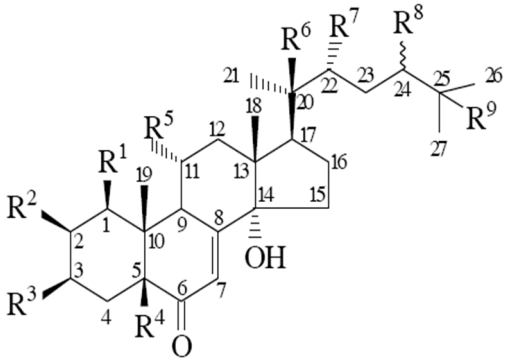
carthamoides, *S. oleracea* and *C. album* and *L. alba* Hartw. ex Benth (Vereskovskii, Chekalinskaya *et al*. 1983; Varga, Szendrei *et al*. 1986; Ripa, Martin *et al*. 1990; Grebenok and Adler 1991; Savchenko, Blackford *et al*. 2001; Preston-Mafham and Dinan 2002). The distribution of PEs within an individual plant was reported in some plant and related to the organ type and position as well as the state of development of organ. The fluctuation and distribution of PEs within plant was known to involve biosynthesis and transport, and highest concentration of PEs was little elucidated. In spinach, it was reported that active biosynthesis of PEs was occurred in development tissue and these compounds were transported to other organs.

The biosynthesis pathway was studied by feeding of radiolabelled precursor. The MVA, Lathosterol, 7-dehydrocholestrol, CHR, and E have been elucidated as the precursors of PEs biosynthesis in some plant (Sauer, Bennett *et al*. 1968; De Souza, Ghisalberti *et al*. 1970; Lloyd-Jones, Rees *et al*. 1973; Grebenok and Adler 1993; Adler and Grebenok 1995; Devarenne, Sen-Michael *et al*. 1995; Reixach, Irurre-Santilari *et al*. 1996; Ohyama, Kushiro *et al*. 1999; Fujimoto, Ohyama *et al*. 2000). However, the biosynthesis pathway of PEs is still difficult to address and fully understand.

Therefore, to isolate the genes and analyze the relationship between cloned genes and PEs, understanding of PEs biosynthesis in plant material is most important. Especially, expression analysis system based on PEs biosynthesis is necessary for cloning of genes related to PEs.



20E



PEs

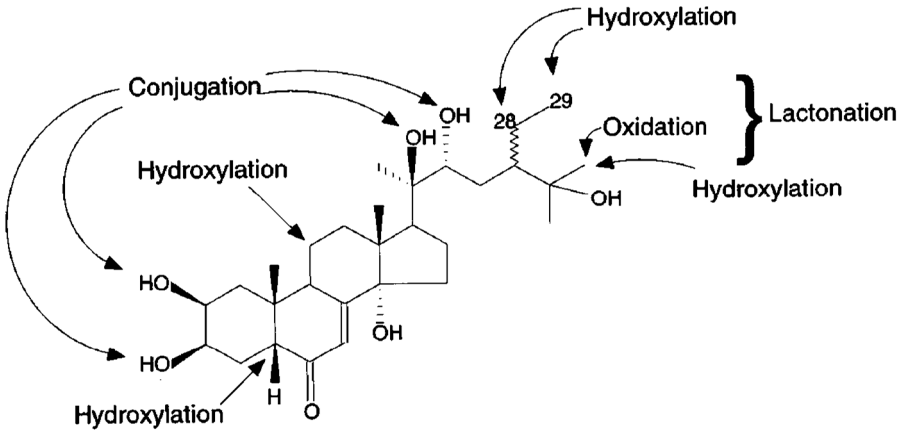


Figure 1. Structures and sites of biochemical modification of PEs. 20E: the structures of the most commonly reported phytoecdysteroid; PEs: the structure of PEs and some common sites of biochemical modifications reported for PEs.

Function of PEs

The role of PEs in plant has not been fully elucidated. The mainly presumed hypothesis concerning the role of PEs in plant is to contribute to the defense of plants against non-adapted phytophagous insect (Bergamasco and Horn 1983; Lafont, Bouthier *et al*. 1991). This hypothesis is supported by the fact that major ecdysteroid is identical in both of most PE-containing plants and insects. Also, the tissues containing highest concentration of 20E are most important for the survival of the plant and these levels are changed throughout plant development, and some non-adapted insect species is deterred on low concentration of 20E (Klein 2004). However, there are a number of insect species which can feed and survive on plants containing very high concentration of PEs, and these resistant species is a rapid detoxification and/or excretion of ingested PEs (Blackford and Dinan 1997; Zeleny, Havelka *et al*. 1997). Hence, PEs can be considered as chemical defense against certain, but not all, herbivorous insects (Dinan 2001).

Other hypothesis of concerning the role of PEs in plant is a hormonal role. The weak gibberellin-like activity was identified in dwarf mung bean and rice bioassays (Dreier and Towers 1988; Machackova, Vagner *et al*. 1995). However, these activities and a hormonal role of PEs have been unconvincing, because other bioassays to demonstrate the gibberellin activity were assessed as negative or not confirmed, and there is lots evidence against its hypothesis (Carlisle, Osborne *et al*. 1963; Jacobs and Suthers 1971; Hendrix and Jones 1972; Felippe 1980).

Purpose of this study

In insects, introduction of hydroxyl group at C_2, C_{20}, C_{22} and C_{25} of PEs have been identified as being catalyzed by CYP and the genes encoding those four enzymes were cloned in the fruit fly *Drosophila melanogaster* (Warren, Petryk *et al*. 2002; Petryk, Warren *et al*. 2003; Warren, Petryk *et al*. 2004). The hydroxylations at C25, C22 and C2 are sequentially catalyzed by CYP306A1 (Phantom: Phm), CYP302A1 (Disembodied: Dib) and CYP315A1 (Shadow: Sad), respectively. Final conversion of E into 20E is catalyzed by CYP314A1 hydroxylase (Shade: Shd).

However, study of PEs biosynthesis genes and enzymes is still primitive. So far, CYP was reported as ecdysone C20 hydroxylase in some plant, even then the information of gene encoding to those enzymes was not known yet (Grebenok, Galbraith *et al*. 1996; Alekseeva 2004; Canals, Irurre-Santilari *et al*. 2005). That reason is assumed that many different CYP families are present in plant and the functions are also extremely diverse.

Phytosterols are synthesized via the MVA pathway (Goad and Goodwin 1966). Phytosterols divided into C27, C28 and C29 sterol in dependence on the number of carbon atoms in their molecules. Phytosterol of C27 structural type may come from CHR and those of C28 and C29 structural type may be derived from campesterol and sitosterol, respectively. Difference of these three intermediate is only alkyl group at C24. Campesterol and sitosterol contain the methyl and ethyl groups at C24, respectively, and CHR is absent. Cycloartenol is the first branch point to convert into CHR, campesterol and sitosterol (Diener, Li *et al*. 2000; Moreau, Whitaker *et al*. 2002; Schaller 2003).

With campesterol and sitosterol biosynthesis, CHR biosynthesis pathway and relevant genes in plant were also indentified well as follow. HMG-CoA is the precursor for CHR biosynthesis. HMG-CoA reductase catalyzes production of MVA from HMG-CoA, which is rate-limiting step for phytosterol biosynthesis. MVA is transformed into isopentenyl diphosphate (IPP) via three sequential steps involving phosphorylation and decarboxylation. IPP is converted into several compounds such as rubber, geranyl pyrophosphate (GPP), geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP). FPP involved from IPP or GPP by FPP synthase is served as intermediate of CHR biosynthesis. Squalene is synthesized from FPP by squalene synthase, and then this product is converted into cycloartenol via two sequential steps involving oxidation and cyclization. After that, cycloartenol is convert into CHR involving eight reactions including CHR 7, 8 delta isomerization and 7-dehydrocholestrol reduction (KEGG 2011).

According to previous reports, the PEs (C27 structural type) were synthesized from CHR, whereas those of C28 and C29 structural type were not served with CHR as precursor (Lloyd-Jones, Rees *et al*. 1973; Grebenok and Adler 1993; Devarenne, Sen-Michael *et al*. 1995; Reixach, Lafont *et al*. 1999; Fujimoto, Ohyama *et al*. 2000; Okuzumi, Hara *et al*. 2003). The most BRs (C28 structural type) that are structurally very similar with PEs were derived from compesterol. Also, it is suggested that the other two groups, C27 and C29 structural type, might be derived from CHR and sitosterol, respectively (Nomura, Sato *et al*. 2001; Fujioka, Takatsuto *et al*. 2002; Fujioka and Yokota 2003; Nomura and Bishop 2006). Therefore, although the number of carbon atoms in major product derived from CHR (C27),

campesterol (C28) and sitosterol (C29), the biosynthesis pathway of those phytosterol is probably similar. This is supported by the fact as follow. The representative enzymes in BR biosynthesis were Arabidopsis *CYP85* and *CYP90* family. Among them, Arabidopsis CYP85 known as C6 oxydase of BR was shown to catalyze the C6 oxidation of multiple BR intermediate (Shimada, Fujioka *et al*. 2001; Kim, Hwang *et al*. 2005). And Arabidopsis *CYP90B* subfamilies catalyzing C22 hydroxylation in BR was revealed to catalyze the C22 hydroxylation of various C27 CHR, C28 campesterol and C29 sitosterol (Bach 1986; Fujita, Ohnishi *et al*. 2006). Additionally, the biosynthesis of C27 BRs was kown to proceed in a similar way to that of C28. In order to understand clearly about PEs in plant and application to agriculture, it is necessary to clarify the biosynthesis pathway and identify the biosynthesis genes together with understanding of distribution pattern within different parts of plants and extending of the search for new plant sources and new PEs. Especially, cloning of genes in key step of PEs biosynthesis will be critical cue for understanding and application of PEs.

Although first PE was isolated at mid-1960s, the discovery of genes related to PEs biosynthesis was not achieved due to the lack of significant intermediates pool, sequential pathway of biosynthesis, and available biosynthesis characterization concerning their molecules in plant.

The objects of this study were to understand the characteristic of PEs biosynthesis and fluctuation in *S. oleracea* and to establish the expression analysis system based on PEs biosynthesis for cloning of genes related to PEs.

PART I Effects of Phytoecdysteroids on Insects

Introduction

At present, the control of pest is primarily dependent upon chemical pesticides. The repeated use of chemical pesticides for several decades can lead to outbreaks of secondary insect pests, widespread development of resistance and undesirable effects on non-target organisms, and fostered environmental and human health concerns. Owing to the abundant unreasonable application of chemical pesticides, 3R (resistance, resurgence, and residue) problem has aroused wide attention among researchers. Therefore, seeking and exploring new types of botanical pesticide in natural plant has been becoming the focus for research (Yan-Zhang, Chang-Ju *et al*. 2007).

Zooecdysteroids are the steroid hormones of arthropods, where they regulate moulting, metamorphosis, reproduction and diapauses. They probably fulfill similar roles in many other invertebrate phyla, but these have not been so extensively investigated. PEs is analogues occurring to deterrence of phytophagous invertebrates. PEs are present in 5-6% of plant species, generally at far higher concentrations than those typically found in arthropods, where they are regarded as contributing to the deterrence of invertebrate predators (Dinan and Lafont 2006). The archetypal ecdysteroid in both arthropods and plants is 20-hydroxyecdysone (20E), since it is the most commonly occurring and the most abundant, but a very wide range of structural analogues has been elucidated, especially from plant sources (Dinan and Lafont 2006).

20E has the bioactivity to pest, which has obvious suppressant activity including antifeedant activity, repellent activity and insecticidal activity. It has been reported that there are at least 32 pests having been tested on these bioactivity. (See **Table 1**)

Several insect species possess deterrent taste receptors that can detect 20E, allowing them to avoid ingesting this molecule. However, a number of insects have been shown to be resistant to 20E. Blackford and Dinan suggested that polyphagous insects are resistant to 20E, while oligophagous insects are more sensitive (Blackford and Dinan 1997).

The objects of this study were to further confirm biological activity of PE, including antifeedant activity, repellent activity and insecticidal activity.

Table 1. The pests have been tested using 20E up to now

No.	Species name	Oder of insect	References	Type of test
1	*Aglais urticae*	Lepidoptera	(Blackford and Dinan 1997)	Insecticidal activity
2	*Aphrophora*	Homoptera	(Defu, Jingwen *et al*. 1997)	Insecticidal activity
3	*Aporia crataegi*	Lepidoptera	(Jingwen, Defu *et al*. 1997)	Insecticidal activity
4	*Bombyx mori*	Lepidoptera	(Kubo, Klocke *et al*. 1983; Nair, Yun-Gen *et al*. 2006)	Insecticidal activity
5	*Bradysia impatiens*	Diptera	(Schmelz, Grebenok *et al*. 2002)	Antifeedant & insecticidal
6	*Chilo partellus*	Lepidoptera	(Jones and Firn 1978)	Antifeedant activity
7	*Chironomus tentans*	Diptera	(Smagghe, Dhadialla *et al*. 2002)	Insecticidal activity
8	*Clostera anastomosis*	Lepidoptera	(Darva, Toledano *et al*. 1997)	Insecticidal activity
9	*Cnaphalocrocis*	Lepidoptera	(Institute of Botany 1978)	Insecticidal activity
10	*Cryptorrhynchus*	Coleoptera	(De-fu, Ming-xue *et al*. 2002)	Insecticidal activity
11	*Cynthia cardui*	Lepidoptera	(Blackford and Dinan 1997)	Insecticidal activity
12	*Helicoverpa Armigera*	Lepidoptera	(Sciences (Wuhan) 1980)	Insecticidal activity
13	*Hyphantria cunea*	Lepidoptera	(Defu, Bela *et al*. 1997)	Insecticidal activity
14	*Hyponomeuta*	Lepidoptera	(Jones and Firn 1978)	Antifeedant activity
15	*Inachis io*	Lepidoptera	(Blackford and Dinan 1997)	Insecticidal activity
16	*Leis axyridis*	Coleoptera	(Defu, Jingwen *et al*. 1997)	Insecticidal activity
17	*Lymantria dispar*	Lepidoptera	(Jingwen, Defu *et al*. 1997)	Insecticidal activity
18	*Malacosoma neustria*	Lepidoptera	(Jingwen, Defu *et al*. 1997)	Insecticidal activity
19	*Myzus persicae*	Homoptera	(Defu, Jingwen *et al*. 1997; Malausa, Salles *et al*. 2006)	Insecticidal activity
20	*Naranga aenescens*	Lepidoptera	(Sciences (Wuhan) 1980)	Insecticidal activity
21	*Parnara guttata*	Lepidoptera	(Sciences (Wuhan) 1980)	Insecticidal activity
22	*Parthenolecanium corni*	Homoptera	(Defu, Jingwen *et al*. 1997)	Insecticidal activity
23	*Pectinophora*	Lepidoptera	(Kubo, Klocke *et al*. 1983)	Antifeedant activity
24	*Phyllobius argentatus*	Coleoptera	(Jones and Firn 1978)	Antifeedant activity
25	*Phyllobius pyri*	Coleoptera	(Jones and Firn 1978)	Antifeedant activity
26	*Pieris brassicae*	Lepidoptera	(Jones and Firn 1978)	Antifeedant activity
27	*Pieris rapae*	Lepidoptera	(Sciences (Wuhan) 1980)	Insecticidal activity
28	*Schistocerca gregaria*	Orthoptera	(Jones and Firn 1978)	Antifeedant activity
29	*Spodoptera littoralis*	Lepidoptera	(Jones and Firn 1978; Sciences (Wuhan) 1980)	Antifeedant activity
30	*Stilpnotia candida*	Lepidoptera	(Darva, Toledano *et al*. 1997)	Insecticidal activity
31	*Tuberolachnus salignus*	Homoptera	(Darva, Toledano *et al*. 1997)	Insecticidal activity
32	*Tyria jacobaeae*	Lepidoptera	(Blackford and Dinan 1997)	Insecticidal activity

Materials and methods

Seed materials

Achyranthes japonica Nakai and spinach (*Spinacia oleracea* L.) seeds were used for extracting PEs. *A. japonica* seeds were collected from around of Jeju national university. Spinach (*Spinacia oleracea* L.) seeds were purchased from commercial market in Jeju, Korea.

PEs Extraction

One kilogram of ground dried samples were extracted 2 times with 10L methanol by shaking for 3 hr at 40℃. Extracts were concentrated by rotary evaporator at 50℃ and dissolved with 1L of 80% methanol. Then, Extracts were partitioned 2 times with 1L of hexane. The aqueous methanol phases were concentrated by rotary evaporator at 50℃ and dissolved with water. After that the solution was filtered with filter paper (Boo 2007).

High performance liquid chromatography (HPLC)

Analytical HPLC Content of 20E and profiles of PEs analyzed by HPLC. The HPLC equipment consisted of Spectra System vacuum degasser, P4000 pump, AS1000 auto sampler with column oven, and UV6000LP Photo Diode Array detector. The extracts were separated on ODS column (Phenomenex, 250×4.6mm ID, 5μ) using 10% 2-propanol containing 0.1% trifluoroactetic acid (TAF) as mobile phase at a flow rate of 1.2ml/min. Ultraviolet (UV) detection for measurement of 20E was set at 242 nm. The profiles of PEs in

A. japonica and *S. oleracea* were identified by comparing HPLC retention time and UV absorption spectra of reference compounds. Detailed HPLC condition was described in **Table 2** (Boo 2007).

20E were used as standard for HPLC analysis and purchased from Sigma Chemical Co.

Table 2. Operation condition of HPLC for PEs analysis

Parameter	Condition
Instruments	Vacuum degasser, P4000 pump, AS1000 auto sampler, UV6000LP Photo Diode Array detector
Column	ODS column (250×4.6 mm ID, 5μ)
Mobile phase	10% 2-propanol (0.1% TFA)
Flow rate	1.2 ml/min
Detection wavelength	242 nm
Scan wavelength	200-400 nm
Injection volume	20 μl
Column temp.	40℃
Run time	45.0 min

Antifeedant test

Three insect pests - *Anomala albopilosa,* beet armyworm (*Spodoptera exigua* (Hübner)) and diamondback moth (DBM) (*Plutella xylostella* L.) - were used for antifeedant activity test of PE.

Anomala albopilosa *A. albopilosa* was collected from around of Jeju national university.

The insects were put into the centre of the cage containing cabbage spayed with PE or water, and then checked it out after 48 hours.

Spodoptera exigua *S. exigua* was obtained from Dr. Jeong-Heub Song in Jeju Agricultural Research and Extension Services. The healthy and normal pupae were selected from the soil after 2d pupation. After distinguishing the sex, the pupae were disinfected for 15 minutes using 0.2% NaOCl solution and rinsed by sterilized water. The Seven pairs of pupa were put into the cylindrical plastic arena until the adult emergence in the custody pest control room under 27±1℃ temperature, 70±10% relative humidity, and 14:10 (L: D) photoperiod.

To obtain the eggs, adult moths were fed with 10% sugar solution, and the cage was changed every day. The artificial diet (See **Table 3.**) was supplied before a egg hatching of day. The diet was replaced with every 2 to 3 day. To pupate, the mature larvae were transferred to other cage with some sterilized soil.

For choice test, the artificial diet (treatment and control feed) was weighed before putting into the plastic rearing cage and the remaining diet after 24 hours. 10 heads of 3rd instars larvae was applied for each treatment and repeated three times.

Antifeedant rate was calculated as follow;

$$\text{Antifeedant ratio (\%)} = (C-T)/(C+T)\times 100\% \text{ ---------- Eq. 1.}$$

which C and T was the weight of control diet and PE treated diet, respectively.

Plutella xylostella *P. xylostella* was obtained from Dr. Jeong-Heub Song in Jeju Agricultural Research and Extension Services. The test method was same with *S. exigua* test.

The adults were fed with 10% sugar solution. Each day moved the adults to the new plastic cage. And the egg-laid plastic dish maintained in the same room. The day before egg hatching, the common head cabbage (*Brassica oleracea* L. var. *capitata*) was put into the cage. Feed plant was replaced at every 2 or 3 days until pupated.

For choice test, firstly, make the sequential series of concentration by geometric progression pharmacy dilution method of the compound. Fresh cabbage leaf discs, which were cut off by puncher with diameter 21mm, were dipped in tested solution for 5. After natural dry, the leaf discs of treatment and of control were randomly placed on the moisture filter paper in petri dish. There were placed 3 heads of 2nd instar larvae on a plate. Each test was repeated three times. After 48 hours, the weight of leaf disc was measured. Calculate the antifeedant ratio by different concentration by Eq. 1.

Table 3. Main components of the artificial diet for the larvae of *S. exigua*

Main components	**Dosage**
Agar	*25g*
Distilled water	*1000ml*
Boiling	
Wheat germ	*240g*
Yeast extract	*32g*
Casein	*32g*
MPH	*1.25g*
Mix up, cooling until 55℃	
Vanderzent vitamine mixture	*4g*
Sorbic acid	*1g*
Ascorbic acid（Vc）	*4g*
Wesson salt mixture	*6g*
Linseed oil	*2ml*
Kanamycin sulfate250ppm	*2ml*

Repellent test

Cryptotympana dubia larva was used for testing repellent activity of PE.

100ml of PE extract from *A. japonica* (1000μg/ml) as treatment and water as the control was mixed with 400g soil, and then put into the cage. 20 larvae of white grub were placed on the middle of treatment and control soil. After 48 hours, the number of staying white grub was counted.

Insecticidal test

Culex pipiens pallens larva was used for testing insecticidal activity of PE.

First, make the sequential series of concentration by geometric progression Pharmacy dilution method of the compound. 20 heads of 1st instar larva were tested in a cage. Each treatment repeated three times. The number of live larva was counted on 2 day intervals for total 7 days.

Results

Antifeedant activity of PEs

The antifeedant activity of PE was tested for *Anomala albopilosa* by choice test (See **Figure 2**). The insects were put into the centre of the box containing cabbage spayed with PE or water, and then check it out after 48 hours. As the result, leave of control cabbage were almost eaten up, however treatment cabbage were not. This result showed PE has very high antifeedant activity on this insect.

The antifeedant activity of PE was also tested for *Spodoptera exigua* by choice test (See **Figure 3**, **Figure 4**). The antifeedant ratio of the 3rd instar larvae that fed on the diets with addition of 100, 200, 400, 800 μg/ml of 20E standard, were up to 20.5%, 20.7%, 45.5% and 42.1%, respectively. And similarly, the antifeedant ratio of the 3rd instar larvae that fed on the diets with addition of 100, 200, 400, 800 μg/ml of PE extract from *A. japonica*, were up to 18.1%, 27.3%, 23.1% and 39.1%, respectively. This result showed PE has very high antifeedant activity on this insect. The highest antifeedant activity was observed at 400μg/ml of PE.

Finally, the antifeedant activity of PE was also tested for *Plutella xylostella* by choice test (See **Figure 5**, **Figure 6**). The antifeedant ratio of the 2rd instar larvae that fed on the diets with addition of 100, 200, 400, 800 μg/ml of 20E standard, were up to 42.1%, 57.0%, 72.6% and 100%, respectively. And similarly, the antifeedant ratio of the 3rd instars larvae that fed on the diets with addition of 100, 200, 400, 800 μg/ml of PE extract from *A. japonica*, were up to 24.1%, 42.5%, 45.5% and 64.3%, respectively. This result showed PE

has very high antifeedant activity on this insect. The higher dosages of PE shows the hihger antifeedant activity.

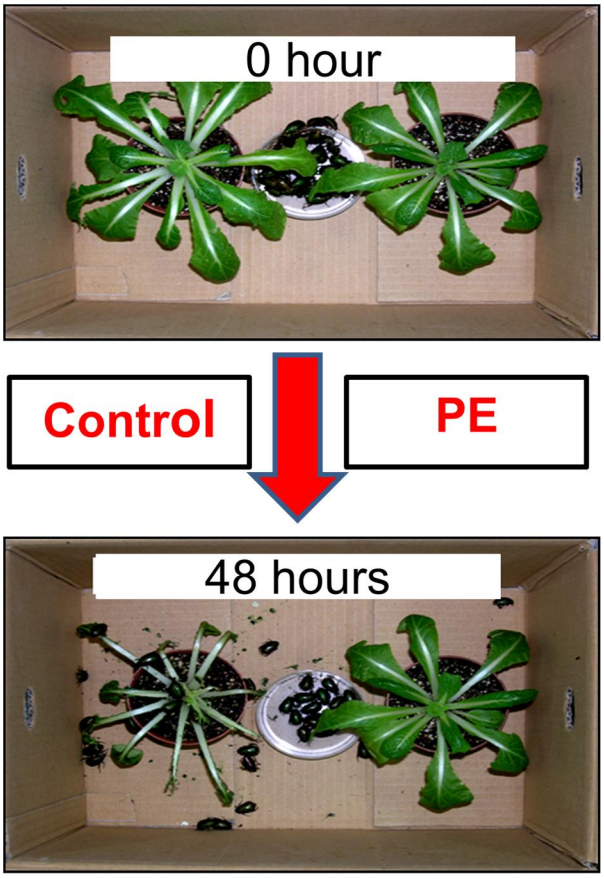


Figure 2. Choice test of PE extract from *A. japonica* for *Anomala albopilosa* adults.

