CHAPTER 4
DISCUSSIONS

4.1 1-Deoxy-D-xylulose 5-phosphate reductoisomerase from *C. stellatopilosus* Ohba

1-Deoxy-D-xylulose 5-phosphate reductoisomerase (EC 1.1.1.267) catalyzes the conversion of 1-deoxy-D-xylulose 5-phosphate to 2*C*-methyl-D-erythritol 4-phosphate in the presence of NADPH and Mg^{2+} (Takahashi *et al.*, 1998). In this study, the gene encoding 1-deoxy-D-xylulose 5-phosphate.reductoisomerase was cloned and functionally characterized from *C. stellatopilosus* Ohba young leaves. The full-length of *dxr* gene had an open reading frame of 1,404 base pairs, starting with an initiation codon ATG and ending with a termination codon TGA. The deduced amino acid encoding by this cDNA has 468 amino acid residues with a predicted molecular mass of 50.6 kDa and isoelectric point of 5.64.

The *dxr* gene have been firstly cloned and characterized from *E.coli* (Takahashi *et al.*, 1998; Kuzuyama *et al.*, 2000a). In higher plants, *dxr* genes have been reported and the mRNA sequence available at National Center of Bioinformatic Information Center (NCBI) more than 20 plant species. Only 9 plants were characterized in detail such as *Arabidopsis thaliana* (Schwender *et al.*, 1999; Rohdich *et al.*, 2006), *Mentha piperita* (Lange and Croteau, 1999), *Catharanthus roseus* (Veau *et al.*, 2000), *Lycopersicon esculentum* (Rodriguez-Concepcion *et al.*, 2001), *Stevia rebaudiana* (Totte *et al.*, 2003), *Zea mays* (Hans *et al.*, 2004), *Coleus forskohlii* (Engprasert *et al.*, 2005), *Gingko biloba* (Kim, *et al.*, 2006) and *Campthotheca acuminata* (Yao *et al.*, 2006). All the *dxr* nucleotide sequences were about 1,242-1,434 base pairs encoded 406-486 amino acid residues and three domains were found in all plants *dxr* sequence: domain I was chloroplast transit peptide in N-terminal of Dxr, which directed the enzyme to plastids where the DXP pathway operated in plants, domain II was proline-rich motif P(P/Q)PAWPG(R/T) in N-terminal region of Dxr, which was not present in prokaryotic Dxrs (Carretero-Paulet *et al.*, 2002), The last domain was NADPH binding site (GSTGSIGT) in N-terminal region of all plants Dxr (Rane and Calvo, 1997; Lange and Croteau, 1999; Schwender *et al.*, 1999; Kuzuyama *et al.*, 2000b).

The deduced amino acid was analyzed using TargetP (Nielsen *et al.*, 1997; Nielsen *et al.*, 1990). Three domains: chloroplast transit peptide, proline-rich motif and NADPH binding site were found in CsDxr sequence. The premature CsDxr contained the chloroplast transit peptide at the N-terminal of 44 amino acid sequences. Thus, the mature CsDxr after cleave the leader sequence consisted of 424 amino acids. Alignment of the deduced amino acid sequence of CsDxr obtained from this study with the amino acid sequence of several representative Dxrs from other plants, malaria parasite and *E. coli* revealed high identity more than 76% to higher plants, especially *Hevea braziliensis* (90% identity). But it exhibited low similarity to Dxr protein of malaria parasite and *E. coli.* Therefore, *dxr* gene obtained from this study, is belonging to the reductoisomerase family. It can be noted that *dxr* gene of *C. stellatopilosus* located in chloroplast indicated by the chloroplast transit peptide. The results is in line with the investigation of the isoprenoid biosynthesis (Wungsintaweekul and De-Eknamkul,

2005) in *C. stellatopilosus* young shoots and localization of plaunotol in its leaves (Sitthithaworn *et al.*, 2006). Moreover, gene encoding geranylgeranyl diphosphate synthase was shown to be located in the chloroplast (Sitthithaworn *et al.*, 2001).

The catalytic residues of Dxr protein were reported from the *E. coli dxr*-gene deficient mutants (Kuzuyama *et al.*, 2000b). CsDxr amino acid sequence has Glu-231 that important in the conversion of 1-deoxy-D-xylulose 5-phosphate into 2*C*-methyl-D-erythritol 4-phosphate (Kuzuyama *et al.*, 2000b). The amino acid residues of His-153, His-209 and His-257 were shown as part of the binding sites of 1-deoxy-D-xylulose 5-phosphate (Kuzuyama *et al.*, 2000b). Fig. 4.1 showed the location of the catalytic residues, also present in the deduced amino acid of CsDxr.

4.2 1-Deoxy-D-xylulose 5-phosphate reductoisomerase in *C. stellatopilosus* is not rate-limiting step enzyme in plaunotol biosynthesis

The mRNA transcription level of *dxr* gene from *C. stellatopilosus* was determined using semiquantitative RT-PCR. This technique had high reproducibility and reflected trends in gene expression as observed by Northern blot (Burleigh, 2001). Comparison to household gene (18S rRNA) indicated that the *dxr* transcripts in leaves were found in relation to plaunotol accumulation. The result showed that *dxr* mRNA were detected only in shoot and leaves, with the strong expression in leaves and weak expression in shoot. That was found to be associated with plaunotol production. However, the stem *dxr* mRNA was not detected but the plaunotol accumulation was found. It is probably because of two specific primers that used to amplify the part of *dxr* gene were designed from CsDxr from leaves. The mRNA expression in leaves indicated that chloroplast organelle plays an important role in plaunotol biosynthesis. In addition, none of plaunotol was found in root as well as absence of *dxr* gene transcript. From the accumulation curve of plaunotol indicated that plaunotol is not the end product of terpenoid biosynthesis in *C. stellatopilosus*. Plaunotol is accumulated highly in young leaves and decreased gradually. Probably, downstream intermediates such as GGPP and GGOH, serve as substrate for cyclic diterpenoid compounds such as plaunolide, plaunol A, B, etc. and latex.

Previously, several studies demonstrated that gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase play a role in terpenoid biosynthesis (Veau *et al.*, 2000; Walter *et al.*, 2000; Mahmoud and Croteau, 2001). Tomato cDNA encoding a Dxr protein was cloned and the levels of mRNA related the plastid isoprenoid biosynthesis during tomato fruit ripening (Rodriguez-Concepcion *et al.*, 2001). Metabolic engineering of 1-deoxy-D-xylulose 5-phosphate reductoisomerase and menthofuran synthase in *Mentha piperita* increase the essential oil yield (Mahmoud and Croteau, 2001).

Study on β-carotene production in *Elaeis guineensis* demonstrated that gene encoding 1-deoxy-D-xylulose 5-phosphate synthase has different transcription levels during oil palm fruits ripening following weeks after fertilization. However, 1-deoxy-D-xylulose 5-phosphate reductoisomerase gene transcript occurs in all stages after fertilization (Khemvong and

Suvachittanont, 2005). This data indicated that *dxr* gene is not rate limiting step in β-carotene biosynthesis (Khemvong and Suvachittanont, 2005).

From this study, expression of *dxr* mRNA were constant in 1^{st}-5^{th} leaf and decreased in shoot while the amounts of plaunotol were varied, therefore *dxr* gene of *C. stellatopilosus* is involved in the IPP biosynthesis via the DXP pathway. Moreover, Dxr protein is not rate-limiting step in plaunotol biosynthesis.