CHAPTER VI
DISCUSSION

Plaunotol is an acyclic diterpene alcohol derived from four isoprene units that are biosynthesised exclusively via the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway (Wungsintaweekul et al., 2007). Of the isoprenes, one molecule of dimethylallyl diphosphate (DMAPP) is attached to the other three molecules of isopentenyl diphosphate (IPP) by a head-to-tail condensation, which is catalysed by the enzyme geranylgeranyl diphosphate synthase (GGPPS), yielding geranylgeranyl diphosphate (GGPP) (Nualkaew et al., 2006). GGPP is subsequently converted to geranylgeraniol (GGOH) by a two-step monodephosphorylation [5] catalysed by a membrane-bound GGPP phosphatase (Nualkaew et al., 2006; Nualkaew et al., 2013). In the final step, GGOH is presumably hydroxylated specifically at the C-18 position to form 18-GGOH or plaunotol, the oil-like substance that accumulates in the chloroplast (Sitthithaworn, 2006).

Of these steps, the enzyme that catalyzes the last reaction of GGOH-18-hydroxylation has not been identified in *C. stellatopilosus*. Our previous studies indicated that although the enzyme activity of GGOH-18-hydroxylase was easily detected in the microsomal fraction in the presence of NADPH (Tansakul and De-Eknamkul, 1998) purification of the enzyme to obtain either a membrane-bound or solubilized form for characterization has not yet been achieved. However, partial characterization of the microsomal fraction suggests that the hydroxylase enzyme is a plant cytochrome P450 (Chanama et al., 2009).

To elucidate the entire biosynthetic pathway of plaunotol, the CsCPR and CsGG18H genes potentially involved in the last GGOH-18-hydroxylation step were identified. In this thesis, the results of the cloning, expression and characterisation of both genes from *C. stellatopilosus* Ohba are described. In addition, the levels of the CsCPR and CsGG18H transcripts in the shoots, stalks and young leaves of *C. stellatopilosus* in correlation with other mRNAs involved in the plaunotol biosynthetic pathway are reported.

6.1 Techniques used to isolate the candidate P450 and CPR gene

Plant cytochrome P450s (CYPs) form a large superfamily of heme-containing monooxygenases that catalyze the majority of oxidative reactions, including hydroxylation, epoxidation, dealkylation, dehydration and carbon-carbon bond cleavage (Schuler and Werck-Reichhart, 2003). P450s are located in the endoplasmic reticulum (ER) and their catalytic activities rely strictly upon the supply of electrons from NADPH-cytochrome P450 reductases (CPRs) (Rana et al., 2013). CPRs [EC 1.6.2.4], are also membrane bound proteins localized to the ER and contain an N-terminal positioned FMN binding domain linked to the NADPH binding domain via the FAD domain [13]. Therefore, during the reactions catalyzed by the coordination of CYPs and CPRs, the CYPs provide a wide variety of different regio- and stereospecific reactions, and the CPRs catalyze the transfer of electrons from NADPH via FAD and FMN to the prosthetic heme group of the CYP proteins to complete the reaction. Through these CYP-catalyzed reactions, the diverse oxygenation patterns of natural products generated by secondary metabolic pathways in plants and microorganisms are achieved (Poulos, 2005).

The PCR-based method was successful to isolated plant cytochrome P450, CsG10H and CsGG18H, and one of plant cytochrome P450 reductase, CsCPR by using three strategies of the primer designation. The primer strategy was depended on gene which involved in the last step of the Plaunotol biosynthetic pathway, the GGOH-18-hydroxylase. GGOH-18-hydroxylase gene had been inhibited the Plaunotol product in P450 inhibition reaction so plant cytochrome P450 is the target gene to achived for primer designation. The primers were fished from the plant P450 in each of the 7 clans (Nelson et al., 1996; Nelson et al., 2004) including clan51, clan 71, clan74, clan85, clan86 and clan97. The strategy I was degenerated primer, the aim of this strategy is to amplify the cytochrome P450 in each clan, the primer was designed cover for P450 motif as possible. For this strategy, gene (core fragment) from clan71, clan86 and clan97 could be amplified and all three were classified in hydroxylation reaction. Three core fragments further used as template for strategy II primer designation for each of 5′RACE and 3′-RACE fragment. The 5′-RACE and 3′-RACE fragment from clan71 and clan97 could be amplified and assembly to the core fragment in strategy I. The consensus sequence of each gene was used as a template to strategy III for full-length of the gene. For CPR gene, all of the three primer strategies were the same as for plant P450. However, one isomer of CPR was isolated from *C. stellatopilosus*.

6.2 Sequence variation and function of genes

Though molecular biological techniques, two genes of plant cytochrome P450, *CsGG18H*, and one gene of plant cytochrome P450 reducase, *CsCPR*, isolated from the leaf part were successfully cloned and transformed into *E. coli* BL21(DE3) strain. The sequence encode for the three genes were analyzed and found motif characteristic of the cytochrome P450 and cytochrome P450 reductase:

For cytochrome P450, CsGG18H were had three specific motifs including heme binding, oxygen binding and ERR-triad motif. For cytochrome P450 reductase motifs characterization, the amino acid of CsCPR was represented all the motifs of CPR including two sites of FMN, FAD, and NADPH binding and one site of the P450 binding.

Its means that genes were classify in the P450 group and CPR group according to the motifs characterization. Because of the classification of these enzymes as the microsome type as membrane bound protein. To know the basic of our enzymes, amino acid sequence encode for each gene was submitted into the TargetP 1.1 to predict the location of the enzyme. No enzyme was reported as chloroplast or mitochondrial location and all two enzymes shown one position of the trans-membrane peptide. The results help to understand the native of the enzyme which had necessary for the step of the enzyme preparation and purification.