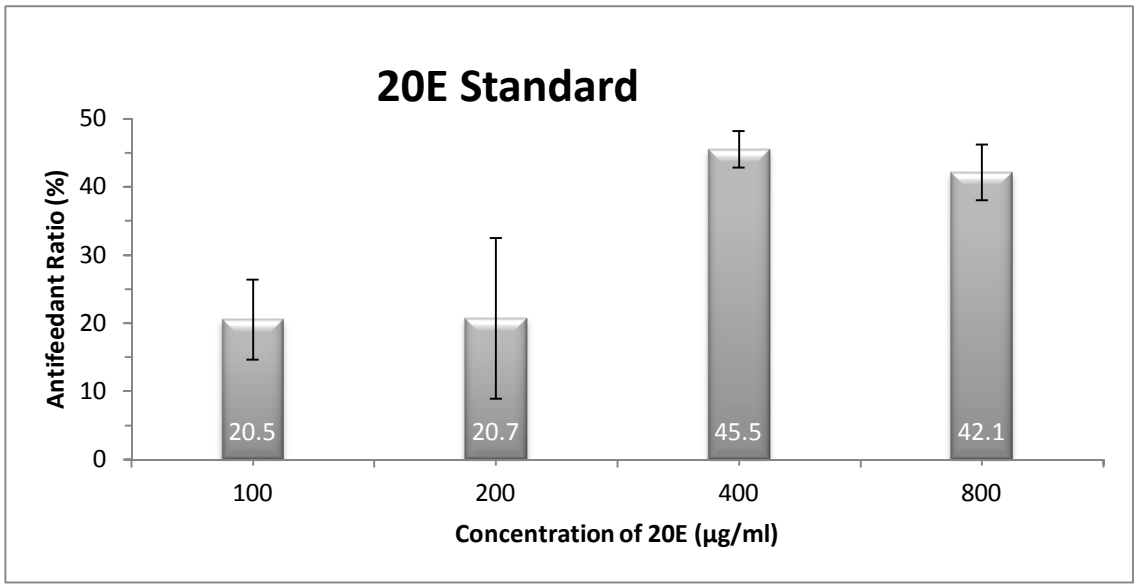


Figure 3. Choice test of 20E standard for the 3rd instar larvae of *Spodoptera exigua*.

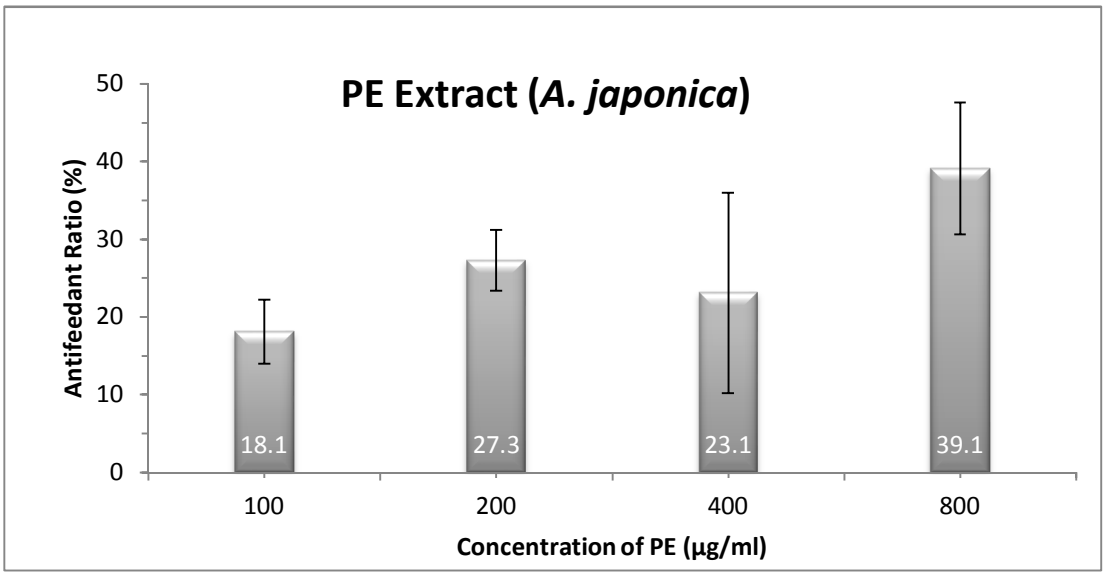


Figure 4. Choice test of PE extract from *A. japonica* for the 3rd instar larvae of *Spodoptera exigua.*

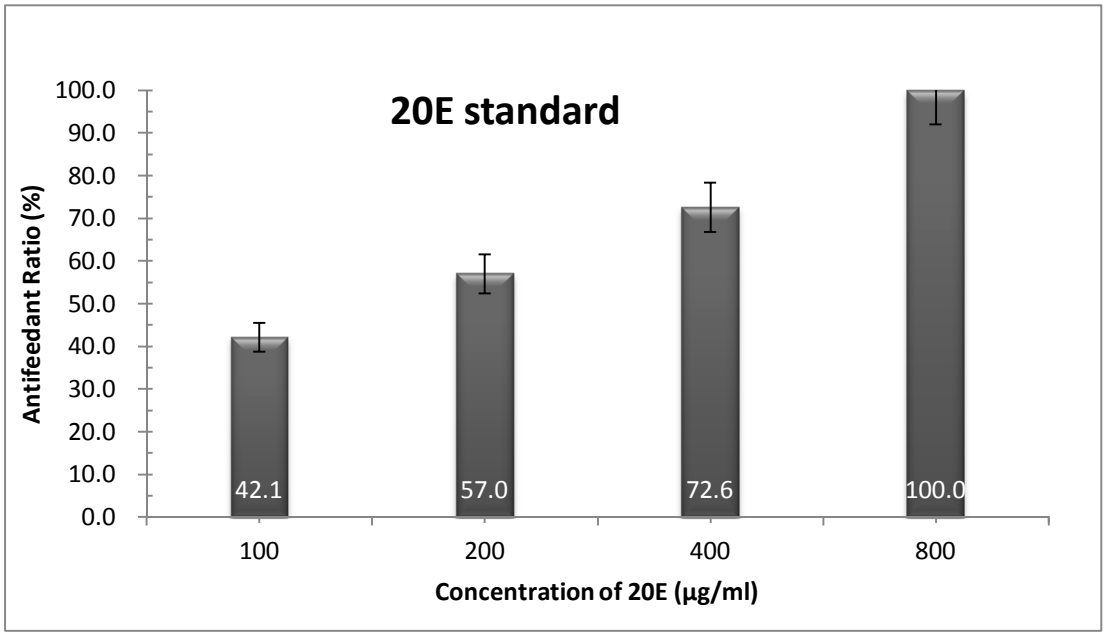


Figure 5. Choice test of 20E standard for the 3rd instar larvae of *Plutella xylostella*.

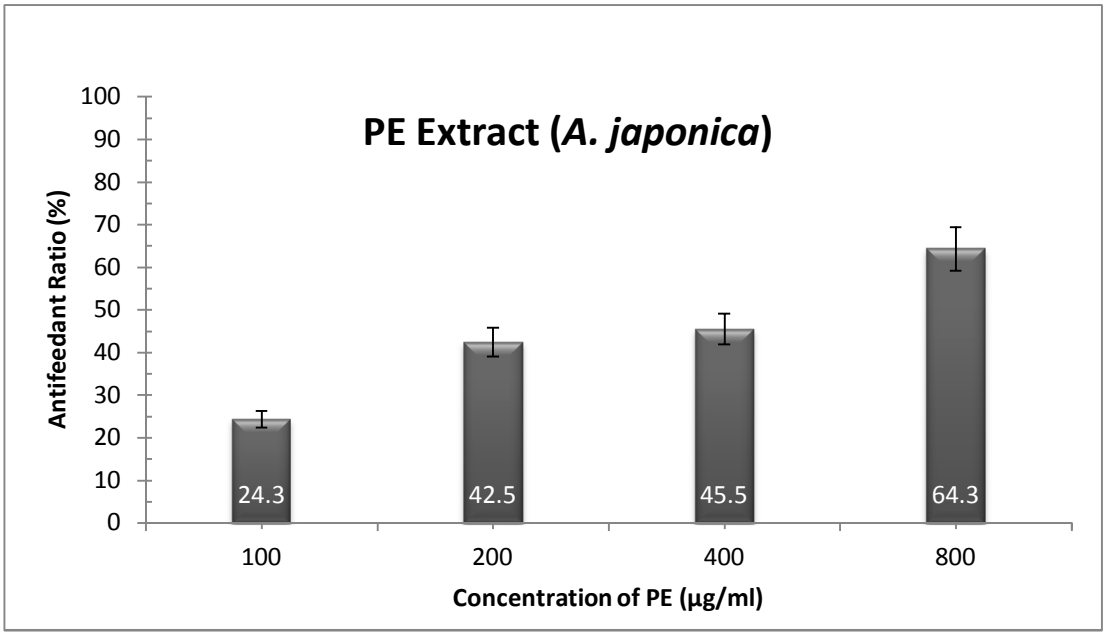


Figure 6. Choice test of PE extract from *A. japonica* for the 3rd instar larvae of *Plutella xylostella.*

Repellent activity of PEs

The repellent activity of PE was tested for *Cryptotympana dubia* by choice test (See **Figure 7**). The insects were put into the centre of the box containing different soil mixed with PE or water, and then check it out after 48 hours. As the result, most of white grub moved into the soil mixed with water (16.3/20). This result showed PE has very high repellent activity on this insect.

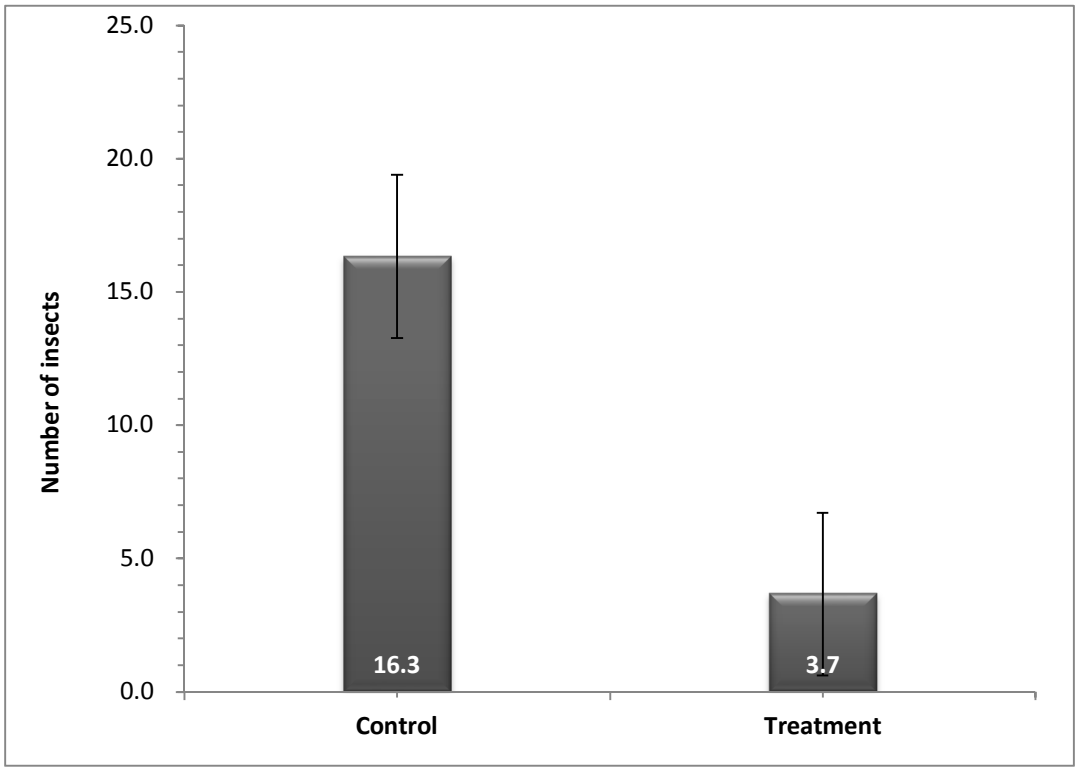


Figure 7. Choice test of PE extract from *A. japonica* for the larvae of *Cryptotympana dubia.*

Insecticidal activity of PEs

The insecticidal activity of PE was tested for *Culex pipiens pallens* by No-choice test (See **Figure 8**). The corrected mortality of 1st instar larvae that reared on the water added with 1, 5, 10, 25 and 50 µg/ml of 20E standard, were up to 32.7%, 40.4%, 76.9%, 98.1% and 100%, respectively. This result showed PE has definitely insecticidal activity on this insect. There was observed 100% insecticidal activity at 50µg/ml of PE.

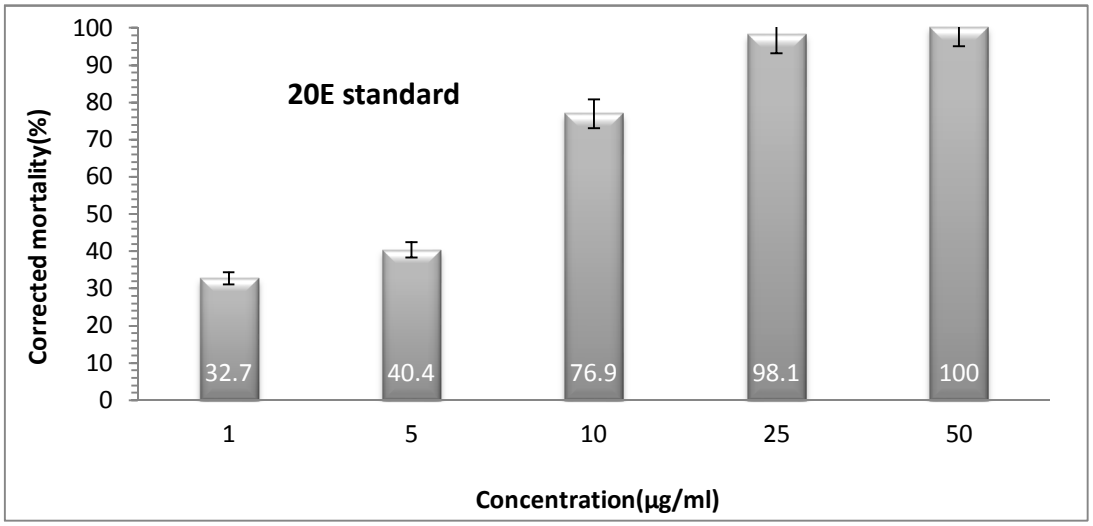


Figure 8. No-choice test of 20E standard for the 1st instar larvae of *Culex pipiens pallens*.

Discussion

In the past, the antifeedant or repellent properties of PEs against insects have a limited researcher's attention. In fact only five studies appear to have touched on the subject (Ma 1972; Schoonhoven and Derksen-Koppers 1973; Jones and Firn 1978; Kubo, Klocke *et al*. 1983; Blackford, Clarke *et al*. 1996). The most extensive study was that of Jones and Firn, in which 20E was shown to deter feeding in *Pieris brassicae* (Lepidoptera) at above 5μg/ml concentration. and *Chilo partellus* (Lepidoptera), *Phyllobius pyri* and *P. argentatus* (Coleoptera) at 60μg/ml concentrations. *P. brassicae* feeds almost exclusively on a range of PE-negative plants and would thus be unlikely to encounter PEs in its normal diet. Jones and Firn also revealed that *S. littoralis* is not deterred from feeding by 50-70μg/ml of 20E. This has been confirmed in a study of Blackford (Blackford, Clarke *et al*. 1996).

Literature precedence has categorized the handful of lepidopteran species studied to date according to their tolerance or susceptibility to exogenously applied PEs, the reasons why some insects are able to thrive on diets containing high levels of ecdysteroid while other species suffer marked abnormalities in growth and development have attracted limited research. The data presented in this report also confirmed the decision. *Spodoptera exigua* and *Plutella xylostella* all belong to order Lepidoptera. So the high concentration of PE was needed to deter feeding the food. These also maybe because the two insect species larvae are able to feed on almost any plant species and are truly polyphagous which contain PE-positive plants within their normal host range such as *S. oleracea.* But the low

concentration of PE was needed to deter feeding the food for *Anomala albopilosa* which belongs to order Coleoptera, as such *Cryptotympana dubia* which belongs to order Hemiptera.

Every way, we can summarize results in this biological activity test part that PE have antifeedant, repellant and insecticidal activity. And these results support PE role in plant is probably to defense plants against non-adapted phytophagous insect.

PART II Cloning of Candidate Phytoecdysteroid genes and Their modification for Enzymatic Study

Introduction

The biosynthesis pathway of ZEs was well characterized in insect. The ingested CHR is known as a precursor in insect and converted into 7-dehydrocholesterol. The conversion of 7-dehydrocholesterol to the Δ4-diketol are commonly retained as the black box, and this is converted first into the diketol and then into E (Ono, Rewitz *et al*. 2006). The final four steps are to convert the ketodiol derived diketol into 2-, 22-dideoxyecdysone, 2-deoxyecdysone, E and 20E, sequentially. The genes related to the final four steps have been identified in *Drosophila*. These four genes encoding ecdysteroids hydroxylase are CYP family (Warren, Petryk *et al*. 2002; Petryk, Warren *et al*. 2003; Warren, Petryk *et al*. 2004).

Studies on PEs biosynthesis started about 30 years ago about the same time as in insects (Dinan, Harmatha *et al*. 2009). Given the large amounts of ecdysteroids found in some plant species, it could be thought that elucidation of the pathway could be easily addressed. In reality, the high amounts are the result of ecdysteroid accumulation over a long period, which does not require such high rates of synthesis when compared to that in insect moulting glands; moreover, the sites of ecdysteroid production have not been defined, and we do not know whether biosynthesis takes place in all, or only is some, specialized cells (Dinan, Harmatha *et al*. 2009). It will become possible to address such a question only with

a molecular biological approach, when genes encoding some of the biosynthetic enzymes have been identified, which is not yet the case for the following reasons: (1) none of the plant species for which genomic sequence information is available accumulate ecdysteroids and (2) unlike brassinosteroids, we cannot expect that mutants will display a specific phenotype. Thus, very few attempts have been made so far to purify biosynthetic enzymes using classical biochemical approaches (Grebenok, Galbraith *et al*. 1996; Canals, Irurre-Santilari *et al*. 2005).

In the plant, the MVA and CHR have been elucidated as the precursors of PEs biosynthesis in *Podocarpus elata*, *Taxus baccata*, *Spinacia oleracea*, *Ajuga reptans* var. *atropurpurea*, *Zea mays* and *Polypodum vulgare* (Sauer, Bennett *et al*. 1968; Lloyd-Jones, Rees *et al*. 1973; Grebenok and Adler 1993; Devarenne, Sen-Michael *et al*. 1995; Reixach, Irurre-Santilari *et al*. 1996; Fujimoto, Ohyama *et al*. 2000). Lathosterol was identified as the precursor of PEs in *Zea mays*, *Spinacia oleracea* and hairy roots of *Ajuga reptans* var. *atropurpurea* (Adler and Grebenok 1995; Devarenne, Sen-Michael *et al*. 1995; Ohyama, Kushiro *et al*. 1999). The conversion of 7-dehydrocholesterol into 20E was also demonstrated in hairy roots of *Ajuga reptans* var. *atropurpurea* and *Polypodium vulgare* (De Souza, Ghisalberti et al. 1970; Ohyama, Kushiro et al. 1999). According to these investigations, the Lathosterol and 7-dehydrocholesterol were suggested as required intermediates are PEs biosynthesis in the early stage. However, in the later studies, incorporation of 3β-hydroxy-5β-cholestan-6-one into 20E and introduction of 7-ene in late stage were indentified in hairy roots of *Ajuga reptans* var. *atropurpurea* (Hyodo and

Fujimoto 2000). These finding suggested that 7-dehydrocholesterol is not obligatory intermediate and alternative pathway may exist in PEs biosynthesis. Therefore, the PEs biosynthesis in the early stage is still difficult to address and fully understand. Moreover, the hydroxylation steps in the late stage are unclear due to the diversity of PEs present among plant species (KEGG 2011).

Reixach *et al*. has demonstrated the biotransformation of putative PEs precursors in tissue cultures of *Polypodium vulgare* (Reixach, Lafont *et al*. 1999). According to these results, it was revealed that (1) 25-deoxy-20-hydroxyecdysone was transformed into 20E, (2) 2-deoxyecdysone produced exclusively E and 20E, (3) 25-hydroxycholesterol and 22R-hydroxycholesterol were incorporated into E and 20E. And also, it was demonstrated that the conversion of E into 20E which is the last step in the biosynthesis pathway of the main PE was catalyzed by CYP enzyme. However, the gene encoding this CYP enzyme was not identified yet.

Acetate and mevalonate are also converted into ecdysteroids, and acetate may give C27-, C28- and C29-ecdysteroids (Tomás, Camps *et al*. 1993). It is conceivable that C28- or C29-sterols are the precursors for the corresponding C28- and C29-ecdysteroids (e.g. clerosterol for cyasterone) (Okuzumi, Hara et al. 2003; Dinan, Harmatha et al. 2009)).

The objects of this study were to clone of genes related to putatively important step in PEs biosynthesis such as *DHCR, CYP85, CYP90* and *CYP92* gene, and to modification of cloned genes for expression in *E. coli* and further study (See **Figure 9**).

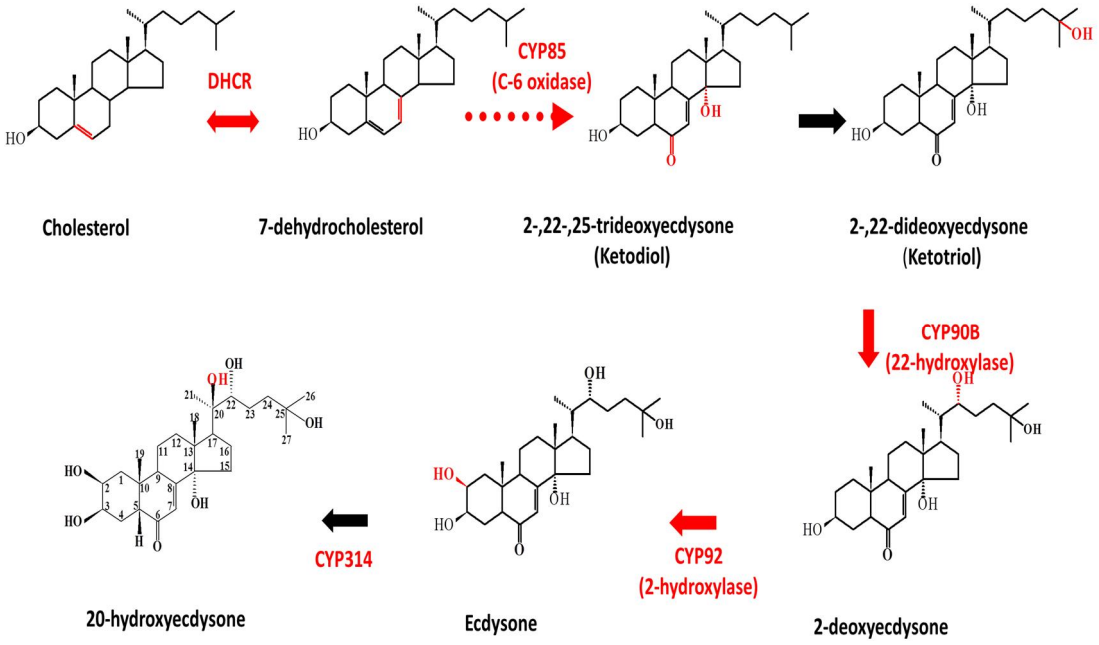


Figure 9. Proposed biosynthesis of PE in plant and candidate genes focused in this thesis.

Materials and methods

Plant materials

Spinach (*Spinacia oleracea* L.) seeds were purchased from Jeju city in Korea. Seeds were sown on damp paper and germinated seedlings were transplanted individually at the cotyledon stage into pots of compost (three seeds per pot). Plant were grown in a greenhouse at 20℃ and under a short (8L:16D) photoperiod at 50% relative humidity (Bakrim, Guittard *et al*. 2009).

Bacterial strains and plasmids

E. coli TOP10 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, Taiwan) vector was used for cloning of PCR product which is for T/A-type cloning.

Isolation of total RNA from plant

Total RNA was extracted using the Easy – BLUE kit (Intron Biotech, Korea) 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 μl of 1× MOPS buffer and 1.25μl 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 (Qiagen, USA) 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 Maxime^{TM} RT PreMix Kit (Intron biotech, Korea) and oligo (dT) primer according to the manufacturer's instruction. RT-PCR amplification was carried out using Ex-Taq^{TM} DNA polymerase (Takara, Japan) using the first strand cDNA as templates. T-personal Thermal Cycler (Biometra, USA) was used for PCR amplification. 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 (http://bioinformatics.weizmann.ac.il/blocks/blockmkr /www/make_blocks.html) *DHCR*, *CYP85*, *CYP90* and *CYP92* genes comparing the conserved amino acid sequences of previously reported genes. The obtained genes were named as *DHCR*, *CYP85*, *CYP90* and *CYP92*. Primers used in this reaction were synthesized in Bionics Company (Seoul, Korea).

The above isolated mRNA was used to synthesis the first strand cDNA. mRNA was reverse transcribed using Maxime^{TM} RT PreMix Kit (Intron biotech, Korea) and oligo (dT) primer according to the manufacturer's instruction. PCR amplification was carried out using Ex-Taq^{TM} 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 4**. 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 TOP10 competent cell according to the manufacturer's instruction.

The positive recombinant vector (confirmed by electrophoresis) was digested by *Hind*Ⅲ. Empty yT & A vector was used as a control. Four μl 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 sent 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 cDNA was generated by 5′ & 3′ RACE technique using Gene-RACE Kit (Invitrogen, USA) to the supplier's instruction. New specific primers for *DHCR*, *CYP85*, *CYP90B* and *CYP92A* 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 (See **Table 5**). The PCR reaction was performed using T-personal Thermal Cycler (Biometra, Germen).

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

Sequence analysis

Then plasmid was extracted for sequencing using DNA-spin ^{TM} Plasmid Purification Kit (Intron biotech, Korea) according to the manufacturer's instruction. The plasmid was recognizing by electrophoresis and restriction enzyme cut (*Hind Ⅲ*).

Sequencing was carried out using M13F or M13R primer (in yT & A Kit) in sequencing center of CNU. Each partial nucleotide and amino acid sequences was analyzed using NCBI

(http://www.ncbi.nlm.nih.gov/BLAST/), ExPASY (http://us.expasy.org/tools), and CAP3 sequence assembly program (http://pbil.univ-lyon.fr/cap3.php). The partial of each gene was used as designing primer for 5′ & 3′ RACE (Rapid Amplification of cDNA Ends)

Codon optimization for recombinant protein expression in *Escherichia coli*

Codon optimizations for recombinant protein expression were made by GenScript USA Inc (http://www.genscript.com/).

