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Croton stellatopilosus Ohba



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CLONING AND CHARACTERIZATION OF TERPENOID HYDROXYLASE GENE FROM
Croton stellatopilosus Ohba

Mrs. Siriluk Sintupachee

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology

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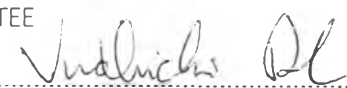


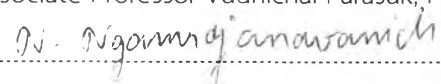
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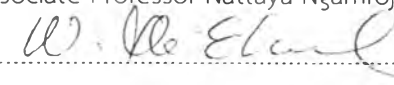
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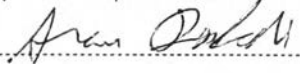
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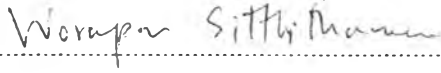
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สิริลักษณ์ สินธุพาชี : การโคลนและลักษณะสมบัติของยีนอะไซคลิกเทอร์ปีนอยด์ไฮดรอกซิเลสจากเปล้าน้อย *Croton stellatopilosus* Ohba. (CLONING AND CHARACTERIZATION OF TERPENOID HYDROXYLASE GENE FROM *Croton stellatopilosus* Ohba) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. นาดยา งามโรจนวิชัย, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. วันชัย ดีเอกนามกุล, 130 หน้า.

ไซโทโครม P450 (CYP หรือ P450) เป็นโปรตีนที่มีเหล็กเป็นส่วนประกอบและเป็นไมโครโซมอลโปรตีนเนื่องจากฝังตัวที่ชั้นเมมเบรนของเอนโดพลาสมิครีติคูลัม ในกระบวนการชีวสังเคราะห์สารประกอบทุติยภูมิในพืช P450 ทำหน้าที่เป็นเอนไซม์เร่งปฏิกิริยาไฮดรอกซิเลชันโดยทำงานร่วมกับไซโทโครม P450 รีดักเทส (cytochrome P450 reductase, CPR) ซึ่งทำหน้าที่ส่งผ่านอิเล็กตรอนให้ P450 เอนไซม์ทั้งสองชนิดมีความเกี่ยวข้องกับกระบวนการชีวสังเคราะห์สารเปลาโนทอล ซึ่งเป็นสารกลุ่มไดเทอร์ปีนที่เป็นองค์ประกอบหลักในเปล้าน้อย โดยที่ในขั้นตอนสุดท้ายของกระบวนการชีวสังเคราะห์จะเป็นการเกิดปฏิกิริยาไฮดรอกซิเลชันของเจอร์รานิลเจอร์รานีโอลเพื่อให้ได้เปลาโนทอล ในการศึกษาจึงใช้เปล้าน้อยเป็นพืชตัวอย่างในการศึกษาเกี่ยวกับยีนที่ควบคุมการสร้าง P450 และ CPR ผลจากการโคลนยีน การศึกษาลำดับกรดอะมิโน และ การวิเคราะห์โดยใช้ความสัมพันธ์ในเชิงวิวัฒนาการ พบยีน P450 ในเปล้าน้อย คือ CsCYP97 และยีนที่มีความสัมพันธ์กับ P450 คือ CsCPR ยีนทั้ง 2 ชนิดแสดงออกใน *E. coli* สายพันธุ์ BL21(DE3) ในพลาสมิด pET32a ผลการตรวจสอบแอคติวิตีพบว่า CsCYP97 สามารถทำงานในปฏิกิริยาที่มีเอนไซม์ CsCPR อยู่เท่านั้น โดย CsCYP97 สามารถเปลี่ยนเจอร์รานิลเจอร์รานีโอลไปเป็นผลิตภัณฑ์ที่เคลื่อนที่ใกล้เคียงกับเปลาโนทอลจากการตรวจสอบบนแผ่นโครมาโทกราฟี เอนไซม์ CsCYP97สามารถแสดงคุณสมบัติเฉพาะของ P450 คือสามารถทำปฏิกิริยากับคาร์บอนมอนอกไซด์ แล้วเปลี่ยนค่าการดูดกลืนแสง จาก 413 นาโนเมตร เป็น 426 นาโนเมตร แอคติวิตีของ CsCPR โดยการตรวจสอบ steady state ของ cytochrome c และ NADPH มีค่า $K_{mcyt C}$, K_{mNADPH} และ V_{max} ตามลำดับ ดังนี้ $10.32 + 2.11 \mu M$, $44.77 + 6.047 \mu M$ และ $0.099 \pm 0.005 \mu M \text{ min}^{-1}$ ผลการแสดงออกของเอ็มอาร์เอ็นเอของยีนทั้ง 2 ชนิดในใบอ่อนของเปล้าน้อย พบว่ามีการแสดงออกที่สัมพันธ์กับยีนอื่นๆในวิถีสังเคราะห์เปลาโนทอล โดยยีนจะแสดงออกในอัตราส่วนสูงในใบที่มีปริมาณสารเปลาโนทอลสูง ซึ่งอาจเป็นไปได้ที่ยีนอาจจะมีส่วนในวิถีสังเคราะห์เปลาโนทอล

