3. Output ที่ได้จากโครงการ

3.1. ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

ยังไม่มี

4. ภาคผนวก

4.1. Reprint/manuscript/บทความสำหรับเผยแพร่/กิจกรรมที่เกี่ยวข้องกับการนำผลงานจากโครงการไปใช้ประโยชน์

ยังไม่มี

4.2. การนำเสนอผลงาน

นำเสนอผลงานรูปแบบ โปสเตอร์ เรื่อง Biosynthesis of triterpenes from Croton stellatopilosus and Centella asiatica leaves: Part I, cDNA cloning of oxidosqualene cyclases ในงานประชุม "นักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว." ครั้งที่ 7 ณ โรงแรมแอมบาสซาเดอร์ ซิตี้ จอมเทียน จังหวัดชลบุรี วันที่ 11 – 13 ตุลาคม 2550

นำเสนอผลงานรูปแบบ โปสเตอร์ เรื่อง The α-amyrin producing oxidosqualene cyclases ในงานประชุม TERPNET 2009 ที่จัดขึ้นที่ มหาวิทยาลัยโตเกียว ประเทศญี่ปุ่น วันที่ 25-29 พฤษภาคม 2552

Biosynthesis of triterpenes from *Croton stellatopilosus* and *Centella asiatica* leaves: Part I, cDNA cloning of oxidosqualene cyclases

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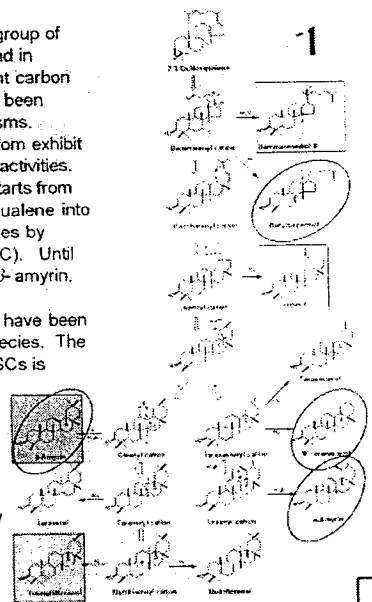
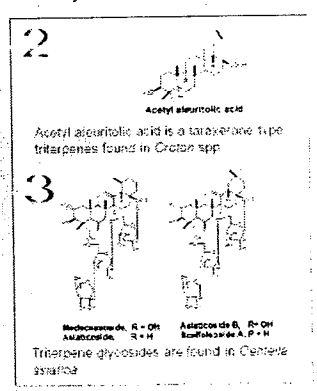
Triterpenoid is a large group of isoprenoidal compounds found in nature. More than 90 different carbon skeletons of triterpenes have been reported from various organisms. Triterpenes in the plant kingdom exhibit a variety of pharmacological activities. Biosynthesis of triterpenes starts from the cyclization of 2,3-oxidosqualene into various skeletons of triterpenes by oxidosqualene cyclases (OSC). Until now several OSCs such as β- amyrin, lupeol, isomultiflorenol, and dammarenediol-II synthases have been cloned from various plant species. The cyclization mechanism of OSCs is shown in **1**.



Cyclization mechanism of plant OSCs

Croton stellatopilosus and *Centella asiatica* were used in this study.

Triterpene found in many *Croton* species is *acetyl* aleuritolic acid (**2**) which in taraxerane skeleton. Major triterpenes in *C. asiatica* (**3**) are asiaticosides and madecassoside (ursane skeleton) which are derived from α-amyrin. It also contains centellasaponin A, centellasaponin B and asiaticoside B (oleanane skeleton) as minor glycosides, which is derived from β-amyrin. Ursane-type glycoside in *C. asiatica* is higher than 30 times to oleanane-type glycoside in this plant.



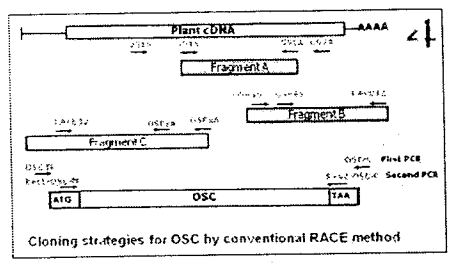
Further works

To reveal the function of these 4 clones, these works will be obtained

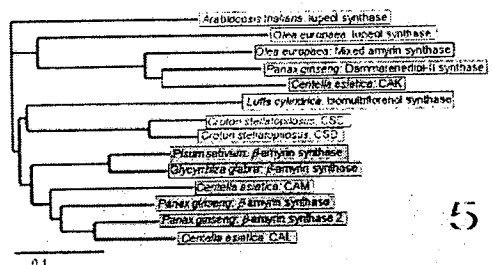
1. Amplification of 5' RACE
2. Amplification of open reading frame of 4 clones
3. Functional analysis of these clones

Experimental

cDNAs were prepared by RT-PCR from total RNA isolated from *Croton sublyratus* and *Centella asiatica* leaves. Homology based PCRs with degenerated primers designed from the known oxidosqualene cyclases were used for amplification of core fragment of triterpene synthase cDNAs. Amplification of 3' end fragments were carried out by RACE technique using specific primers designed from core fragments (**4**). The DNA sequences were analyzed for their homology by DNAsis for window ver. 2.1, Clustal W (1.83) Multiple Sequence Alignments and Treeview 1.6.6.