Table 4. Degenerate primer sequences for cloning of PE biosynthetic genes in spinach

Target gene	Primer sequence
DHCR	F:5′-GAATTTTGATATTAAGGTTTTTACTAATTGTAGATTYGGNATGATG- 3′
	R:5′-TTTTTCACAATACAGCTTCCAATACTTNCCRTAYTT- 3′
CYP85	F: 5′-GGAACTATGGGATGGCCTATTTTYGGNGARAC- 3′
	R:5′-TCTTCCAGAGCCTTAGGATGATCRTGNARRTA- 3′
CYP90	F: 5′- GGAACATTTGGATGGCCTTTTHTNGGNGARAC - 3′
	R:5′- CCCATTCTCAGAGTTTCATTAATAACAYWYTGNGTRAA - 3′
CYP92	F:5′-CCCCGAGGAGTTCAAGTAGATGBTNGAYGA- 3′
	R:5′-GGGTGGACAGCCCGAAGRYYTCNTCCAT- 3′

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

Table 5. Primer sequences of 5′ and 3′ RACEs PCR for cloning genes in spinach

Target gene	Sequences	Description
DHCR-R1	5′-TATACACTGATGGGACCCACACC-3′	Gene specific primer for DHCR 5′-RACE PCR
DHCR-R2	5′-ACAGTGAGGAATATCACGTAG-3′	Gene specific nested primer for DHCR 5′-RACE PCR
DHCR-F1	5′-GGCAGTTTTGGCCTTGACATAC-3′	Gene specific primer for DHCR 3′-RACE PCR
DHCR-F2	5′-TTGGATGGTAAGGTTGCTGAT-3′	Gene specific nested primer for DHCR 3′-RACE PCR
CYP85-R1	5′-GGTTAGTACCAGGGTACCCACAGTC-3′	Gene specific primer for CYP85 5`-RACE PCR
CYP85-R2	5′-GGGGTGTCCAACAATAGTGTC-3′	Gene specific nested primer for CYP85 5′-RACE PCR
CYP85-F1	5′-AGAAGGGCACCCCTCATGTACT-3′	Gene specific primer for CYP85 3′-RACE PCR
CYP85-F2	5′-GTTGGGCAGTTGAGCTTGATT-3′	Gene specific nested primer for CYP85 3`-RACE PCR
CYP90B-R1	5′-ATGCAATGGAAACAGAGGAAGTCTCATG-3′	Gene specific primer for CYP90B 5′-RACE PCR
CYP90B-R2	5′-AGTCTCATGTCCAGCAAACAACAAACTC-3′	Gene specific nested primer for CYP90B 5′-RACE PCR
CYP90B-F1	5′-GTAGTATCAGCTCCTATCAATTTACCAG-3′	Gene specific primer for CYP90B 3′-RACE PCR
CYP90B-F2	5′-ACCAGGAACTCCTTATAGAAGGGCATT-3′	Gene specific nested primer for CYP90B 3`-RACE PCR
CYP92A-R1	5′-ATGAGCCCACTAACACGGGTACT-3′	Gene specific primer for CYP92A 5′-RACE PCR
CYP92A-R2	5′-CACTAACACGGGTACTGACCCATA-3′	Gene specific nested primer for CYP92A 5′-RACE PCR
CYP92A-F1	5′-CACCACTCAATCGACAAGCTCTC-3′	Gene specific primer for CYP92A 3′-RACE PCR
CYP92A-F2	5′-AGTACCCGTGTTAGTGGGCTCAT-3′	Gene specific nested primer for CYP92A 3′-RACE PCR
5' Primer	5′-CGACTGGAGCACGAGGACACTGA-3′	GeneRAGE 5′ Primer
5' nested Primer	5′-GGACACTGACATGGACTGAAGGAGTA-3′	GeneRAGE 5′ nested Primer
3' Primer	5′-GCTGTCAACGATACGCTACGTAACG-3′	GeneRAGE 3′ Primer
3' nested Primer	5′-CGCTACGTAACGGCATGACAGTG-3′	GeneRAGE 3′ nested Primer

Results

Cloning of *DHCR* candidate gene

The clone of *DHCR* was obtained by degenerate RT-PCR and RACE overlapping the 5' and 3' end fragments. The cloning of *DHCR* was confirmed by PCR and then the plasmid was extracted for sequencing. Nucleotide sequence analysis revealed that full length of *DHCR* is 1657 bp nucleotide acids. The coding region (ORF) of this cDNA was 1302 bp nucleotides which encoded a deduced protein of 434 amino acids.

The determined 1657 bp nucleotide sequence corresponding to deduced amino acid of 434 amino acids was subjected to a search against known sequences in databases using the blastp search in NCBI database. The sequence of *DHCR* in spinach showed high homologous with known *DHCR* gene in various plant species. The deduced amino acid sequence of *DHCR* was compared with that in other plants *DHCR* genes Using ClustalW and GeneDoc. The result showed high identities with those of *Arabidopsis thaliana* (86%), *Tropaeolum majus* (85%), *Gossypium hirsutum* (85%) , *Ricinus communis* (86%) and *Achyranthes japonica* (94%) (Zhou 2009). (See **Figure 10**)

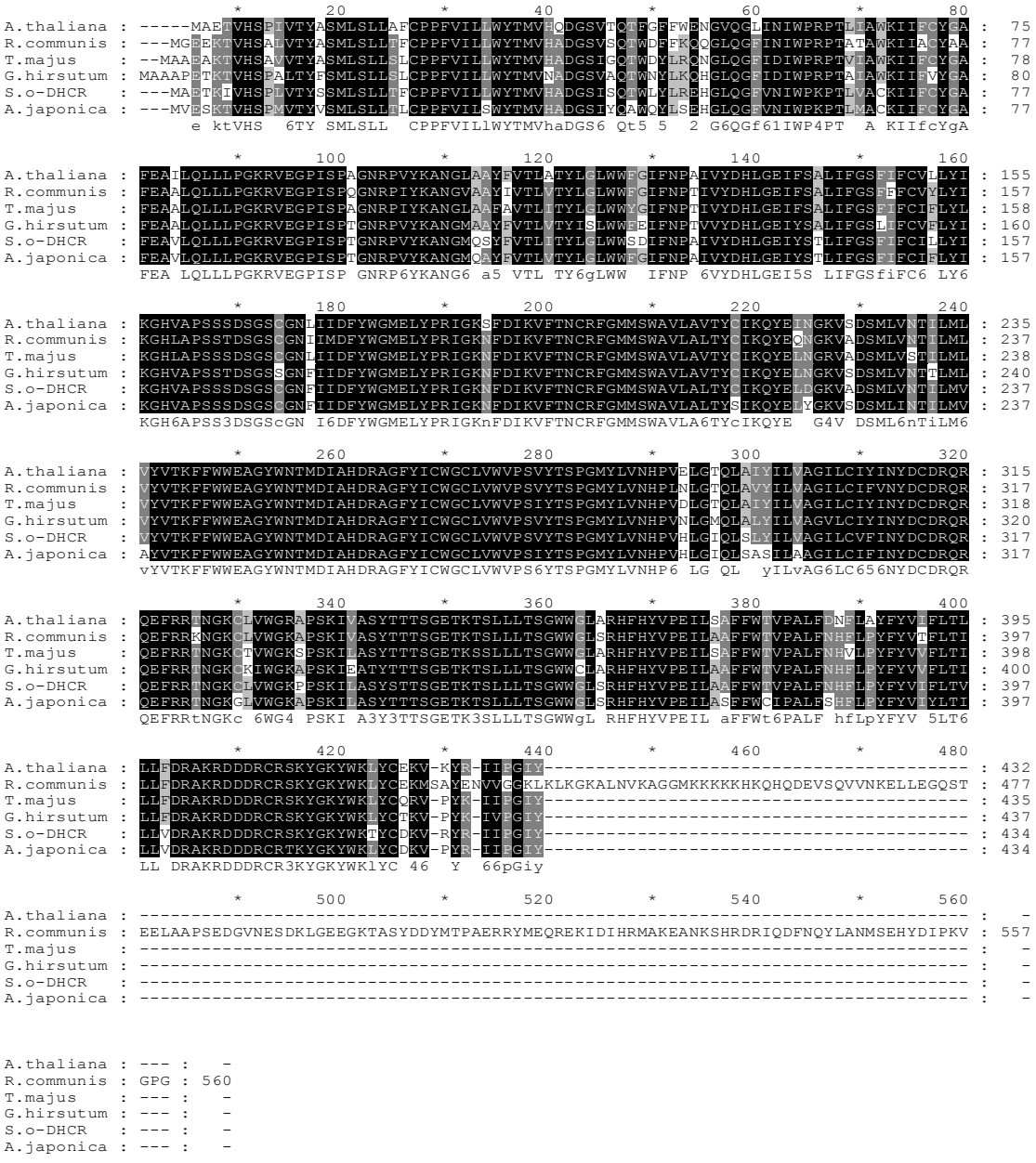


Figure 10. Alignment of deduced amino acid sequences of *DHCR* isolated from *S. oleracea* with other plant in the NCBI database. The deduced amino acid of *DHCR* from *Arabidopsis thaliana* (NP_175460.1), *Ricinus communis* (EEF48723.1) **,** *Tropaeolum majus* (AAR29980.1), *Gossypium hirsutum* (ABA01480.1) and *Achyranthes japonica* are shown. Numbers indicate the position of amino acid residues. Conserved residues are highlighted (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.

Cloning of *CYP85* candidate gene

The clone of *CYP85* was obtained by degenerate RT-PCR and RACE overlapping the 5' and 3' end sequences. The cloning of *CYP85* was confirmed by PCR and then the plasmid was extracted for sequencing. Nucleotide sequence analysis revealed that full length of *S.o-CYP85* is 1669 bp in nucleotide sequence. The coding region (ORF) of this cDNA was 1392 bp nucleotides which encoded a deduced protein of 464 amino acids.

The determined 1669 bp nucleotide sequence corresponding to deduced amino acid of 475 amino acids was subjected to a search against all known sequences in databases using the blastn and blastx search in NCBI database. Both searches showed highly homologous with known *CYP85* gene sequence in various plant species. The deduced amino acid sequence of *CYP85* was compared to other plant's *CYP85* genes Using ClustalW and GeneDoc. The result showed high sequence identities with those of *Achyranthes japonica* (92%), *Nicotiana tabacum* (78%), *Solanum lycopersicum* (76%), *Vitis vinifera* (79%), *Phaseolus vulgaris* (77%), and *Arabidopsis thaliana* (67%) (Zhou 2009). (See

Figure 11)

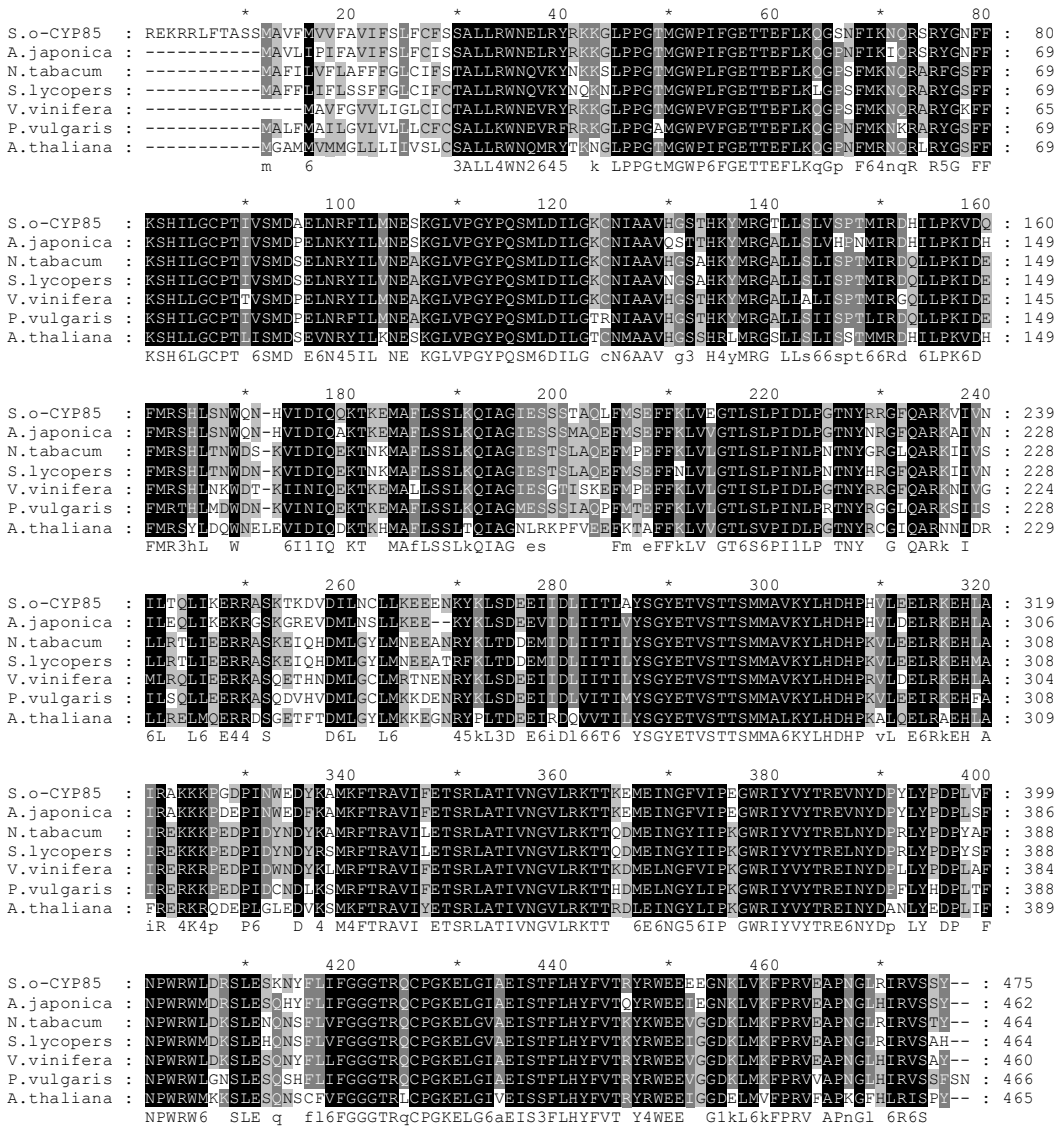


Figure 11. Alignment of deduced amino acid sequences of *CYP85* isolated from *S. oleracea* with other plant in the NCBI database. The deduced amino acid of CYP85A1 from *Achyranthes japonica*, *Nicotiana tabacum* (ABG36709.1), *Solanum lycopersicum* (Q43147.1), *Vitis vinifera* (ABB60086.1), *Phaseolus vulgaris* (Q69F95.2), and *Arabidopsis thaliana* (Q9FMA5.1) are shown. Numbers indicate the position of amino acid residues. Conserved residues are highlighted (in black when present in all sequences). The alignment was created with GeneDoc program. Gaps in the sequences are indicated by dashes were produced by alignment program ClustalW and GeneDoc program automatically. Numbers of nucleotides are indicated on the right margin.

Cloning of *CYP90B* candidate gene

The clone of *CYP90B* was obtained by degenerate RT-PCR and RACE overlapping the 5' and 3' end fragments. The cloning of *CYP90B* was confirmed by PCR and then the plasmid was extracted for sequencing. Nucleotide sequence analysis revealed that full length of *CYP90B* is 2270 bp nucleotide acids. The coding region (ORF) of this cDNA was 1500 bp nucleotides which encoded a deduced protein of 500 amino acids.

The determined 2270 bp nucleotide sequence corresponding to deduced amino acid of 500 amino acids was subjected to a search against known sequences in databases using the blastp search in NCBI database. The sequence of *CYP90B* in spinach showed high homologous with known *CYP90B* gene in various plant species. The deduced amino acid sequence of *CYP90B* was compared with that in other plants *CYP90B* genes Using ClustalW and GeneDoc. The result showed high identities with those of *Pisum sativu*, *Arabidopsis thaliana, Zinnia elegans* and *Zea may* and homologies were 64~70%. (See **Figure 12**)

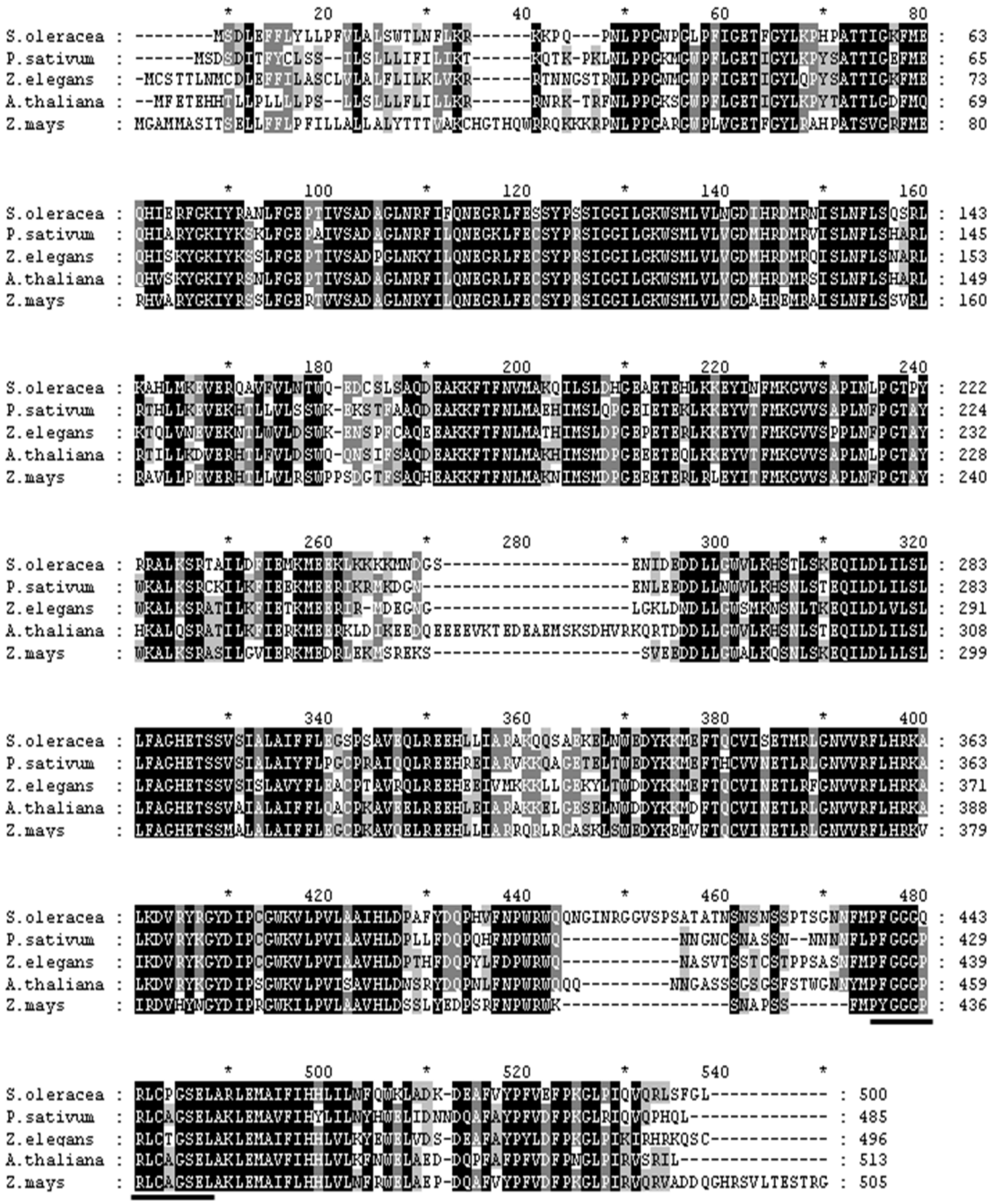


Figure 12. Alignment of deduced amino acid sequences of *CYP90B* isolated from *S. oleracea* with other plant in the NCBI database. The deduced amino acid of *CYP90B* from *Pisum sativu*, *Arabidopsis thaliana Zinnia elegans* and *Zea may* are shown. Numbers indicate the position of amino acid residues. Conserved residues are highlighted (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.

Cloning of *CYP92A* candidate gene

The clone of *CYP92A* was obtained by degenerate RT-PCR and RACE overlapping the 5' and 3' end fragments. The cloning of *CYP92A* was confirmed by PCR and then the plasmid was extracted for sequencing. Nucleotide sequence analysis revealed that full length of *CYP92A* is 1809 bp nucleotide acids. The coding region (ORF) of this cDNA was 1563 bp nucleotides which encoded a deduced protein of 521 amino acids.

The determined 1809 bp nucleotide sequence corresponding to deduced amino acid of 520 amino acids was subjected to a search against known sequences in databases using the blastp search in NCBI database. The sequence of *CYP92A* in spinach showed high homologous with known *CYP92A* gene in various plant species. The deduced amino acid sequence of *CYP92A* was compared with that in other plants *CYP92A* genes Using ClustalW and GeneDoc. The result showed high identities with those of *Gossypium hirsutum* (73%), *Medicago truncatula* (71%) and *Pisum sativum* (71%) and homologies were 71~73%. (See **Figure 13**)

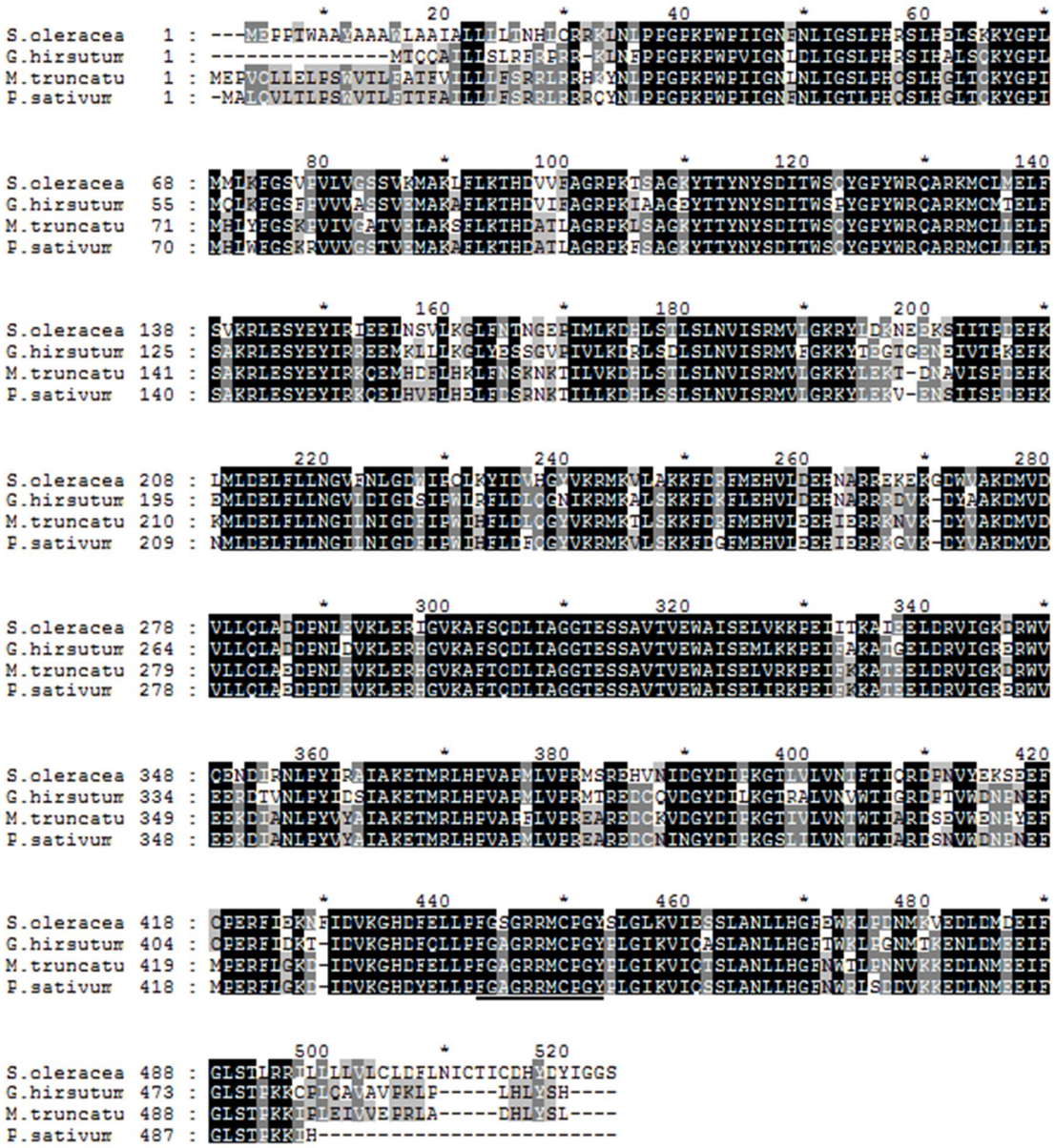


Figure 13. Alignment of deduced amino acid sequences of *CYP92A* isolated from *S. oleracea* with other plant in the NCBI database. The deduced amino acid of *CYP92A* from *Gossypium hirsutum*, *Medicago truncatula*, *Pisum sativum* are shown. Numbers indicate the position of amino acid residues. Conserved residues are highlighted (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.

Optimized sequence of genes

In my study, *CYP85, CYP90, CYP92* and *CYP314* (DNA sequence come from insect information) four genes were optimized for expression in *E. coli*. First, for gene expression, the amino acid sequence were slight modified by adding 7 amino acid residue MAKKTSS in N-terminal to increase expression. In addition, The $[His]_5$ residues (usually $[His]_4$, $[His]_5$, $[His]_6$) which can chelate Ni^{2+} and similar metal ions; thus Ni^{2+}-chelate affinity column can be used for rapid purification were added in C-terminal of the amino acid of *CYP85, CYP90, CYP92* and *CYP314*, respectively.