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SIRILUK SINTUPACHEE: CLONING AND CHARACTERIZATION OF TERPENOID HYDROXYLASE GENE FROM CROTON STELLATOPILOSUS OHBA. ADVISOR: ASSOC. PROF. DR. NATTAYA NGAMROJANAVANICH, Ph.D., CO-ADVISOR: ASSOC. PROF. WANCHAI DE-EKNAMKUL, Ph.D., 130 pp.

Plant cytochrome P450 (CYP or P450) is a heme-containing enzyme located on the membrane of endoplasmic reticulum (ER) as a microsomal type. It plays an important role in catalyzing a vast variety of metabolic reactions. P450s are closely associated with the cytochrome P450 reductases (CPRs), another group of membrane-bound enzymes required for electron transfer to P450s. Among various groups of secondary metabolites, the terpenoids, which represent the largest class of characterized natural plant compounds, are often substrates for the P450s hydroxylation reactions. Since *Croton stellatopilosus* Ohba (Euphorbiaceae), the Thai medicinal plant producing the acyclic diterpenoid plaunotol, has been shown to involve the P450-dependent hydroxylation in the final step of the biosynthetic pathway of plaunotol, the plant was used in this study as a model for searching the involved P450 and CPR genes. Through the steps of gene cloning, characterization, sequence analysis, and phylogenetic analysis, one candidate of the P450, namely CsCYP97 and one of the associated CPR, namely CsCPR were found from the plant. All the three genes could be expressed in *Escherichia coli* (BL21(DE3)) in pET32a. The microsomal fractions of the P450 protein CsCYP97 appeared to have their enzyme activity only in the presence of CsCPR. The CsCYP97 could catalyze the conversion, presumably hydroxylation, of geranylgeraniol to an unknown compound co-chromatographed closely with plaunotol on TLC plate. The CsCYP97 was proved to be P450 as characterized by rate limiting state of the carbon monoxide, with shifting of the absorbance peak from 413 to 426 nm. For the CsCPR, its activity assay at the steady-state for cytochrome c and NADPH was determined to have its kinetic values for K_m cytC, K_m NADPH and V_{max} of $10.32 \pm 2.11 \mu\text{M}$, $44.77 \pm 6.047 \mu\text{M}$ and $0.099 \pm 0.005 \mu\text{M} \cdot \text{min}^{-1}$, respectively. Real-time PCR analysis of transcripts of CsCYP97 and CsCPR from the shoot to leaves in each position indicated that the expression pattern of CsCPR gene is correlated to the expression of the genes involved in the biosynthesis of plaunotol. Until the current report, the whole genome elucidation of *C. stellatopilosus* Ohba is not available in database. Therefore, the cloning and functional studies of the P450 and CPR enzymes will lead to a better understanding of the metabolic control of plaunotol biosynthetic pathway in the future.

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