6.3 Recombinant protein expression

The recombinant enzymes could be expressed in the pET32a system. The pET system is an expression vector which has specific high affinity with T7 promotor for over expression comparing to the usually pCWOri, used for P450 overexpression (Chang et al., 2007; Hull and Celenza, 2000). The pCWOri, developed by FW Dahlquist which is the protein induced under the *Tac* promotor and widely compatible with the protein strain. The heterologous genes were successfully expressed in yeast (Chau and Croteau, 2004; Chau et al., 2004; Tamaki et al., 2005; Teoh et al., 2006; Zhu et al., 2006), however some of the P450 families have been done in *E. coli* such as *CYP74C* in Rice, CYP79B1 in *Pichea sitchensis* (Kuroda et al., 2005; Naur et al., 2003) and the genes *CsG10H*, *CsGG18H*, and *CsCPR* of *C. stellatopilosus* Ohba used in this study. In case of trying to solubilize the enzyme using the lysis buffer, only the lysis buffer containing the Tris-HCl pH7.6 can

solubilized a bit of the enzyme detected by SDS-PAGE. For characteristic of plant P450 and CPR, as a soluble enzyme the solubilized buffer would be in the step of the enzyme preparing.

6.4 Cytochrome P450 and cytochrome P450 Reductase characterization

Both CsG10H and CsGG18H activities were tested for the rate limiting step, the spectrum chromatogram could be shifted from the wavelength of 413 to 420 nm, confirming that it has P450 characterization. Both patterns of the spectrum chromatograms were relatively the same. The rate limiting step described the catalytic mechanism of P450 that heme-posthetic group in the protein can alternate between the ferrous and oxide (Ferric, Fe^{3+}) states during the electron transport of the reduce (Ferrous, Fe^{2+}) form with carbon monoxide binding that give an absorbance at 450 nm. The 450 nm absorbance peck could not detect from both plant P450 isolated from the *C. stellatopilosus* after CO boubling. The possible reason is P450 in this study was alone did not had a couple enzyme as CPR to donor the electron as real. The *in vitro* was set by adding the strong reducing agent as dithionite in order to change the state of the ferrous which show the characterization of the P450 before incorporated with the CO.

For CsCPR, the enzyme was tested for the electron donor between NADP and NADPH to cytochrome c, the reaction strongly transfer electron in NADPH by increase the 550 nm absorbance peck. CPR is belonging to the ferrodoxin superfamily which could bond to the cytochrome c, cytochrome b5 and cytochrome P450 so in this experiment, CPR isolated from *C. stellatopilosus* could be donor the electron using the NADPH to the cytochrome c represented the CPR characterization.

6.5 Enzymatic reaction

The microsome fractions of CsGG18H were further tested for their functional activities using thin layer chromatography. The products could only produce when the reaction is added with the associated enzyme CsCPR and NADPH as co-enzyme. This means that the P450 needs the associated enzyme CPR to catalyze by donor the electron from NADPH to produce the products. However the co-expression of the P450 and CPR will be need to confirm on the same plasmid.

Thorough sequence comparison confirmed that *CsGG18H* and *CsCPR* can be classified into the CYP97C subfamily, and CPR class I, respectively, according to the previous reports. Within CYP97 family, the enzyme function is still not clear but has been proposed to be involved in the carotenoid pathway, tentatively is classified in the CYP97C which is epsilon-ring carotenoid catalytic group. The closely related CYP97C in *V. vininfera* has not yet reported the exact catalytic reaction.

For CPR, CsCPR is classified in class I with the other dicotyledon plant group and only one isoform from *C. stellatopilosus* Ohba could be isolated.

Plant CRPs are grouped as either A-type or non-A-type based on both the available sequences in 11 phylogenetically distinct clans (Bak et al., 2011) and the phylogenetic trees (Durst and Nelson, 1995). The A-type P450s constitute a monophyletic clade are currently the CYP71 clans whereas the more diverse non-A-type P450s are the remaining 10 clans (Bak et al., 2011). It was originally postulated that the A-type P450s were involved in secondary metabolism, and the non-A-type P450s were involved in primary metabolism (Chapple, 1998; Werck-Reichhart et al., 2002). However, this view is over simplified because there are several non-A-type CYPs that have been categorized in the secondary plant metabolism group (Werck-Reichhart et al., 2002). Biochemically, plant P450s are involved in a wide range of secondary metabolite biosynthetic reactions, which are grouped into three major classes, terpenoids, phenylpropanoids and nitrogen-containing compounds, including alkaloids, cyanogenic glucosides and glucosinolates (Hamberger and Bak, 2013).

6.6 Transcripts of genes

The involvement of *CsGG18H* and *CsCPR* in the biosynthesis of plaunotol is also supported by the results of the real-time expression of the two genes in the shoots, leaves and stalks. The expression of *CsGG18H* clearly showed their correlations with the accumulated content of plaunotol in these plant parts, although it appeared that *GGPPS* had limited expression in the shoots, and *DXS* and *DXP* had limited expression in the twig as well. However, the observed in-parallel levels of *CsGG18H* and *CsCPR* expression in the shoot and twig also suggested that both genes are not closely associated. It is likely that that *CsGG18H* specifically functions in plaunotol biosynthesis, whereas CsCPR has a more common function with other CYPs in the same plant.