Because a wide variety of factors regulate and influence gene expression levels, so using OptimumGene^{TM} algorithm takes into consideration as many of them as possible, producing the single gene that can reach the highest possible level of expression. In this case, the native gene employs tandem rare codons that can reduce the efficiency of translation or even disengage the translational machinery. We changed the codon usage bias in *E. coli* by upgrading the CAI (Codon adaptation Index) from low to high. GC content and unfavorable peaks have been optimized to prolong the half-life of the mRNA. The Stem-Loop structures, which impact ribosomal binding and stability of mRNA, were broken. In addition, the optimization process has screened and successfully modified those negative cis-acting sites as listed in the introduction. (See **Figure 14**, **Figure 15**, **Figure 16** and **Figure 17**)

```
Optimized 4 ATGGCTAAAAAAACCTCATCAACGAATCATCTGTGTCGCCGCAAACTGAATCTGCCGCCG
 Original 4 ATGGCTAAGAAAACGTCTTCCACCAACCACCTCTGCCGCCGTAAACTCAACCTACCACCA

Optimized 64 GGTCCGAAACCGTGGCCGATCATTGGTAACTTCAATCTGATTGGCAGTCTGCCGCATCGT
 Original 64 GGGCCAAAACCATGGCCCATCATTGGGAACTTCAATCTTATTGGGTCGCTCCCCCACCGC

Optimized 124 TCCCTGCACGAACTGAGCAAAAAATATGGTCCGCTGATGATGCTGAAATTTGGCTCCGTC
 Original 124 TCTCTCCATGAACTCTCTAAGAAATATGGGCCTCTAATGATGCTCAAATTCGGGTCAGTC

Optimized 184 CCGGTGCTGGTTGGTAGCTCTGTGAAAATGGCCAAACTGTTCCTGAAAACCCATGACGTG
 Original 184 CCTGTATTAGTGGGCTCATCAGTGAAAATGGCAAAACTTTTCCTTAAAACTCACGATGTA
```

Figure 14. The demonstration of optimization for target gene sequence. Original: the part original gene sequence; Optimized: the part optimized gene sequence; the red color condons were changed according to codon usage bias and GC content.

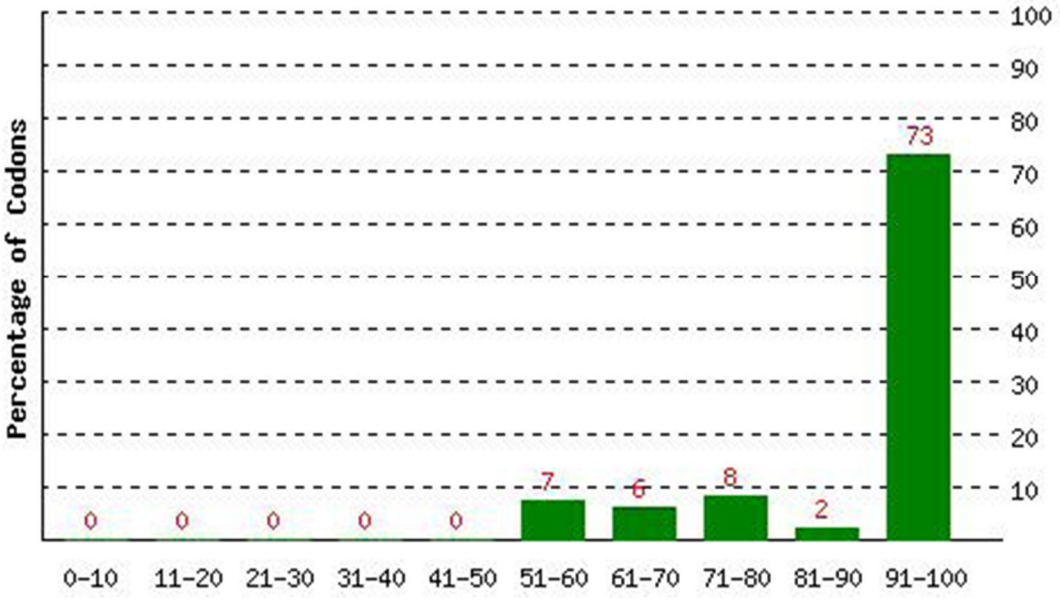


Figure 15. The percentage distribution of codons in computed codon quality groups after codon optimization of CYP85. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism.

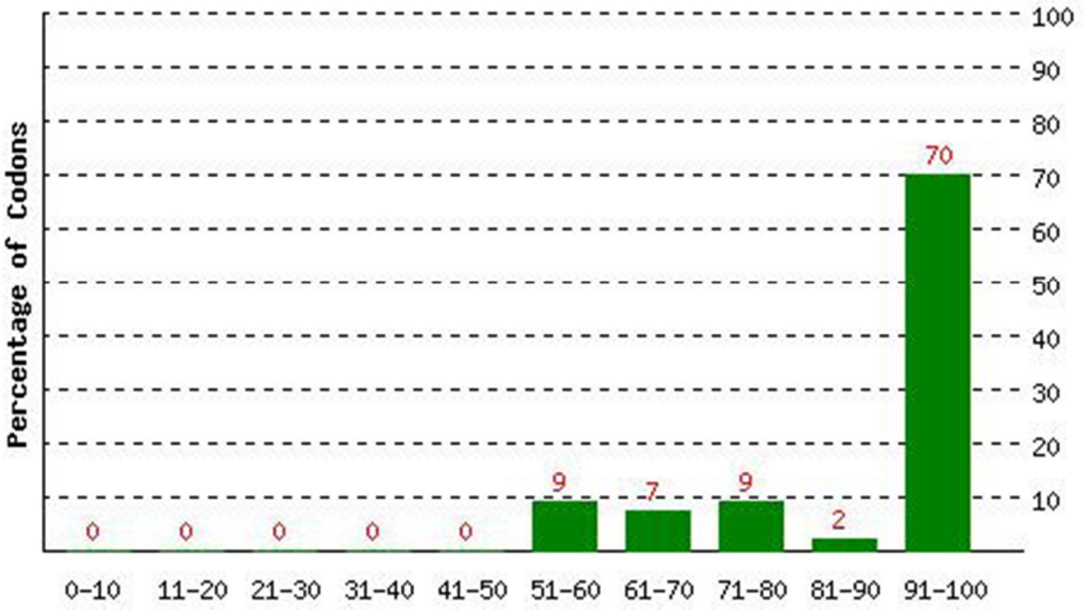


Figure 16. The percentage distribution of codons in computed codon quality groups after codon optimization of CYP90B. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism.

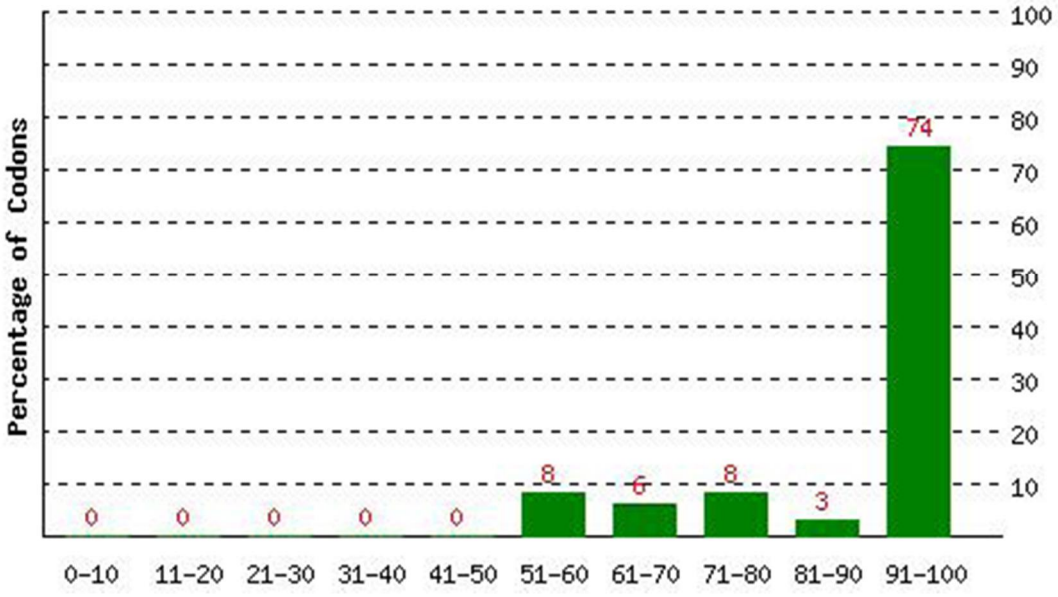


Figure 17. The percentage distribution of codons in computed codon quality groups after codon optimization of CYP92A. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism.

Discussion

The biosynthesis pathway of ZEs was well characterized in insect. The ingested CHR is known as a precursor in insect and converted into 7-dehydrocholesterol. The conversion of 7-dehydrocholesterol to the Δ4-diketol are commonly retained as the black box, and this is converted first into the diketol and then into E (Ono, Rewitz *et al*. 2006). The final four steps are to convert the ketodiol derived diketol into 2-, 22-dideoxyecdysone, 2-deoxyecdysone, E and 20E, sequentially. The genes related to the final four steps have been identified in *Drosophila*. These four genes encoding ecdysteroids hydroxylase are CYP family (Warren, Petryk et al. 2002; Petryk, Warren et al. 2003; Warren, Petryk et al. 2004). But Until now, biosynthesis pathway of PE is not clear. Based on metabolomic studies, putative pathway from phytosterol to 20E is like **Figure 9**. In this study, four candidate genes of *DHCR, CYP85, CYP90B* and *CYP92* were cloned, and four candidate genes of *CYP85*, *CYP90B, CYP92* and *CYP314* were modified for recombinant protein expression in *E. coli.*

Characteristics of the putative *DHCR* gene

Previous papers have revealed that cholesterol is a direct precursor of 7-dehydrocholesterol in the early steps of PEs biosynthesis (Ohyama, Kushiro *et al*. 1999; Fujimoto, Ohyama *et al*. 2000). 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 synthase. 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* as PE-positive plant. The high sequence similarity in ORF between DHCRs in different species of PE-positive plant suggests that they probably take part in the same catalytic process of the C7-C8 double bond formation in PEs biosynthesis.

Characteristics of the putative *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. It has been studied that CYPs take part in the last 4 steps of 20E biosynthesis in Drosophila. 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 family, containing *CYP85, CYP90* and *CYP92* families (Nomura and Bishop 2006).

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 *CYP85A1,A2* subfamily (Nomura, Kushiro *et al*. 2005). Similar, the production typhasterol, 3-dehydroteasterone and teasterone was converted from 6-deoxotyphasterol, 3-dehydro-6-deoxoteasterone, and 6-deoxoteasterone via *CYP85A1, A2*, respectively.

The structure of PE 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 PE. The study of *CYP85* is benefit to understand the biosynthesis of PE in plant. In this study, a full length of gene *CYP85* was cloned in spinach. The deduce amino acid showed 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 *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 CYP85 gene maybe function as a C-6 oxidation in the PE biosynthesis process in spinach.

Characteristics of the putative *CYP90B* gene

The Arabidopsis *dwarf4* (dwf4) mutant displays a dwarf phenotype with dark-green, small and rounded leaves, and endogenous levels of the downstream compounds after the C-22 hydroxylation step are extremely reduced relative to the wild-type (Choe, Dilkes *et al*. 1998; Choe, Fujioka *et al*. 2001). Genetic analysis revealed that the DWF4 gene encodes *CYP90B1*, which shares 43% homology with *CYP90A1*. *CYP90B1* could catalyse the C-22 hydroxylation step in BR biosynthesis. Because the structure of PE is similar to brassinolide, therefore, *CYP90B* was hypothesized to catalyse the C-22 hydroxylation step in the biosynthesis of PE.

The study of *CYP90B* is benefit to understand the biosynthesis of PE in plant. In this study, a full length of gene *CYP90B* was cloned in spinach. The deduce amino acid showed 64~70% identity with that in *Pisum sativu*, *Arabidopsis thaliana*, *Zinnia elegans* and *Zea may*. And also, a heme-binding site with FxxGxRxCxG (Werck-Reichhart, Bak *et al*. 2002) was contained in the amino acid sequence encoded by spinach *CYP90B*. Therefore, this gene cloned in spinach was identified belong to *CYP90B* subfamily in CYP450. And the molecular weight of amino acid encoded by this gene was about 56.9 kDa. Because the high identity on amino acid with *Zea may* as PE-positive plant so they probably take part in the same catalytic process of the C-22 hydroxylation in PEs biosynthesis.

Characteristics of the putative *CYP92A* gene

Kang *et al*. (2001) have detected steroid C-2 hydroxylase activity of DDWF1

(dark-inducedDWF-like protein 1), a pea P450 designated *CYP92A6* (Kang, Yun *et al*. 2001). Because the structure of PE is similar to brassinolide, therefore, *CYP92A* was hypothesized to catalyse the C-2 hydroxylation step in the biosynthesis of PE.

The study of *CYP92A* is benefit to understand the biosynthesis of PE in plant. In this study, a full length of gene *CYP92A* was cloned in spinach. The deduce amino acid showed 71~73% identity with that in *Gossypium hirsutum* (73%), *Medicago truncatula* (71%) and *Pisum sativum* (71%). And also, a heme-binding site with FxxGxRxCxG (Werck-Reichhart, Bak *et al*. 2002) was contained in the amino acid sequence encoded by spinach *CYP92A*. Therefore, this gene cloned in spinach was identified belong to *CYP92A* subfamily in CYP450. And the molecular weight of amino acid encoded by this gene was about 60 kDa.

Consequently, Most of the previous works in PE biosynthesis have been investigated by metabolomic approach. However there is limited knowledge in genomic approach. In this work a genomic approach was used to identify genes which are essential for molecular biological analysis of PE biosynthesis in plants.

Because on the above results of part Ⅰ, the PE extract from *S. oleracea* had very marked biological activity against herbivores insect, so in this part, I chose the spinach for coning of genes related to PE biosynthesis.

Four genes related to PE biosynthesis, *DHCR, CYP85, CYP90B* and *CYP92A* were isolated from *S. oleracea*. All of these genes were homologous to other plant genes identified with same function and shared more than 60% identities at the amino acid level.

Because high-level expression of plant P450s in bacteria is difficult to achieve without

modifications of the amino-terminal region of the enzyme, so for enhance over expression in *E. coli*, I optimized and synthesized Four CYP genes, *CYP85, CYP90B, CYP92A* and *CYP314* thought adding the MAKKTSS residues in the N-terminal. At the same time I attached the $[His]_5$ residues to C-terminus for Ni^{2+}-chelate affinity column to rapid purification.

In conclusion of part Ⅱ, total of four candidate genes were isolated from spinach. And then, its codon were optimized and synthesized for over expression in *E. coli* or plant.

PART III Construction of Biological System for Functional Analysis of Candidate Gene Related to Phytoecdysteroids Biosynthesis

Introduction

Cytochrome P450 expression systems

Catalytically active Cytochrome P450 enzymes have been successfully expressed in bacterial, yeast, and mammalian cells. A variety of expression vectors have been used, resulting in both transient and stable expression. The system of choice depends on the goals of a particular project. Factors such as expense, ease of use, and yields required should govern the decision whether to use bacterial, yeast, insect or mammalian cDNA expression. High-level expression of mammalian P450s in bacteria usually requires modifications of the amino-terminal region of the enzyme. The *E. coli* P450-OR fusion proteins may also come of age for use in fermentation-production processes for the chemical industry. Many cytochromes P450 have been expressed in yeast, with variable levels of expression. Baculovirus, albeit somewhat tedious in having to individualize expression conditions, can produce high levels of enzyme (Gonzalez and Korzekwa 1995).

Cytochrome P450s are membrane proteins. Determination of a 3D structure is an important tool for understanding a protein function and mechanism of action. There are four main methods for determining 3D structure: X-ray diffraction, nuclear magnetic resonance

(NMR), atomic force microscopy (AFM) and electron diffraction(Leviatan, Sawada *et al*. 2010). X-ray diffraction, based on protein crystallization, is currently the most widely used method (Caffrey 2003). However, only a small number of high-resolution 3D structures of membrane proteins have been solved, most of them from bacteria. One reason for this dearth of information is that it is difficult to obtain a sufficient amount of the protein of interest for crystallography, since membrane proteins are usually present at minute levels in natural biological membranes (Tate 2001). Hence, structural studies of membrane proteins usually require overexpression of the membrane protein.

Over the last decade, methods have been developed to enhance overexpression in *E. coli*. High-level expression of mammalian P450s in bacteria is difficult to achieve without modifications of the amino-terminal region of the enzyme. A native rabbit CYP2E1 was produced in a catalytically active form. Levels of expression were reported to be 0.3% of total *E. coli* protein (Gonzalez and Korzekwa 1995). The cholesterol 7α-hydroxylase CYP7A1 was expressed in *E. coli* after slight modification of the amino terminal sequence. When amino acids 2 through 24 of CYP7A1 were deleted, expression increased 10-fold, and the enzyme was preferentially found in the soluble portion of the cellular extract, in contrast to the full-length P450, which was found in the membrane fraction. A modified form of bovine CYP17A1 was produced in *E. coli*. In this case, the second was changed to a GCT-encoding alanine, and codons 4 and 5 were modified to TTA in order to make the 5′ end of the mRNA AT rich, similar to other *E. coli* mRNAs. The last nucleotides of codons 6 and 7 were also changed to A and T, respectively, to minimize the possibility of secondary

structure. In the absence of these modifications, apoprotein was not found in the cellular extracts. The modified version was produced as holoenzyme and could be reconstituted with OR to generate the proper catalytic activities. Levels of expression were up to 16mg P450 per liter of culture (Barnes, Arlotto *et al*. 1991).

Attachment of an oligo-His region, usually at the N- or C-terminus, has been used to facilitate protein purification (Porath 1992). The free His residues (usually $[His]_4$, $[His]_5$, $[His]_6$) can chelate Ni^{2+} and similar metal ions; thus Ni^{2+}-chelate affinity column can be used for rapid purification (Porath, Carlsson *et al*. 1975). Such approaches have been used with P450s, with His tags at either the C- or N-terminus (Imai, Globerman *et al*. 1993; Jenkins and Waterman 1994; Kempf, Zanger *et al*. 1995; Hanna, Dawling *et al*. 2000). Detergent is needed to solubilize the membranes and keep the proteins disaggregated during chromatography, and the detergent must be removed in a subsequent step.

Non-A-type P450s mediating steroid biosynthesis

Like vertebrates and fungi, plants produce polyhydroxylated steroidal hormones to regulate and control tissue morphology. In plants these types of hormones are designated brassinosteroids and they are built on a campestanol carbon skeleton. The brassinosteroids are nonessential phytohormones with impact on morphological characteristics, for example, leaf shape and dwarfism. Biological functions of brassinosteroids are controlled by specific receptors and suppressors. These mediate signal transduction and control regulation of target genes including those for brassinosteroid biosynthesis. Brassinosteroids may potentiate plant

fitness and defense in response to pathogen attack, since brassinosteroids induce systemic defense responses in tobacco and rice (De Montellano 2005).

The biosynthetic pathway for brassinosteroids has not yet been fully elucidated. Models as presented for two parallel pathways assigned as “the early C-6-oxidation” and “the late C-6-oxidation” pathways have been suggested. Non-A-type plant P450s participate in the production of the plant sterols that are brassinosteroids precursors. A key enzyme is obtusifoliol 14a-demethylase. This belongs to the CYP51 family and also assigned as BAS1 belonging to the A-type family and encoding a brassinolide 26-hydroxylase, has been shown to regulate light perception and control accumulation of brassinosteroids.. But CYP90 and CYP85, participating in brassinosteroids biosynthesis are non-A-type P450 families (De Montellano 2005).

Metabolite analysis of PE

From plant sample to pure compounds, the purification strategy always comprises a multi-step procedure including extraction, prepurification and the one or several chromatographic steps. After the fresh or dry sample is cut up or ground to a powder form, then to do that (1) extraction with organic solvents (perhaps in a sequence of increasing polarity); (2) solvent partitions to remove less polar and /or more polar compounds; (3) initial chromatographic steps (flash chromatography, counter-current chromatography or low-pressure column chromatography on silica or alumina and (4) final purification by TLC and /or HPLC.

Extraction can be performed using a large range of solvents. For polar steroids it is possible to first extract with apolar solvents, which will extract non-polar compounds, and then with a more polar one. After concentration, a second step usually involves a partition between two non-miscible solvents. The purpose of such a step is that it can be used whatever the sample size and it can be very efficient in removing both more polar and less polar contaminants, if two complementary partition steps are being used (Ghosh and Laddha 2006).

The uniformity of PEs was controlled by various analytical procedures that included TLC and HPLC. Purity control was accomplished using both NP-TLC and RP-TLC. Both separation and detection methods were used to determine the PEs content of the plant extracts. Chromatographic and related separation methods served to differentiate the PEs from each other and also from the other compounds. The most popular method for PEs analysis was HPLC combined with UV detection at 254 nm. The detectable signal was the consequence of 7-en-6-one conjugation. As the maximum of UV absorbance was really at 240 through 245 nm, the specificity could be slightly increased to performed detection somewhere at that wavelength.

Purpose of this study

The objects of this study were to optimize recombinant protein expression in *E. coli*, and to optimize gene transformation into plant.

Materials and methods

Recombinant Protein Expression of PEs Related CYP450 in *Escherichia coli*

Bacterial strains. The *E. coli* strains, C41 (DE3)-pLysS and DH5α-pGro12 were used in this study.

Transformation of pCW vector with target gene to *E. coli.* The $CaCl_2$-treated cells were taken out from at -70℃, and were thawed in ice. After 5μl ligation mix was added to the cells, the tube were swirled and placed on ice for 30min. And then the cells were heated shock by placing tubes for 45sec in 42℃ water bath. Immediately the tube was placed on ice for 5 min. After that, 1ml LB medium was added to tube and was incubated for 1 hr at 37℃. The cells were spined down for 5min at 13000rpm. After discard most of the media, The cells were resuspended and smeared to the medium plate (LB medium with ampicillin and chloramphenicol), then were incubated 12 to 16hr at 37℃ or overnight. A single colony was picked out and transferred into single 5 ml of LB liquid medium with 100μg/ml ampicillin. And the stock cells were stored at -70℃.

Induction culture. For C41(DE3)pLysS cell, the tube of stock cells with target gene was take out from at -70℃ and the cells were thawed in ice. Each triangular flask with LB media (*100ml, with Ampicillin 50 μg/ml and chloramphenicol 30 μg/ml)* was added in 100μl cell. And the culture was inoculated with shaking about 180rpm at 37℃ overnight. Each 800ml TB medium with δ-ALA 0.5mM, $MgCl_2$ 1mM, Ampicillin 130 μg/ml was added 40ml transgenic cell. The culture was inoculated with shaking 180rpm, at 37℃, to an OD_{600}

of 0.6~0.8. after adding IPTG 0.5mM, the culture was incubated for 3 days at 27-28℃, 120rpm.

For DH5α-pGro12 cell, the tube of stock cells with target gene was take out from at -70℃ and the cells were thawed in ice. Each triangular flask with LB media (100ml, with ampicillin 50μg/ml and kanamycin 30μg/ml) was added in 100μl cell. And the culture was inoculated with shaking about 180rpm at 37℃ overnight. Each 800ml TB medium with δ-ALA 0.5mM, $MgCl_2$ 1mM, Ampicillin 130 μg/ml was added 40ml transgenic cell. The culture was inoculated with shaking 180rpm, at 37℃, to an OD_{600} of 0.6~0.8. After adding IPTG 0.5mM, the culture was incubated for 3 days at 27-28℃, 120rpm.

Protein extraction. After 3 days, the cells were harvested from 1600ml liquid culture by centrifugation at 5,000 rpm for 12 min using a 250ml Centrifuge bottles. The cell pellet was suspended in 60 ml Ni-NTA washing buffer (lysis buffer). And was incubated with gentle stirring on magnetic stir (1~2hours, 4℃). And then, After the cells were sonicated (10 sec pulse, 55 sec resting, and 6 min), the little DNase, 1.2% O.G (octyl-β-D-glucopyranoside), 30μl Mercaptoethanol and 0.5% Sodium cholate were added. The cells incubated with gentle stirring on magnetic stir (Overnight, 4℃). The protein was obtained through centrifuging (15,000 rpm for 1 hour, 4℃).

Ni^{2+}-NTA agarose affinity column chromatography. Ni^{2+}-NTA resin was used to purify the target expression protein. First, the column was washed with 50ml Lysis buffer. Then the protein was applied to the Ni-NTA column. Through washing the column with 50ml O.G 0.5% buffer, 50ml High salt buffer, 50ml 20mM Imidazole buffer, 70ml 30mM

Imidazole buffer and 150ml 50mM Imidazole buffer, the target protein was obtained by adding 50ml elution buffer.

Protein separation. The target recombinant expression protein fraction was selected by UV spectrophotometer (UV1800 Shimadzu, UV probe version 2.34, JAPAN) and SDS electrophoresis. The best fraction were combined and centrifuged at 5,500 rpm for 40min using Millipore tube. Further, the fraction was purified by AKTA prime plus (Hiprep 16/60 Sephacryl S-300 HR).

CYP450-CO complex assay

Briefly in this method, the isolated CYP450 protein was placed in the CO chamber, that CO gas was allowed to flow into the chamber. After 20 second in incubated condition, the sample was measured in absorbance from 400nm to 480nm by UV spectrophotometer (UV1800 Shimadzu, UV probe version 2.34, JAPAN).

Table 6. The buffers for Ni^{2+}-NTA agarose affinity column chromatography

Buffer	Reagent	Stocks	Final Concentration
Lysis buffer (pH 7.5)	*Tris-HCl pH 7.5*		*0.1M*
	NaCl		*0.2M*
	Glycerol		*20%*
O.G 0.5% buffer	*Tris-HCl (pH 7.5)*		*0.1M*
	O.G(octyl- β-D-glucopyranoside)		*0.5%*
	Glycerol		*20%*
	NaCl		*0.2M*
High salt buffer (pH 7.5)	*Tris-HCl pH 7.5*		*0.1M*
	NaCl		*1M*
	Glycerol		*20%*
20mM Imidazole buffer(pH7.5)	*Imidazole*	*1M*	*20mM*
	Tris-HCl pH 7.5		*0.1M*
	NaCl		*0.2M*
	Glycerol		*20%*
30mM Imidazole buffer(pH7.5)	*Imidazole*	*1M*	*30mM*
	Tris-HCl pH 7.5		*0.1M*
	NaCl		*0.2M*
	Glycerol		*20%*
50mM Imidazole buffer(pH7.5)	*Imidazole*	*1M*	*50mM*
	Tris-HCl pH 7.5		*0.1M*
	NaCl		*0.2M*
	Glycerol		*20%*
Elution buffer	*Imidazole*	*1M*	*200mM*
	Tris-HCl pH 7.5		*0.1M*
	NaCl		*0.2M*
	Glycerol		*20%*
	O.G(octyl- β-D-glucopyranoside)		*0.5%*

Plant materials

The tobacco (*Nicotiana tobacum* cv. NC) was used in this study. The seeds were sterilized by immersion in 2% sodium hypochlorite solution for 10 min and were rinsed three times with sterile distilled water. The sterilized seeds were dried on sterile filter paper for 5 min. The seeds were then placed on petri dishes containing hormone-free MS medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose and solidified with 8 g/L plant agar. Each culture plate was incubated at 25℃ under a 16/8 hr (day/night) photoperiod.

Spinach (*Spinacia oleracea* L.) seeds (Korean cultivar Kyowoonae) were purchased from Nongwoo Bio Co., LTD.

Arabidopsis (*Arabidopsis thaliana,* Columbia ecotypes) seeds were treated at 4℃ for 3days and grown in a temperature controlled chamber at 22-24℃ under a 16/8 hr (day/night) photoperiod.

***Agrobacterium* strain**

The *Agrobacterium tumefaciens* strain LBA4404 and EHA105 were used. *Agrobacterium* was cultured on YEP medium (AN 1987).

Construction of expression vector in plant

Gateway™ LR reaction kit (Invitrogen, USA) was used for construction of expression vector in plant. pB7WG2D,1 vector were used for over-expression of target gene. And pK7GWIWG2D(II),0 vector was used for knock-down of target gene (See **Figure 18**).

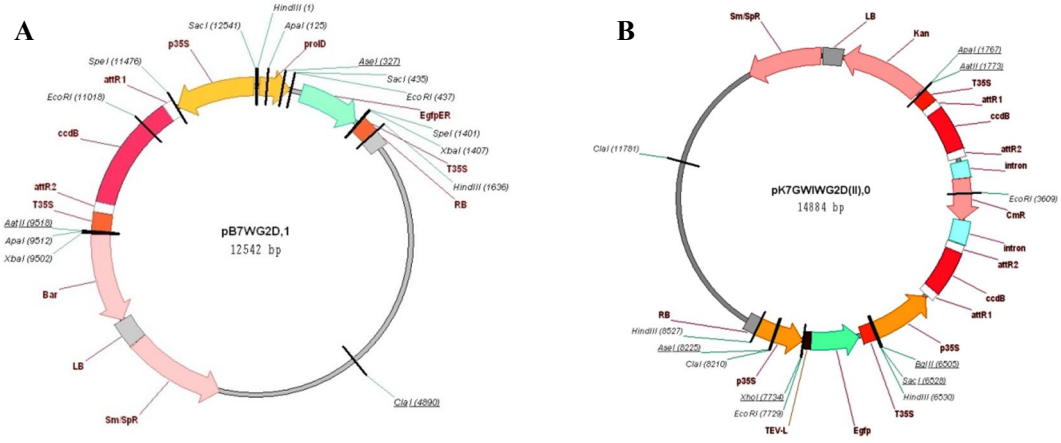


Figure 18. Gateway binary vectors were used for over-expression and knock-down in plant. A: pB7WG2D,1; B: pK7GWIWG2D(II),0

Construction of recombinant protein expression vector in *Escherichia coli*

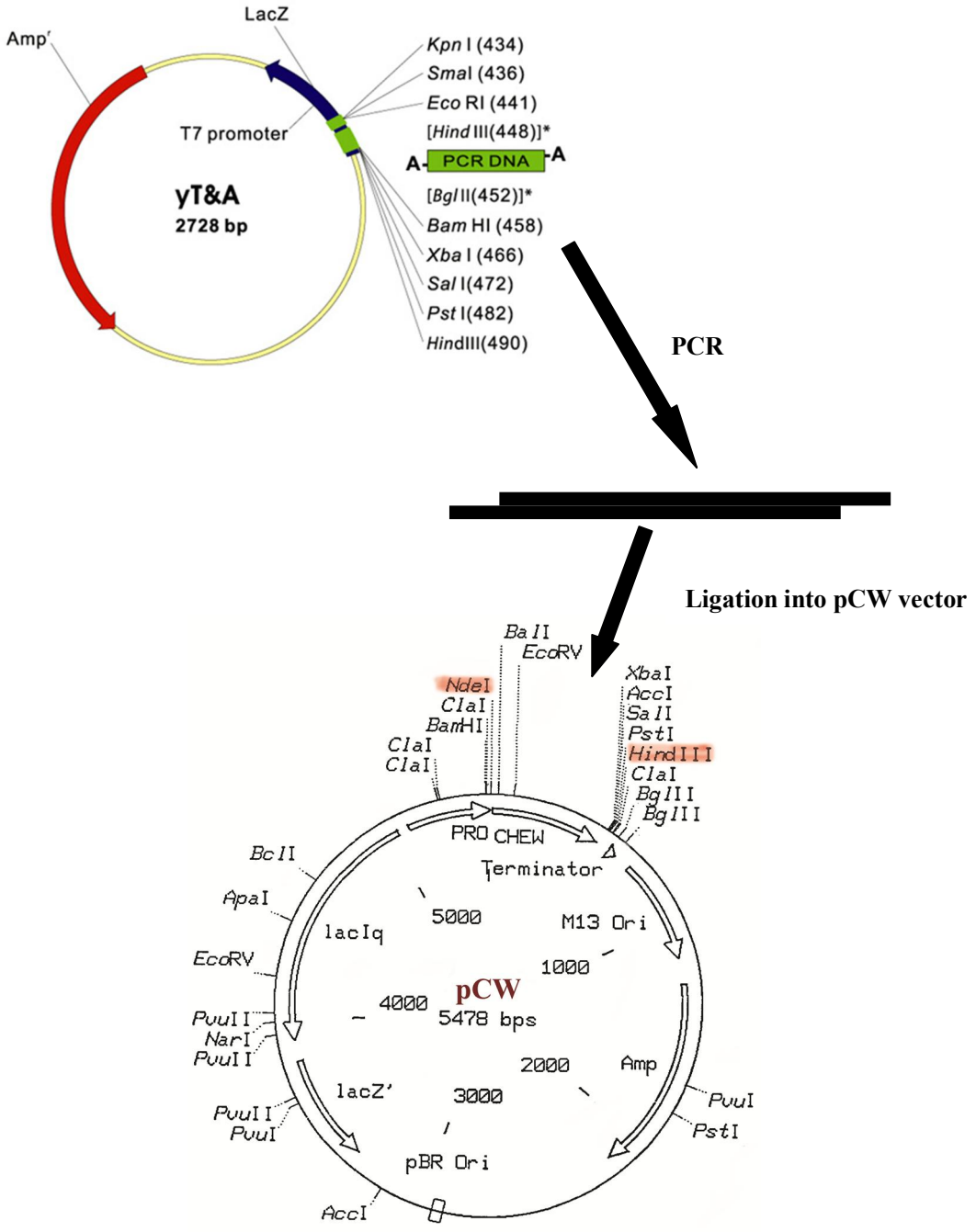


Figure 19. Construction scheme of pCW expression plasmid showing the insertion position for target gene between the *Ndel* and *Hind III* restriction sites.

Transformation system using tobacco for stable gene expression

Agrobacterium-mediated transformation of *N. tobaccum* cv. nc was conducted (Horsch, Fry et al. 1985). Leaves were cut into around 0.5cm discs. The explants were inoculated with *A. tumefaciens* suspensions for 5 to 10 min. Then the leaves were transferred to Co-cultivation medium. After 3 days of co-culture, explants were transferred to degermation medium. After 7 days of degermation, explants were transferred to selection medium. The developing shoots were transferred to regeneration medium. The rooted plants (T0) were transferred to soil and grown in a growth chamber (See **Figure 20**).

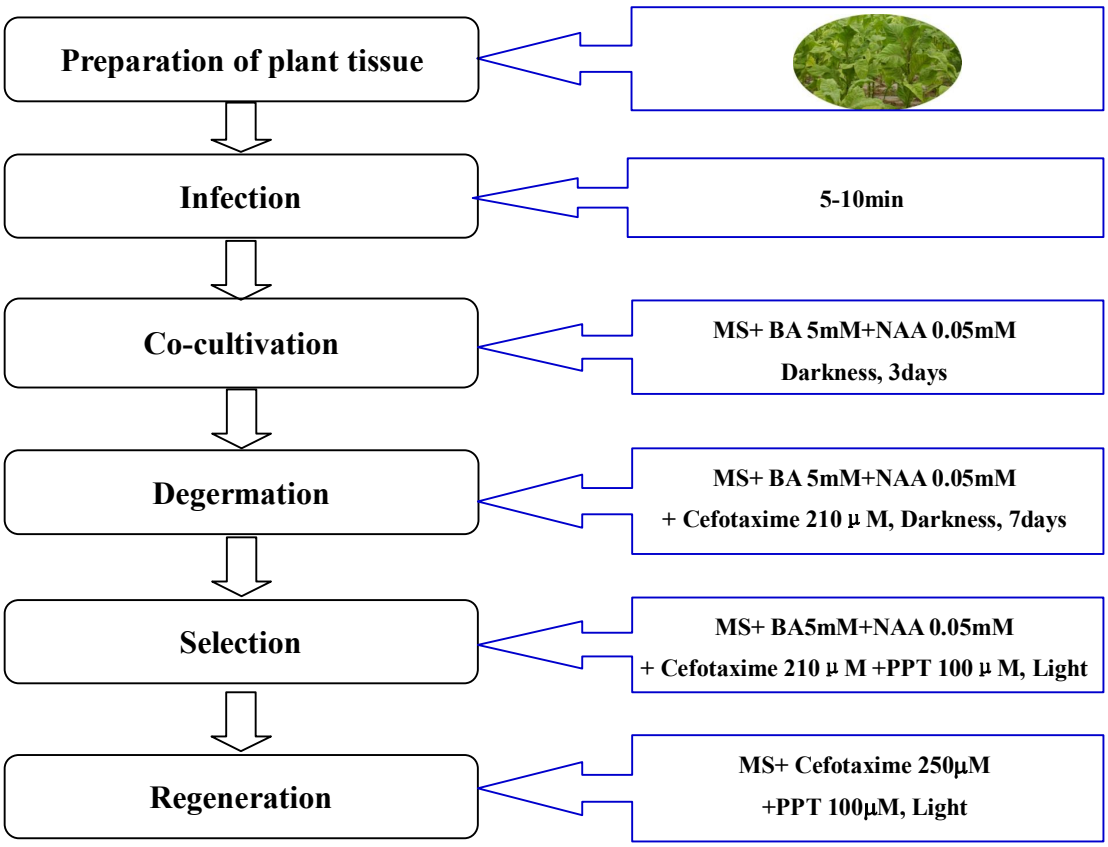


Figure 20. Transformation system using tobacco for stable gene expression.

Transformation system using Arabidopsis for stable gene expression

Transgenic Arabidopsis plants were generated by agro-infiltration using the floral dip method (Clough and Bent 1998). (See **Figure 21**)

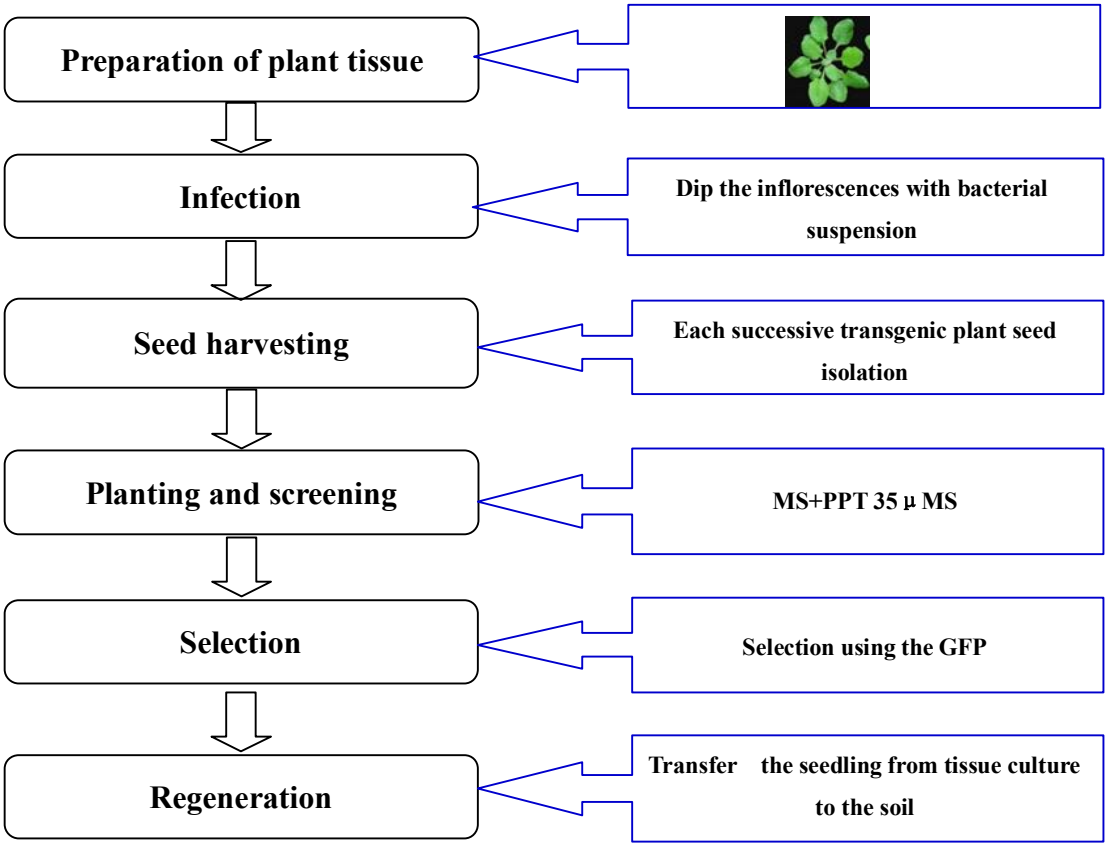


Figure 21. Transformation system using Arabidopsis for stable gene expression.

Transformation system using spinach for stable gene expression

Spinach seeds were surface sterilized by 5% Sodium hypochlorite (NaOCl) solution (diluted from 12% NaOCl with SDW) with some drops of Tween 20 for 20 minutes with stirring, followed by 70% ethanol for 20 minutes. Seeds were then rinsed 3 times by SDW and blotted on filter paper in clean bench for vacuum. The seed coats were then taken out. Seeds were germinated on onto ½ MS salts, MS vitamins (G medium) supplemented with 20 g/L sucrose in short day condition (8hours photoperiod).

Root explants were excised (3-5 mm) from 2-4-week-old seedlings. Explants were then cultured onto ½ MS salts, MS vitamins, supplemented with 5 μM BA, 2 μM NAA, 0.1 μM GA_3 (added after autoclave), 20 g/L sucrose (pH 5.8). Explants were transferred to fresh medium every 2 weeks.

Root explants were excised 0.5 cm in length then were immersed in *Agrobacterium* suspension for 10-20 minutes. Explants were then blotted on filter paper then cultured in co-cultivation medium (½ MS supplemented with 5 μM BA, 2 μM NAA, 0.1 μM GA3, 20 g/L sucrose (pH5.4), 200 μM acetosyringone) in darkness for 3 days. After co-cultivation, explants were washed by 250 μM cefotaxime solution and cultured in elimination medium with 250 μM cefotaxime. 2 weeks after, 5 μM PPT were added for transgenic selection. The transformation procedure is showed in following figure (See **Figure 22**).

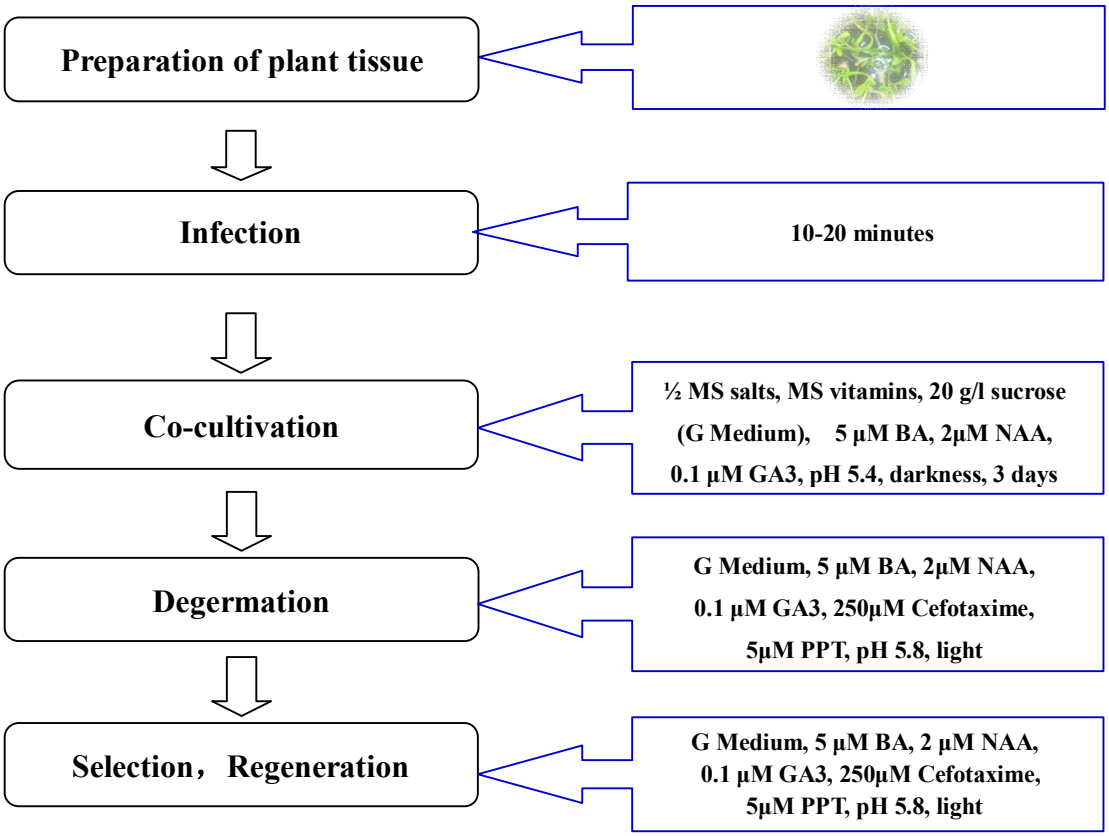


Figure 22. Transformation system using spinach for stable gene expression.

Transformation system using spinach for transient gene expression

Transgenic plant leaf was generated by agro-infiltration using the injection syringe method. After agro-infiltration, during 5 days, GFP expression was checked. Then the transgenic leave were harvested and stored at -80℃.

DNA isolation and confirmation of transgenic plants by PCR

DNA isolation used the Protocol for GENE ALL™ Plant SV mini kit (GENEALL, Korea). For PCR analysis, DNA was isolated from the young leaves of putative transgenic plants according to select using PPT and to check GFP expression. Genomic DNA was amplified by PCR using target gene specific primers. (See **Table 7**)

RNA extraction and reverse transcription (RT)-PCR analysis

RNA extraction used the RNAiso Plus (Total RNA extraction reagent) kit (TaKaRa, Korea). And cDNA was made using Maxime™ RT PreMix Kit (Intron biotech, Korea). For PCR analysis, RNA was isolated from the young leaves of putative transgenic plants according to select using PPT and to check GFP expression. The cDNA was amplified by PCR using target gene specific primers. (See **Table 7**)

Table 7. Primer sequences for cloning of PE biosynthetic genes

Target gene	Primer sequence
DHCR(So)	F:5′-ATGGCGGAAACAAAGATAGTGCA- 3′
	R:5′- TCAATAGATTCCTGGTATAATCC - 3′
CYP85(So)	F: 5′- CACCATGGCCGTTTTTATGGTGGTTTTTGCTGTGATTTT - 3′
	R:5′- CTAATAACTCGAAACTCGAATGC - 3′
CYP90B(So)	F: 5′- CACCATGTCTGACTTAGAATT - 3′
	R:5′- TCAGAGTCCAAAGCTTAAGCG - 3′
CYP92A(So)	F:5′-CACCATGGAGCCACCACCTTGGGCC- 3′
	R:5′-TTAACTACCTCCAATATAATCATAA- 3′
DHCR(Aj)	F:5′- ATGGTGGAATCAAAGACAGTT - 3′
	R:5′- TCAATAGATTCCAGGTATAAT - 3′
CYP85(Aj)	F:5′- CACCATGGCTGTTTTGATTCCAA - 3′
	R:5′- TTCGAGTATCGAGTTATTAA - 3′

So: gene cloned from *S. oleracea; Aj*: gene cloned from *A. japonica*

Extraction of PEs

Collected plant materials were grinded to a fine powder, using a pestle and mortar under liquid nitrogen. And samples (ca. 25mg) were extracted 3 times, each for 3 hr, with 1ml of methanol at 55℃. The extracts were mixed with 1.3ml of water and partitioned 2 times with 2ml of hexane. The aqueous methanol phases were dried at 55℃ and dissolved with 1ml of methanol (Boo 2007).

Results

Construction of vectors for gene expression

Vectors for gene expression in plant The full-length of *DHCR, CYP85, CYP90B, CYP92A, Aj-CYP85* and *Aj-DHCR* genes were introduced into the pB7WG2D, 1 vector at the attR1 and attR2 site to replace the ccdB gene for gene over-expression. The resulting construct were named *pB-DHCR, pB-CYP85, pB-CYP90B, pB-CYP92A, pB-Aj-CYP85* and *pB-Aj-DHCR,* respectively. These vectors were introduced into *A. tumefaciens* strain LBA4404 (CLONTECH, USA), respectively. (See **Figure 23**)

The hair-pin sequence were designed based on full-length of *DHCR, CYP85, CYP90B* and *CYP92A* genes and also were introduced into the pK7GWIWG2D(II),0 vector at the attR1 and attR2 site to replace the ccdB gene for gene knock-down expression. The resulting construct were named *pK-DHCR, pK-CYP85, pK-CYP90B* and *pK-CYP92A,* respectively. These vectors were introduced into *A. tumefaciens* strain LBA4404 (CLONTECH, USA), respectively. (See **Figure 24)**

Vectors for recombinant protein expression in *Escherichia coli* The full lengths of *CYP85, CYP90B, CYP92A and CYP314* were introduced into the pCW vector by double-enzyme digestion with *NdeI* and *HindIII* for gene over-expression. The resulting constructs were named *pCW-CYP85, pCW-CYP90B, pCW-CYP92A, pCW-CYP314,* respectively. These vectors were introduced into *E. coli*, respectively. (See **Figure 25**)

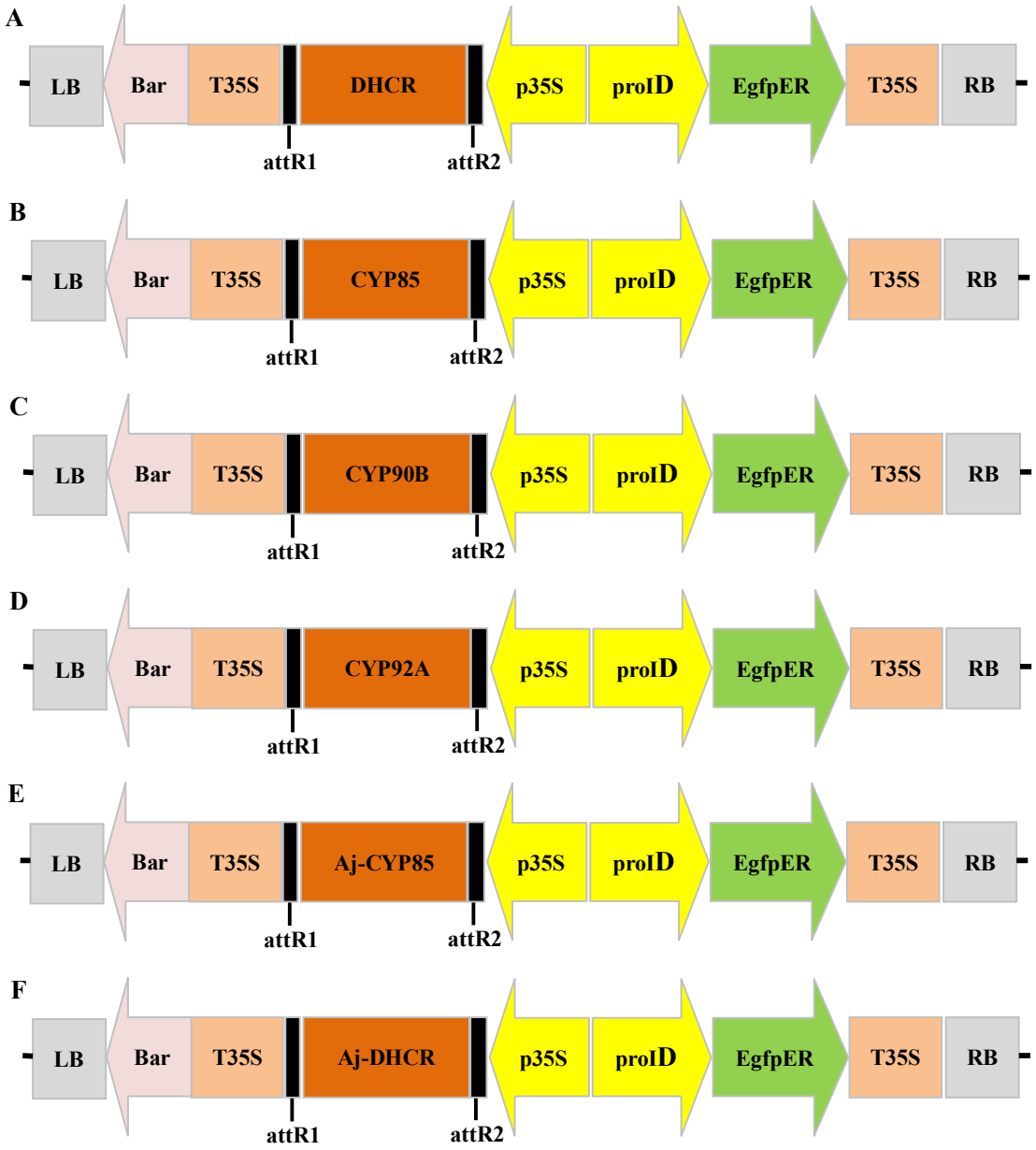


Figure 23. Recombinant plasmid for gene over-expression bearing *DHCR*(A), *CYP85*(B), *CYP90B*(C), *CYP92A*(D), *Aj-CYP85*(E) and *Aj-DHCR*(F) genes.

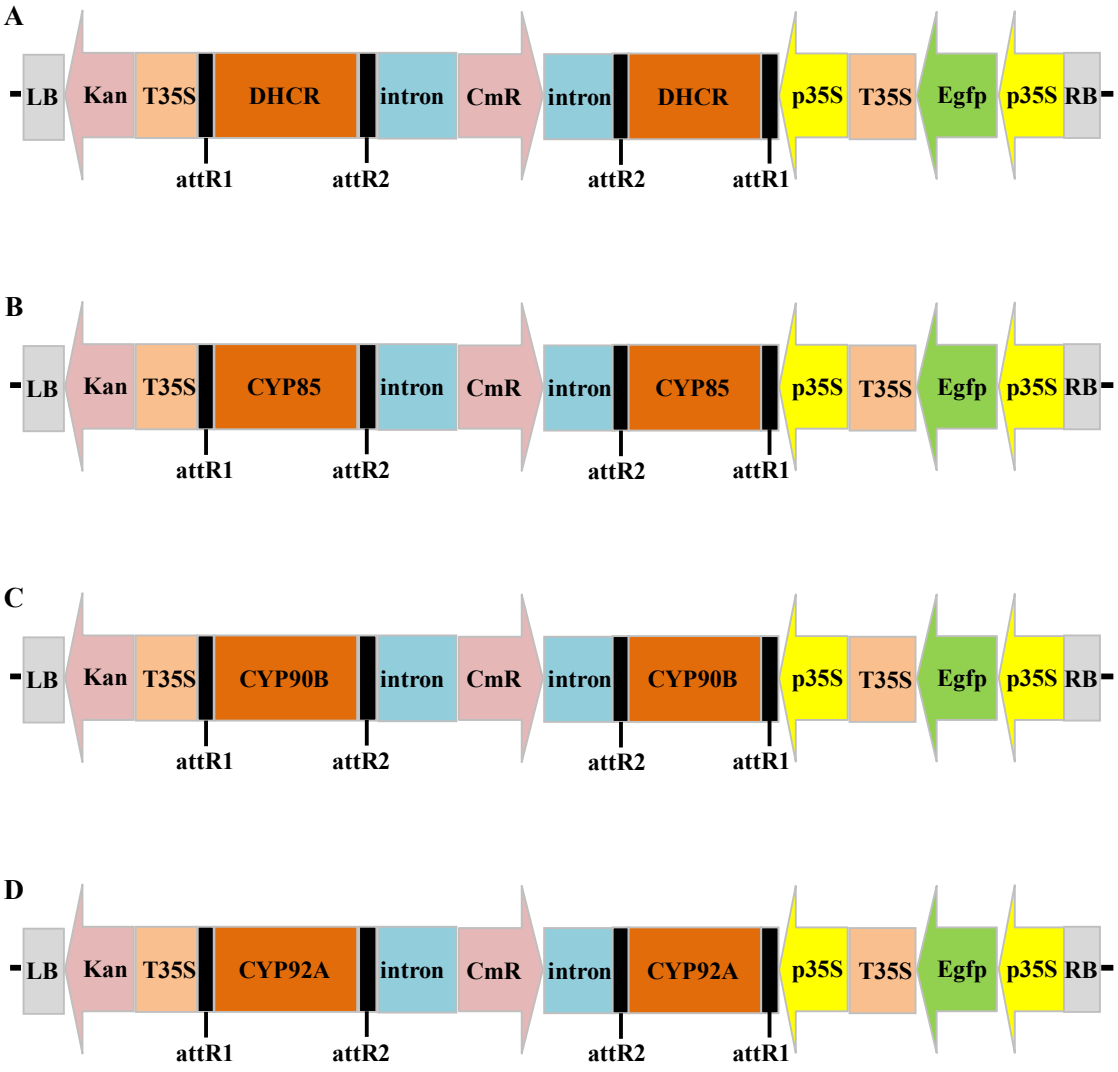


Figure 24. Recombinant plasmid for gene knock-down expression bearing *DHCR*(A), *CYP85*(B), *CYP90B*(C) and *CYP92A*(D) genes.

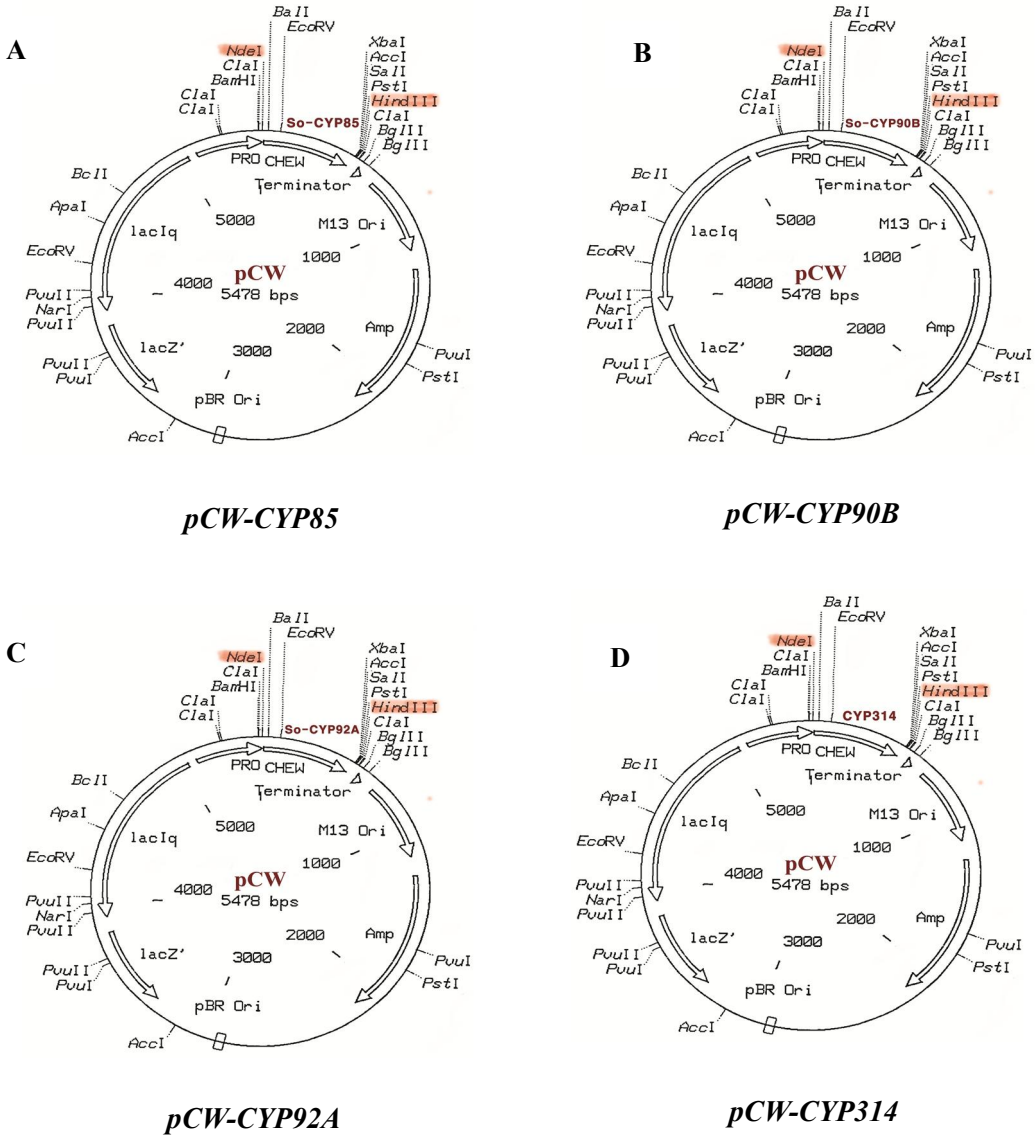


Figure 25. Recombinant protein expression vector construction for *CYP85*(A), *CYP90B*(B), *CYP92A*(C) and *CYP314*(D) genes in *E. coli*.

Expression and purification of recombinant CYP450 enzymes in *Escherichia coli*

Through to optimize the condition of recombinant protein expression in *E. coli,* Enzymatically active CYP85, CYP90B, CYP92A and CYP314 have been expressed in and purified from *E. coli*. The target proteins were detected spectrophotometrically and were found to be integrated into *E. coli* membranes. These proteins were purified to electrophoretic homogeneity from solubilize bacterial membranes using two sequential chromatographic steps, Ni^{2+}-NTA agarose affinity column chromatograph followed by AKTA prime plus (Hiprep 16/60 Sephacryl S-300 HR, USA). (See from **Figure 26** to **Figure 37**)

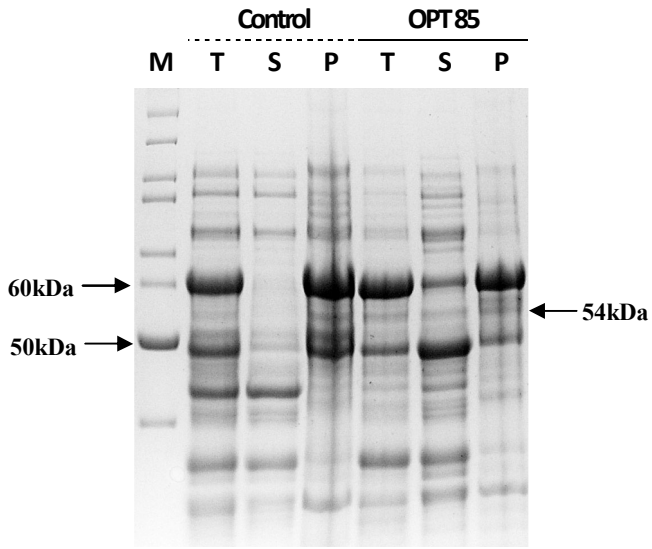


Figure 26. SDS-PAGE of CYP85 recombinant protein extracted from *E. coli*. The SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue R250. T: crude cell extract, S: supernatant, P: pellet. Molecular mass (kDa) of standard proteins are shown on the left.

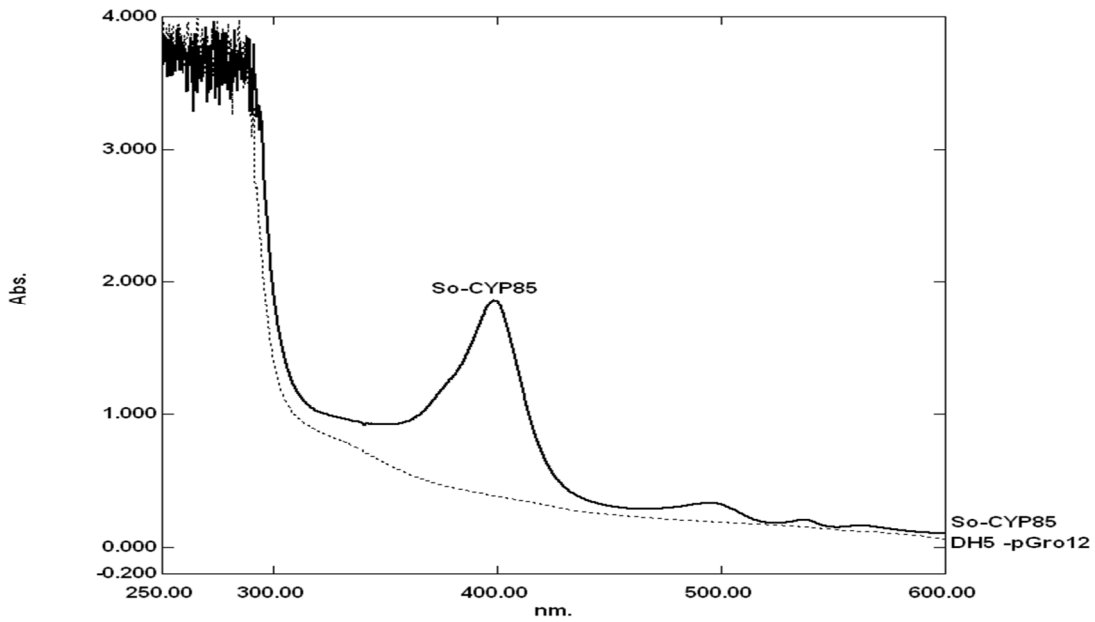


Figure 27. UV-visible spectrum of purified CYP85.

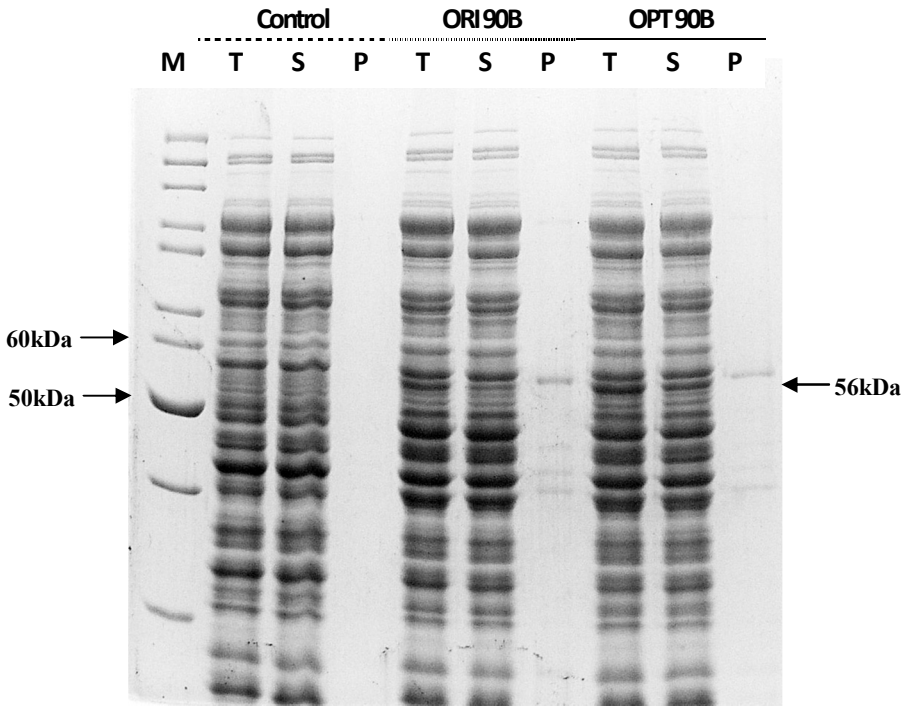


Figure 28. SDS-PAGE of CYP90B recombinant protein extracted from *E. coli.* The SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue R250. T: crude cell extract, S: supernatant, P: pellet. Molecular mass (kDa) of standard proteins are shown on the left.

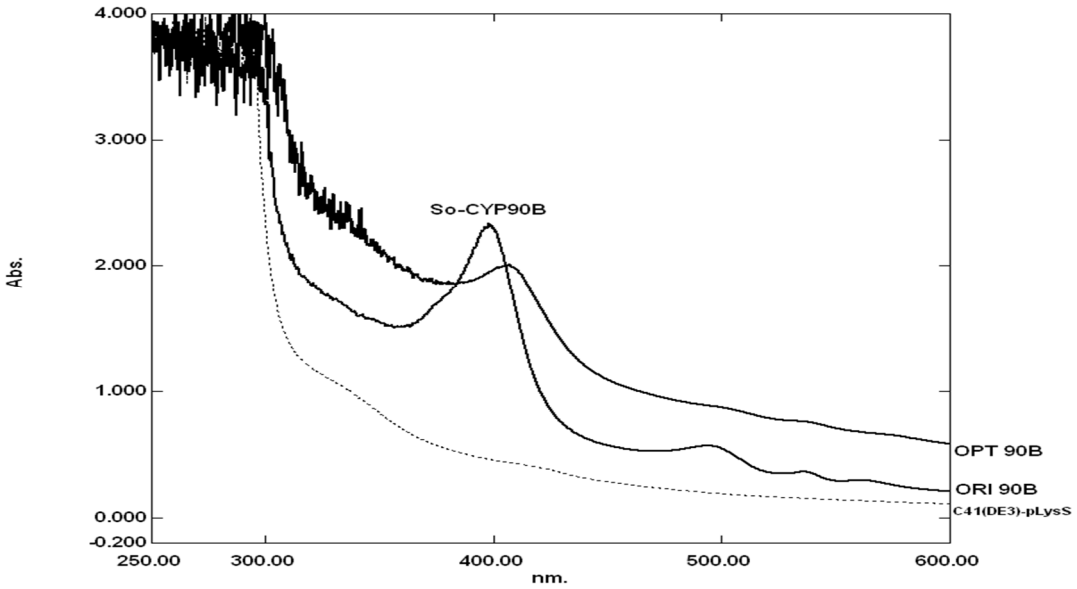


Figure 29. UV-visible spectrum of purified CYP90B.

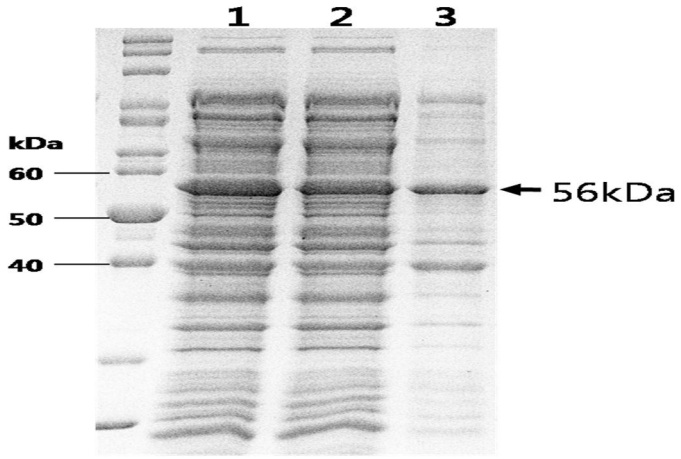


Figure 30. SDS-PAGE of CYP90B recombinant protein extracted from *E. coli*. The SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue R250. Line 1: crude cell extract, 2: supernatant, 3: pellet. Molecular mass (kDa) of standard proteins are shown on the left.

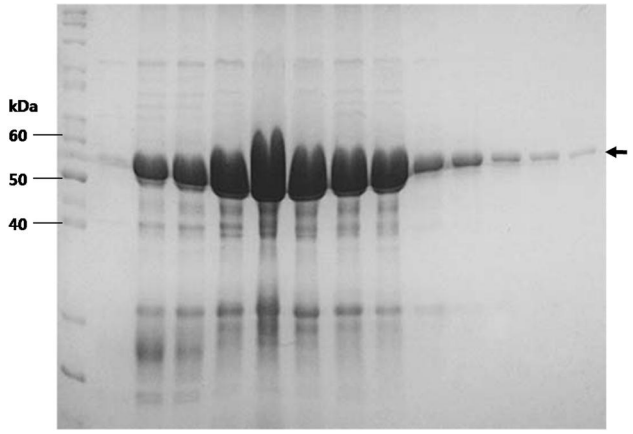


Figure 31. SDS-PAGE of purified CYP90B recombinant protein using Ni-NTA His-tag affinity chromatography. CYP90B protein was eluted from Ni^{2+}-NTA-agarose with 200 mM imidazole contained 0.5% octyl-β-D-glucopyranoside, pH 8.0. The SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue R250. Molecular mass (kDa) of standard proteins are shown on the left.

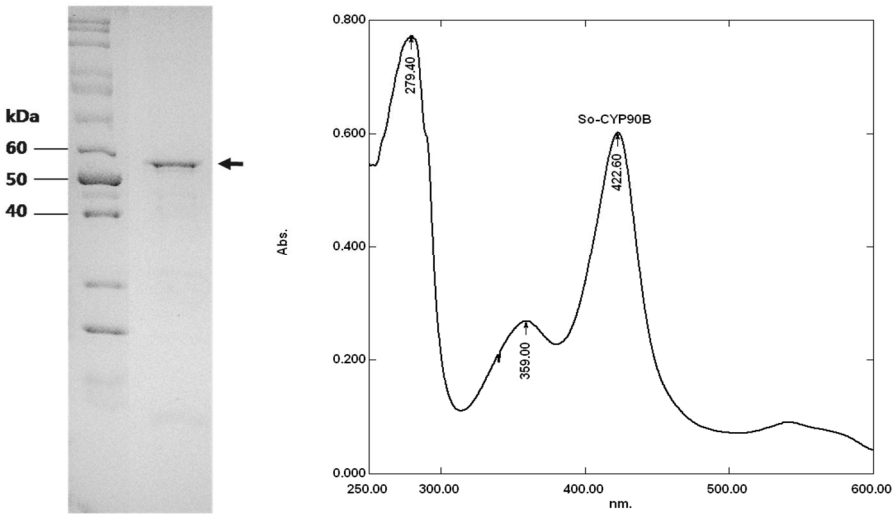


Figure 32. UV-visible spectrum and SDS-PAGE of purified CYP90B. **Left,** SDS-PAGE of the purified CYP90 using Gel filtration. The SDS -polyacrylamide gel was stained with Coomassie Brilliant Blue R250. Molecular mass (kDa) of standard proteins are shown on the left. **Right,** UV-visible spectrum of purified CYP90B.

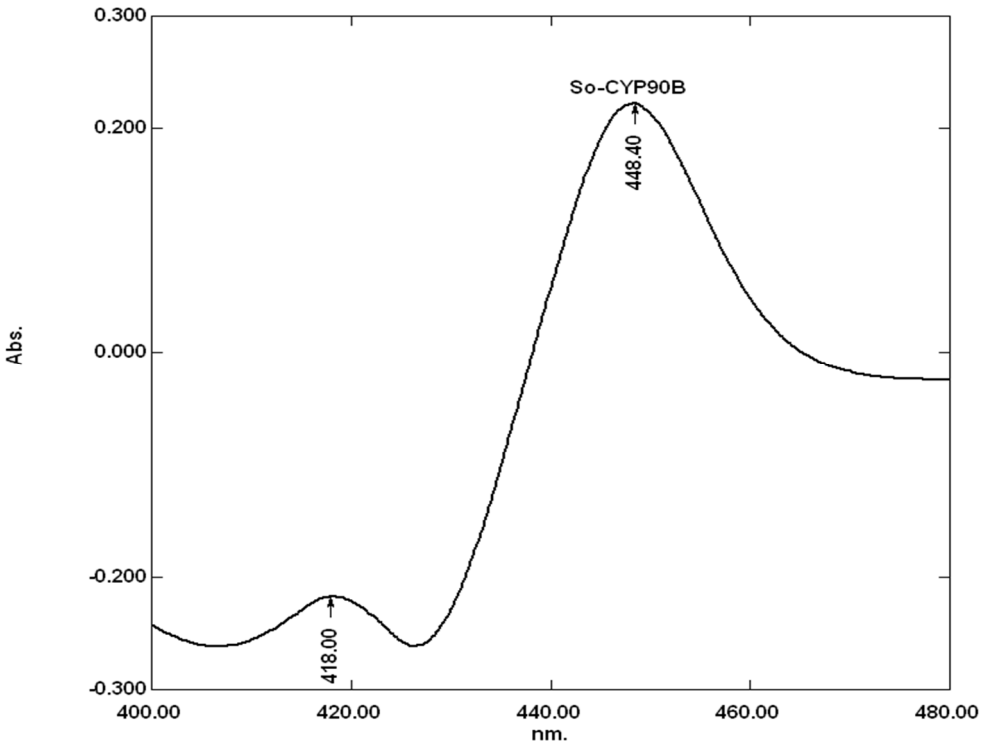


Figure 33. UV-visible spectrum of purified CYP90B after flushing the sample with CO gas.

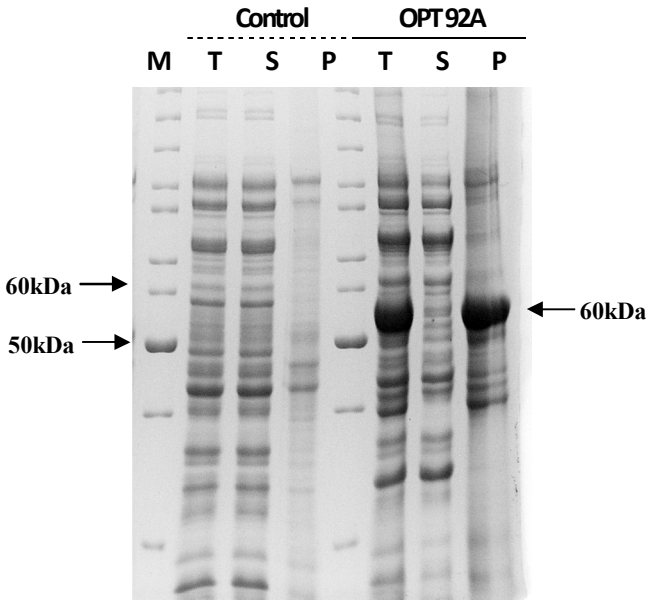


Figure 34. SDS-PAGE of CYP92A recombinant protein extracted from *E. coli.* The SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue R250. T: crude cell extract, S: supernatant, P: pellet. Molecular mass (kDa) of standard proteins are shown on the left.

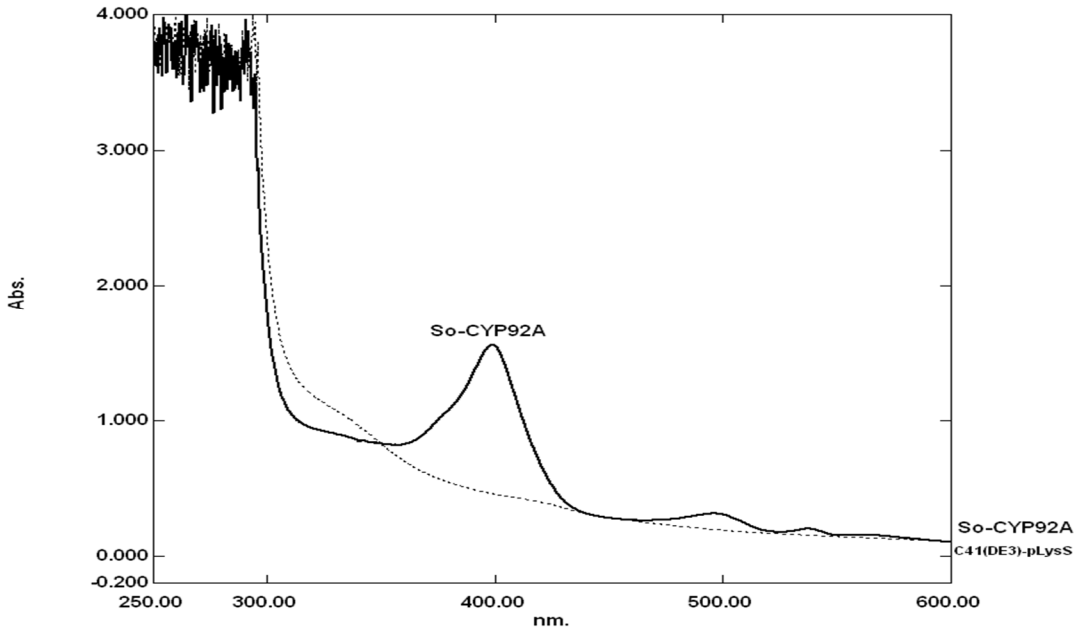


Figure 35. UV-visible spectrum of purified CYP92A.

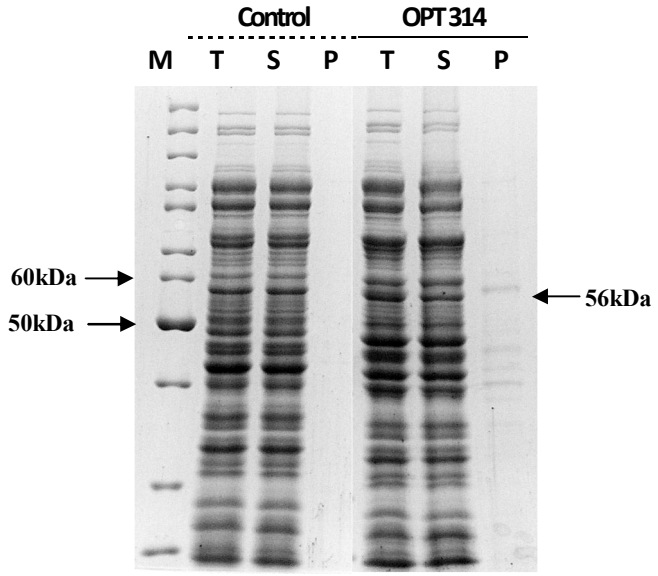


Figure 36. SDS-PAGE of CYP314 recombinant protein extracted from *E. coli.* The SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue R250. T: crude cell extract, S: supernatant, P: pellet. Molecular mass (kDa) of standard proteins are shown on the left.

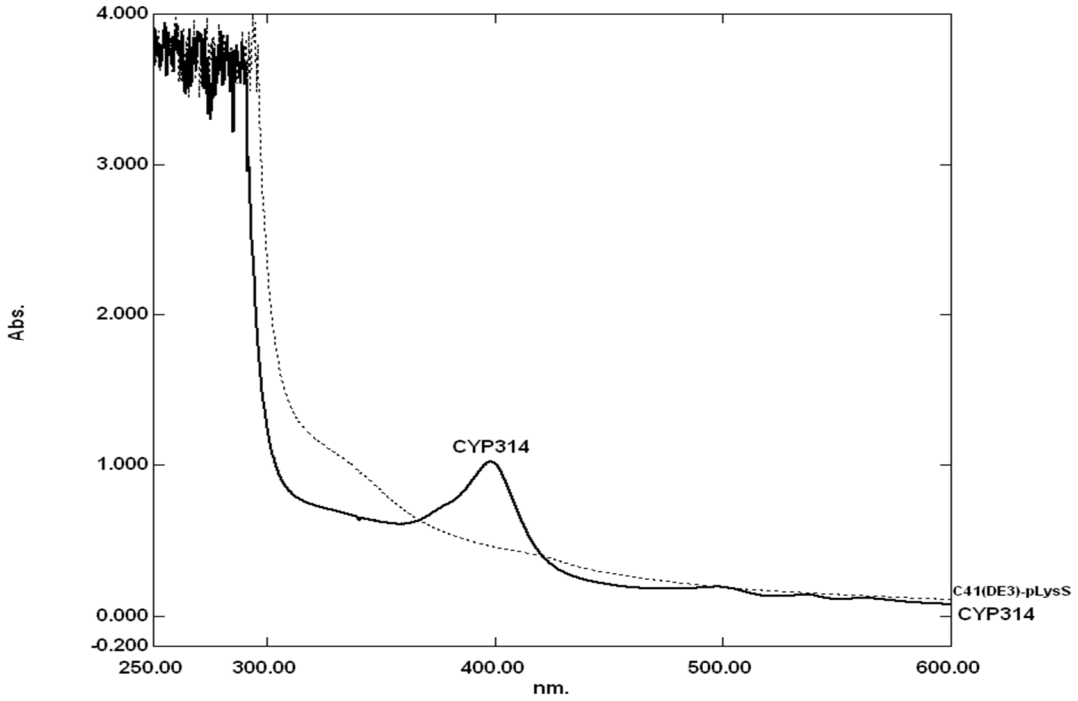


Figure 37. UV-visible spectrum of purified CYP314.

Construction of transgenic line for transient gene expression in plant

Construction transgenic line for transient gene over-expression Transgenic plant leaves of spinach were generated by agro-infiltration using the injection syringe method using *A. tumefaciens* strain LBA4404 with *pB-CYP85, pB-CYP90B* and *pB-CYP92A* vector, respectively. After agro-infiltration, during 5 days, GFP expression was checked. Then the transgenic leaves were harvested and stored at -80℃. (See **Figure 38**)

Construction transgenic line for transient gene knock-down expression Transgenic plant leave of spinach were generated by agro-infiltration using the injection syringe method using *A. tumefaciens* strain LBA4404 with *pK-CYP85, pK-CYP90B* and *pK-CYP92A* vector, respectively. After agro-infiltration, during 5 days, GFP expression was checked. Then the transgenic leaves were harvested and stored at -80℃. (See **Figure 39**)

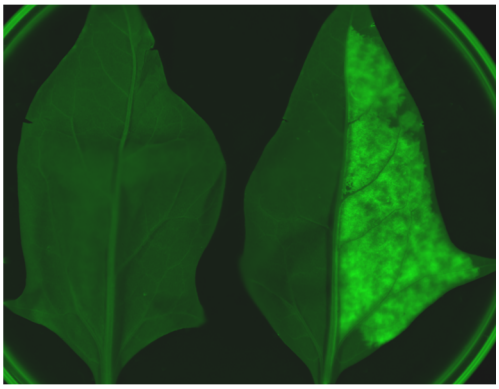


Figure 38. Detection of the green fluorescent protein (GFP) in transgenic plant leaves for transient gene over-expression. Spinach leaves of control (left) and transgenic leaf (right).

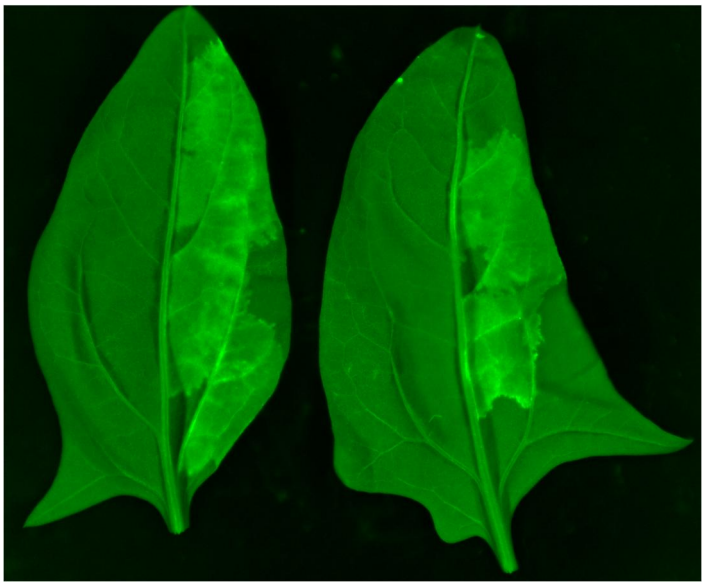


Figure 39. Detection of the green fluorescent protein (GFP) in transgenic plant leaves of spinach for transient gene knock-down expression.

Construction of transgenic line for stable gene expression in plant

In these study, transformation system in PE-positive and negative plants were constructed. Eight kinds of transgenic plants were obtained.

For spinach, *Aj-CYP85* cDNA integration into the spinach genome was confirmed by PCR analysis. To check the expression of the target gene at the transcription level, we performed RT-PCR using total mRNA from transgenic plants, and the analysis showed that the transcription of the target gene occurred indeed in transgenic plant. We confirmed marker gene of Bar in genome by PCR analysis and transcription level by RT-PCR and PPT selection. At the same, we also confirmed marker gene of EgfpER gene in genome by PCR analysis and transcription level by RT-PCR and GFP detection by Fujifilm LAS-4000 Super CCD Remote Control Science Imaging System.(See **Figure 40**)

For *Arabidopsis*, *DHCR, CYP85, CYP90B and CYP92A* cDNA integration into the *Arabidopsis* genome were confirmed by PCR analysis, respectively. We confirmed marker gene of Bar in transcription by PPT selection. At the same, we also confirmed marker gene of EgfpER gene in transcription level by GFP detection by Fujifilm LAS-4000 Super CCD Remote Control Science Imaging System. (See **Figure 41**, **Figure 42**, **Figure 43** and **Figure 44**)

For tobacco, *Aj-CYP85*, *Aj-DHCR* and *CYP92A* cDNA integration into the tobacco genome was confirmed by PCR analysis. To check the expression of the target gene at the transcription level, we performed RT-PCR using total mRNA from transgenic plants, and the analysis showed that the transcription of the target gene occurred indeed in transgenic

plant. We confirmed marker gene of Bar in genome by PCR analysis and transcription level by RT-PCR and PPT selection. At the same, we also confirmed marker gene of EgfpER gene in genome by PCR analysis and transcription level by RT-PCR and GFP detection by Fujifilm LAS-4000 Super CCD Remote Control Science Imaging System. (See **Figure 45**, **Figure 46** and **Figure 47**)

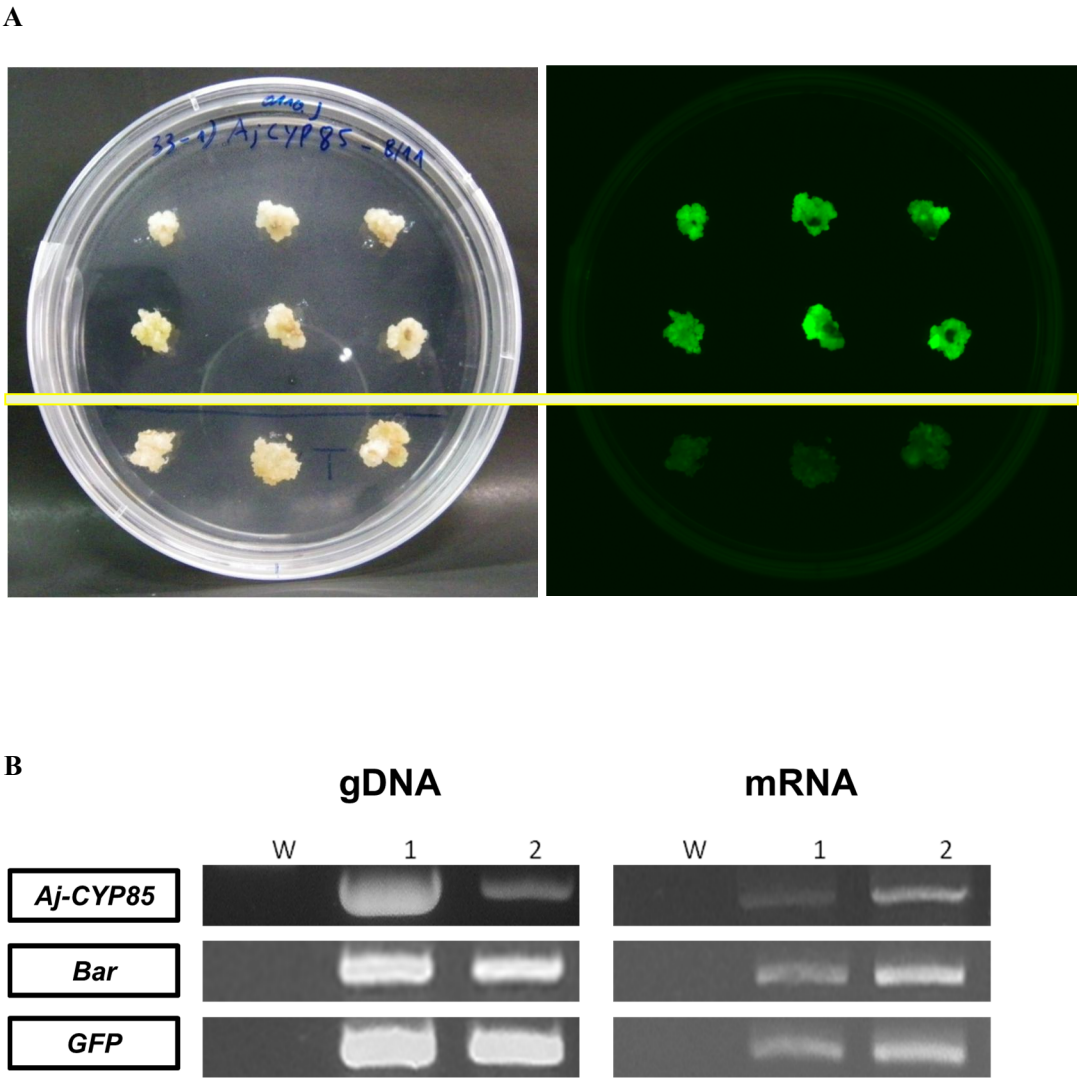


Figure 40. GFP and PCR detection of two transgenic spinach plant lines with over expression of *Aj-CYP85* gene. A: Detection of the green fluorescent protein (GFP) in callus. Transgenic callus(up) and wild type callus(down) in white light (left side) and green fluorescent light (right side). B: PCR analysis of genomic DNAs and mRNAs with the specific primers for *Aj-CYP85* gene, BAR gene and GFP gene. PCR amplification of a 1390bp fragment of the Aj-*CYP85* gene, 490bp fragment of the BAR gene and 500bp fragment of the GFP gene in transgenic plant callus. W; Non transgenic callus; 1-2; transgenic callus.

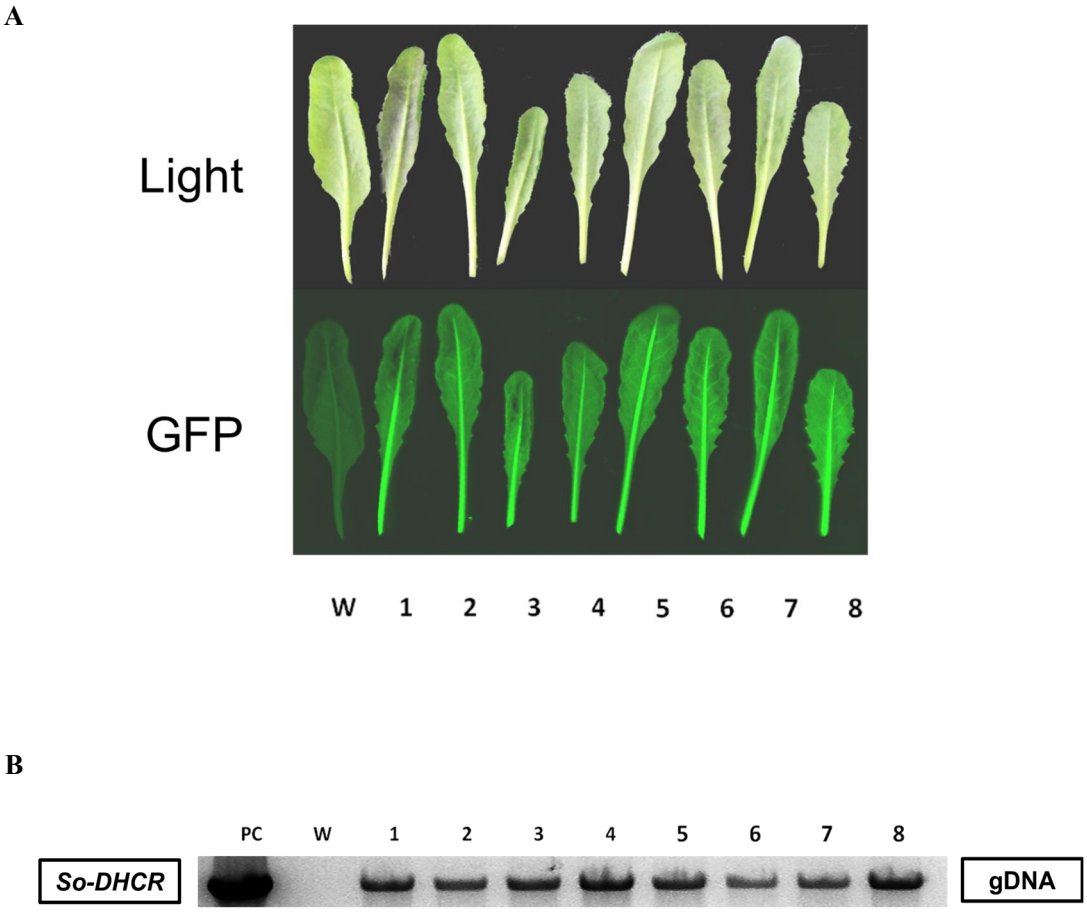


Figure 41. GFP and PCR detection of eight transgenic *Arabidopsis* plant lines with over expression of *DHCR* gene. A: Detection of the green fluorescent protein (GFP). Transgenic plant lines express the GFP (down). W: Wild type plant; 1-8: The transgenic plant lines. B: PCR analysis of genomic DNAs of eight transgenic plant lines. PC, Positive control (Plasmid DNA); W: Non transgenic plant; 1-8: Transgenic plant lines.

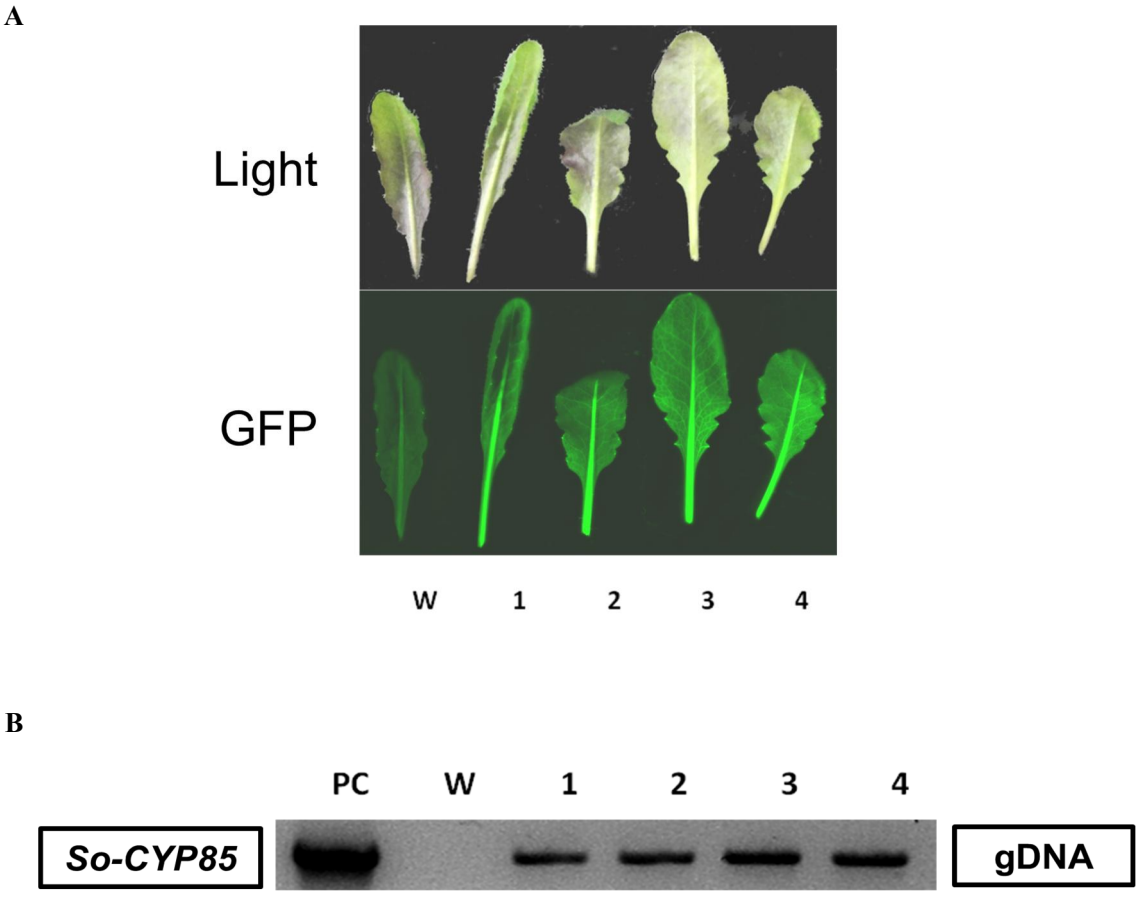


Figure 42. GFP and PCR detection of four transgenic *Arabidopsis* plant lines with over expression of *CYP85* gene. A: Detection of the green fluorescent protein (GFP). Transgenic plant lines express the GFP (down). W: Wild type plant; 1-4: The transgenic plant lines. B: PCR analysis of genomic DNAs of four transgenic plant lines. PC, Positive control (Plasmid DNA); W: Non transgenic plant; 1-4: Transgenic plant lines.

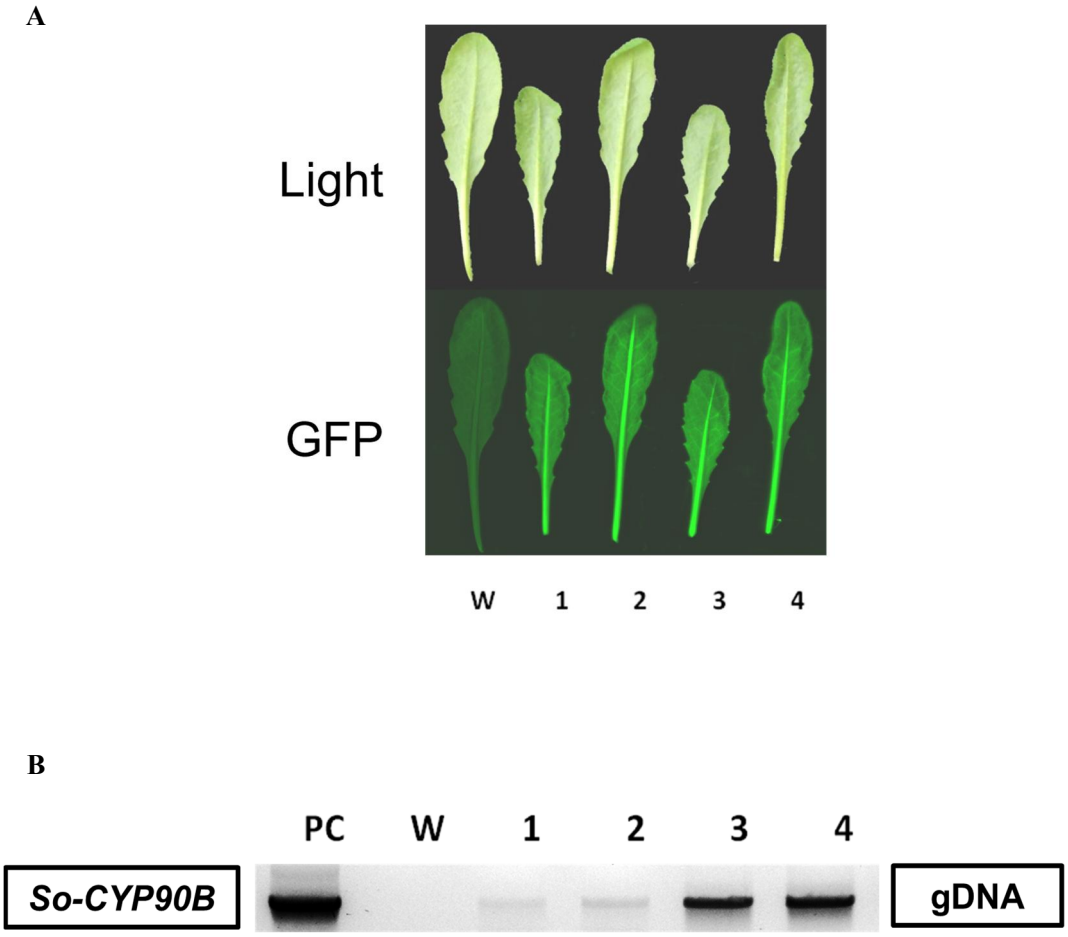


Figure 43. GFP and PCR detection of four transgenic *Arabidopsis* plant lines with over expression of *CYP90B* gene. A: Detection of the green fluorescent protein (GFP). Transgenic plant lines express the GFP (down). W: Wild type plant; 1-4: The transgenic plant lines. B: PCR analysis of genomic DNAs of four transgenic plant lines. PC, Positive control (Plasmid DNA); W: Non transgenic plant; 1-4: Transgenic plant lines.

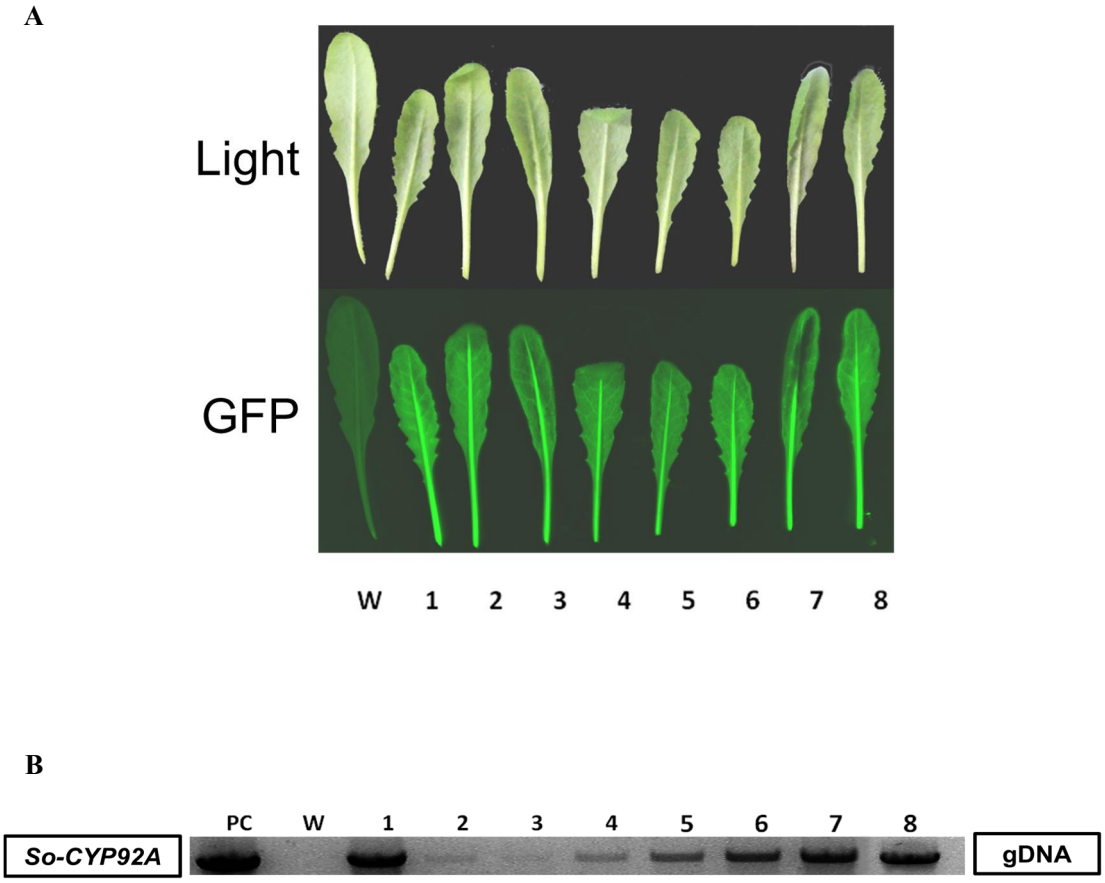


Figure 44. GFP and PCR detection of eight transgenic *Arabidopsis* plant lines with over expression of *CYP92A* gene. A: Detection of the green fluorescent protein (GFP). Transgenic plant lines express the GFP (down). W: Wild type plant; 1-8: The transgenic plant lines. B: PCR analysis of genomic DNAs of eight transgenic plant lines. PC, Positive control (Plasmid DNA); W: Non transgenic plant; 1-8: Transgenic plant lines.

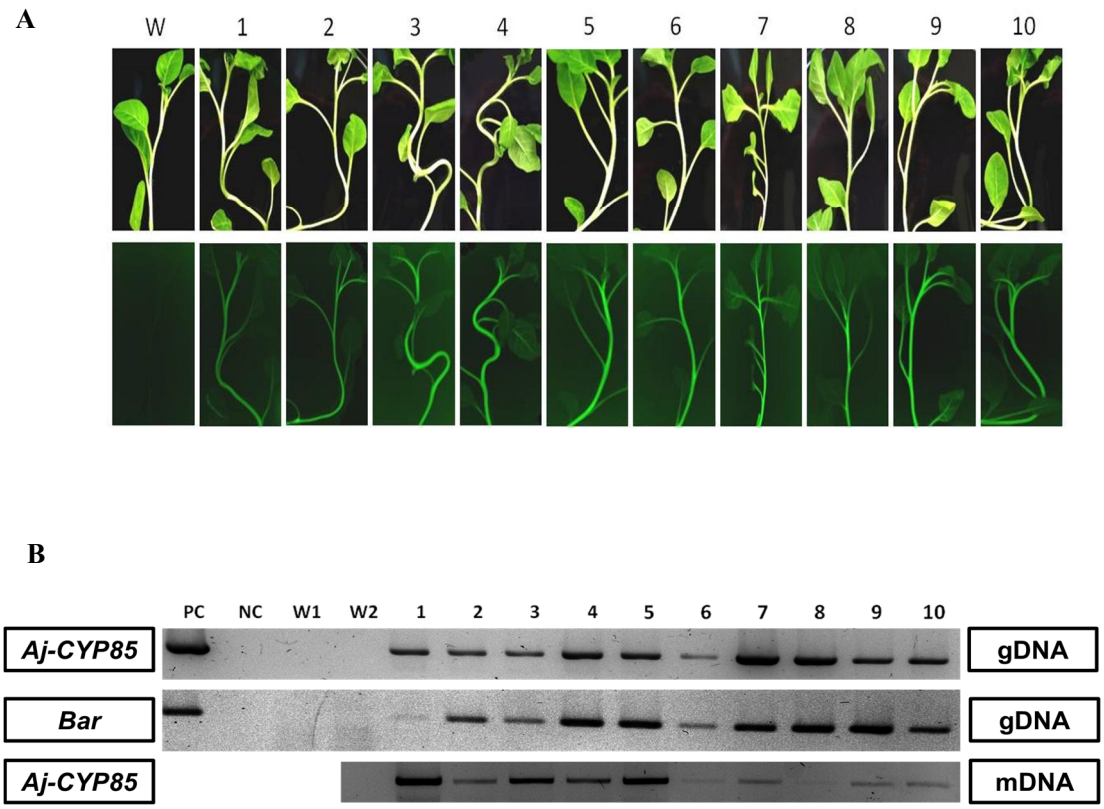


Figure 45. GFP and PCR detection of ten transgenic tobacco plant lines with over expression of *Aj-CYP85* gene. A: Detection of the green fluorescent protein (GFP). Transgenic plant lines express the GFP (down). W: Wild type plant; 1-10: the transgenic plant lines. B: PCR analysis of genomic DNAs or mRNAs of ten transgenic plant lines with the specific primers for *Aj-CYP85* gene and BAR gene. PCR amplification of a 1390bp fragment of the *Aj-CYP85* gene and 490bp fragment of the BAR gene in transgenic plant. PC: Positive control (Plasmid DNA); NC: Negative control; W1, W2: Non transgenic plant; 1-10: Transgenic plant lines.

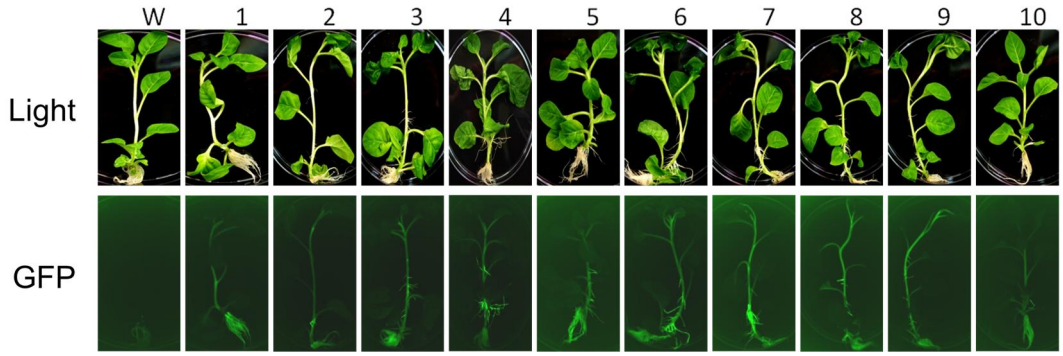


Figure 46. Detection of the green fluorescent protein (GFP) in transgenic tobacco plant lines with over expression *Aj-DHCR* gene. Transgenic plant lines express the GFP (down). W: Wild type plant; 1-10: Transgenic plant lines.

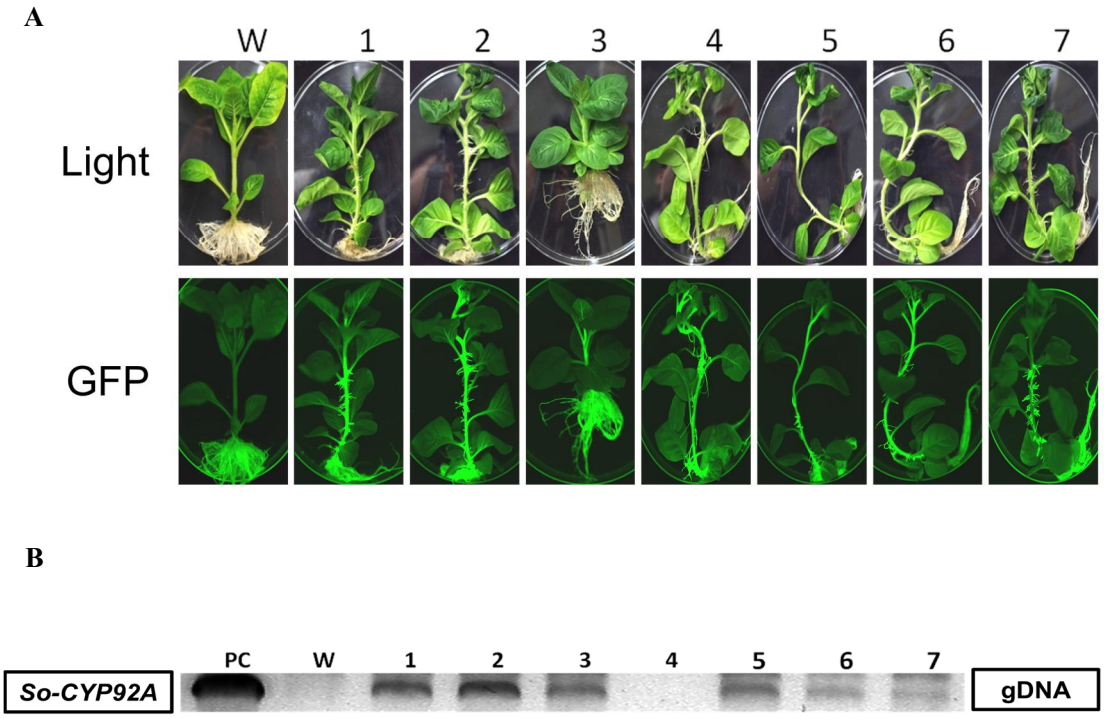


Figure 47. GFP and PCR detection of seven transgenic tobacco plant lines with over expression of *CYP92A* gene. A: Detection of the green fluorescent protein (GFP). Transgenic plant lines express the GFP (down). W: Wild type plant; 1-7: the transgenic plant lines. B: PCR analysis of genomic DNAs of seven transgenic plant lines. PC, Positive control (Plasmid DNA); W: Non transgenic plant; 1-7: Transgenic plant lines.

Construction metabolite profile system by HPLC

For analysis constructed transgenic line, in this study, first, we optimized the HPLC condition like **Table 8**. Using this analysis system, we can separate from 7-DHC to 20E in downstream of putative PEs biosynthesis. So this metabolite profile system was used for analyzing metabolite change in transgenic line.

In transgenic tobacco with *Aj-CYP85* over-expression, there were some increased peaks between 7-DHC and 20E. And transgenic spinach with *CYP90B* or *CYP92A* knock-down expression, there were increased peak in transgenic leaves and decreased peak in wild leaves between 7-DHC and 20E.(See **Figure 49**, **Figure 50** and **Figure 51**)

Table 8. Operation condition of HPLC for PEs metabolites analysis

Parameter	Condition		
Instruments	Vaccum degasser, P4000 pump, AS1000 auto sampler UV6000LP Photo Diode Array detector		
Column	YMC HPLC colum: 250×4.6mm, S-5μm		
Mobile phase	**Time(min)**	**MeOH(%)**	**H_2O (%)**
	0.0	30.0	70.0
	30.0	95.0	5.0
	40.0	100.0	0.0
	60.0	100.0	0.0
	65.0	30.0	70.0
	70.0	30.0	70.0
Other condition	**- Scan wavelength:**	200-600	
	- Wavelength:	242,260nm	
	- Column Temp.:	38 ℃	
	- Flow rate:	1.0 ml/min	
	- Injection volume:	20 μl	
	- Run time:	70.0min	

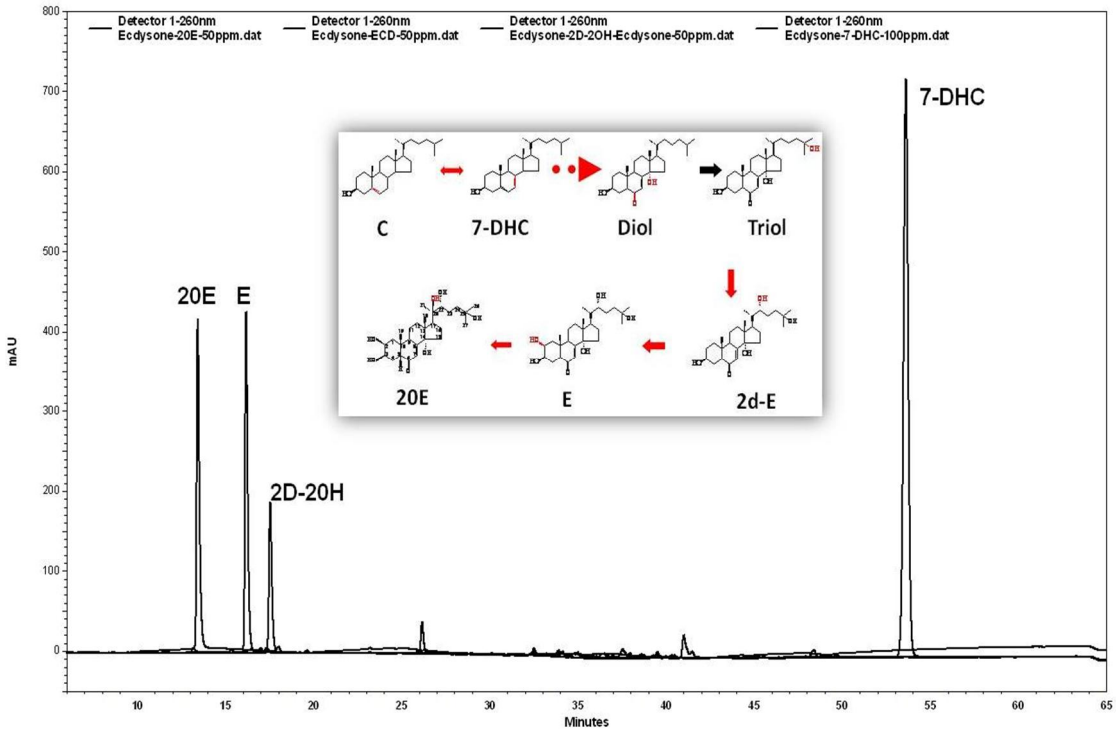


Figure 48. Chromatogram of standard PE metabolites in HPLC. 20E: 20-Hydroxyecdysone, E: Ecdysone, 2D-20H: 2-deoxy-20-Hydroxyecdysone, 7-DHC: 7-Dehydrocholesterol.

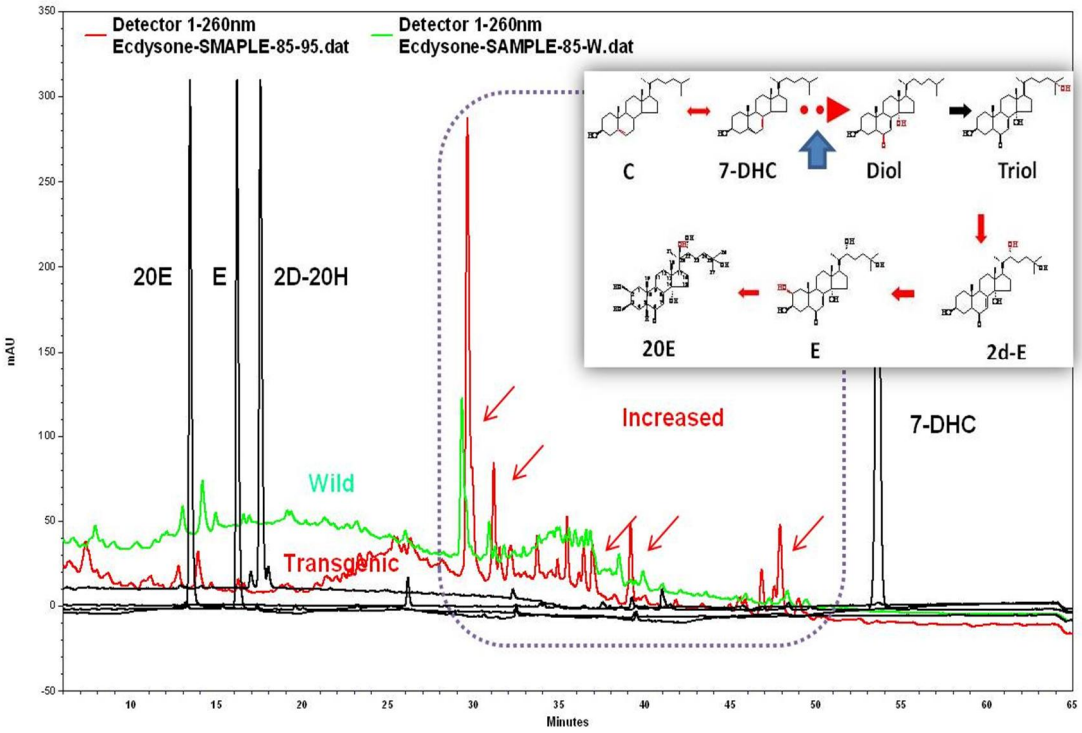


Figure 49. Change of metabolic HPLC profile in transgenic tobacco plant with *Aj-CYP85* over-expression. Green line: Wild type plant; Red line: the transgenic plant. 20E: 20-Hydroxyecdysone, E: Ecdysone, 2D-20H: 2-deoxy-20-Hydroxyecdysone, 7-DHC: 7-Dehydrocholesterol.

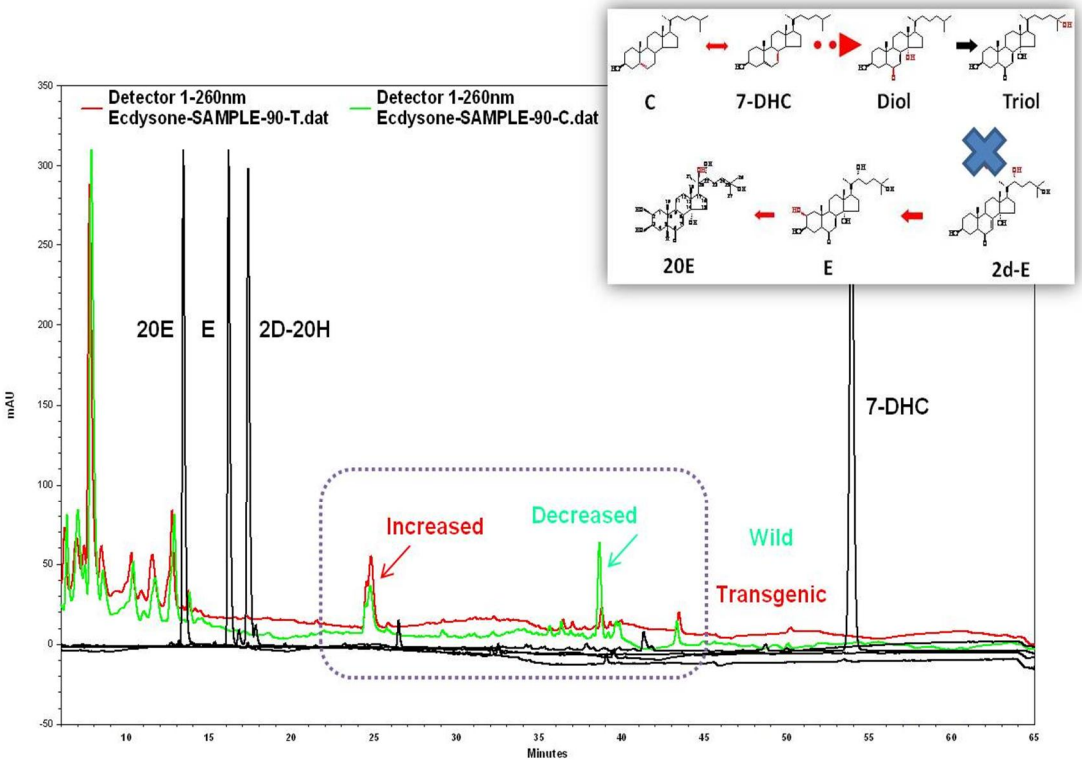


Figure 50. Change of metabolic HPLC profile in transgenic spinach plant with *CYP90B* knock-down expression. Green line: Wild type plant; Red line: the transgenic plant. 20E: 20-Hydroxyecdysone, E: Ecdysone, 2D-20H: 2-deoxy-20-Hydroxyecdysone, 7-DHC: 7-Dehydrocholesterol.

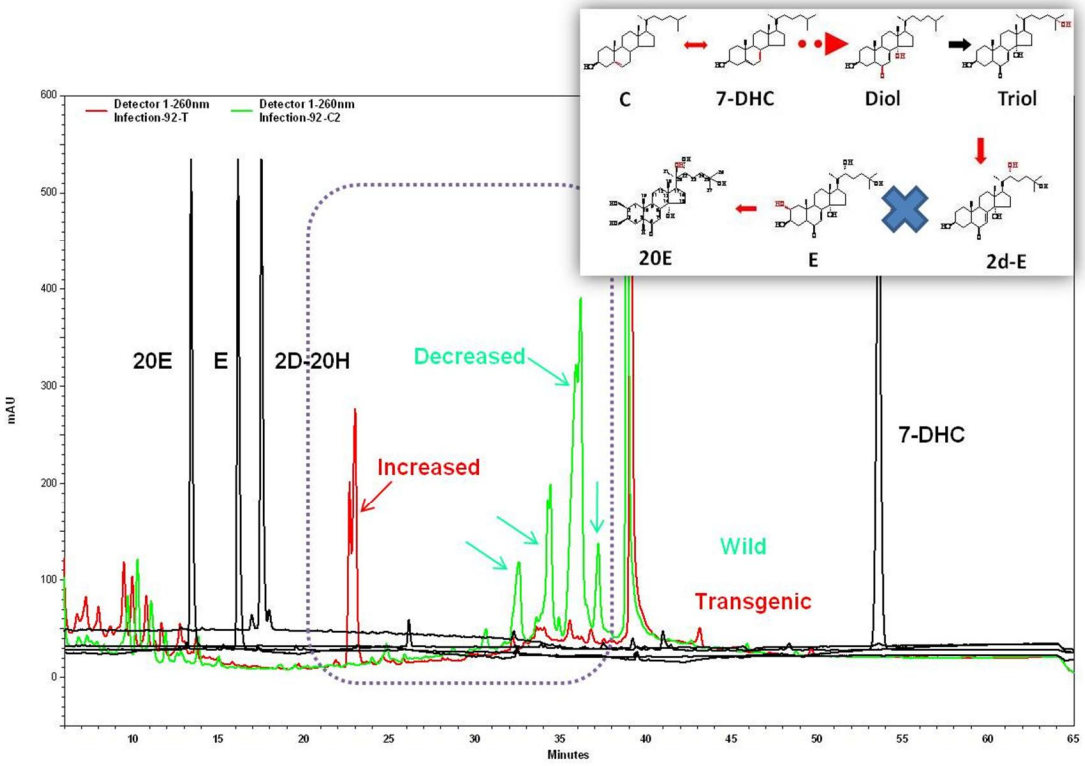


Figure 51. Change of metabolic HPLC profile in transgenic spinach plant with *CYP92A* knock-down expression. Green line: Wild type plant; Red line: the transgenic plant. 20E: 20-Hydroxyecdysone, E: Ecdysone, 2D-20H: 2-deoxy-20-Hydroxyecdysone, 7-DHC: 7-Dehydrocholesterol.

Discussion

Transient and stable expression of genes related PE biosynthesis in plant

PEs are more interesting for their potential role in plant defense mechanism against insects. However, the genes related in PE biosynthesis were unclear in plants until now. For functional studies of candidate genes in PE biosynthesis, an *Agrobacterium*-mediated transformation system was established in *Arabidopsis*, spinach and tobacco for transient and stable expression, respectively. Six transgenic plant lines for transient gene expression were constructed. And eight lines for stable gene expression were constructed. We used a green fluorescent protein (GFP) as a visual selection marker, which allowed us to screen a transgenic line from a large number of untransformed plants. The introduced DNA in putative transgenic plants was confirmed by PCR and RT-PCR, respectively. In conclusion, our results will be provided a potential help to elucidate the PE biosynthetic pathway. Furthermore, these studies may provide means to confer a broad-spectrum insect resistance, which will improve agricultural traits of crop plants.

Over-expression recombinant Cytochrome P450 enzymes in *Escherichia coli*

All P450s, *CYP85, -CYP90B, -CYP92A and CYP314* have been expressed since the codon-usage of four genes was optimized in *E. coli*. The target proteins were purified through two sequential chromatographic steps, Ni^{2+}-NTA agarose affinity and gel-filtration column.

Construction metabolite profile system by HPLC

Metabolite profiling was established by HPLC. This condition could separate metabolites between 7-dehydrocholesterol and 20-hydroxyecdysone in downstream of putative PE pathway. The metabolite changes were found in transgenic tobacco plant for over-expression of *CYP85* gene, leave of spinach for *CYP90B* and *CYP92A*.

CONCLUSION

In this study, the biological activities, biosynthesis and related genes of PEs against herbivorous insect were investigated.

At the conclusion of part Ⅰ, the PE showed a remarkable antifeedant, repellent and insecticidal activity on tested insect pests. These results showed that the PE might operate on the plant defense against non-adapted phytophagous insect.

At the conclusion of part Ⅱ, four candidate genes were isolated from spinach. And then, all codons in each gene were optimized and synthesized for over expression in *E. coli* or plant. Based on the above results, four candidate genes were obtained the over-expression in *E. coli* in part III.

At the conclusion of part Ⅲ, four genes were expressed in *E. coli* for the enzymatic studies. Several transgenic lines against those genes were constructed through the transformation system in PE-positive and negative plants. And finally, metabolite profiling was established by HPLC, and changes of putative PE metabolites were detected in transgenic lines.

Consequently, defensive roles of PE against insects were elucidated, and essential bases (candidate genes, translated proteins & transgenic plant lines) were established for insight into regulation of PE pathway in my thesis.

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