Cloning strategies for OSC by conventional RACE method



Phylogenetic tree of plant OSCs and 4 clones obtained from this study

Results and Discussions

DNA sequences from central regions to 3'-end termini of 4 OSCs were analyzed for the homology analysis with the other plant OSC fragments (**5**). CSC and CSD showed 88% nucleotide identity suggesting that they might be isogenes. CSC and CSD showed 58% identity to isomultiflorenol synthase isolated from *Luffa cylindrical*. Functional analysis of these 2 clones will be studies after obtaining the open reading frame (ORF) of them. CAL and CAM showed 65% nucleotide identity. Both of them are among other β-amyrin synthases. CAL showed 73-85% identity, while CAM showed 65-68% identity to β-amyrin synthases suggesting CAL codes for β-amyrin synthase but CAM may code for other new OSC.

Acknowledgement

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The α-amyrin producing oxidosqualene cyclases

[redacted]

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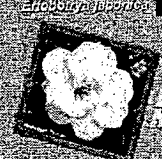
THE UNIVERSITY OF TOKYO


Pisum sativum


Olea europaea


Taraxacum officinale


Eriobotrya japonica


Camellia japonica

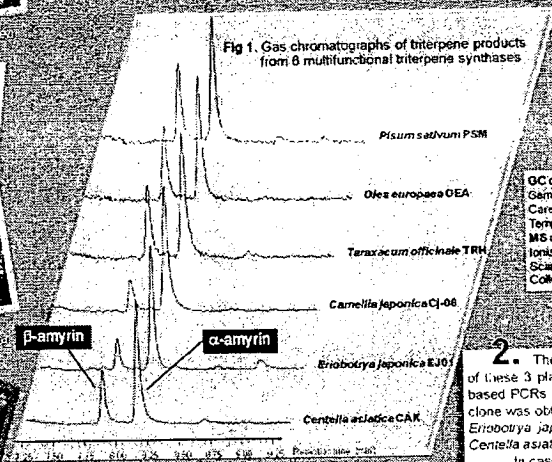

Centella asiatica

1. The cloning of 3 multifunctional triterpene synthases (MTS) genes have been previously reported from higher plants. They are PSM from *Pisum sativum* (Morita et al., 2000), OEA from *Olea europaea* (Saimaru et al., 2007) and TRH from *Taraxacum officinale* (Dokai, 2005). All of these genes encode the enzyme MTS which is responsible for the cyclization of 2,3-oxidosqualene into cyclic triterpenes, which α-amyrin and β-amyrin are main products. The functions of these genes are different from other known OSCs, such as β-amyrin synthases or cycloartenol synthases, which produces sole b-amyrin and cycloartenol, respectively. This leads to the hypothesis that there might be no sole α-amyrin synthase exists in the nature. In order to investigate this hypothesis, three medicinal plants including *Eriobotrya japonica*, *Camelia japonica* and *Centella asiatica*, which are rich in ursane-skeleton triterpenes, were chosen as materials for RNA extraction.

Table 2 Amino acid identity of MTS clones comparison to PSY, β-amyrin synthase from *Pisum sativum*

Clone	CAK	EJ01	CJNEW11	TRH	OEA	PSM
PSY	59	63	60	60	60	79
PSM	63	61	55	56	76	
OEA	74	54	82	81		
TRH	82	54	78			
CJNEW11	76	55				
EJ01	53					

Fig 1. Gas chromatographs of triterpene products from 6 multifunctional triterpene synthases



GC condition
Sampling mode: vaporizer: 250°C, split, sampling 1 min
Career gas: He 45 cm/sec Column: Rtx-5MS
Temp grad: 250°C for 2 min, 10°C/min to 330°C, 330°C for 5 min
MS detector condition
Ionization mode: EI Ion source temp: 250°C
Scan range: 40-500 m/z unit Collection interval: 0.5/s
Collection time: 3-11 min

2. The cDNAs were obtained by reverse transcription from RNA of these 3 plants. The cDNAs of OSCs were cloned using homology based PCRs and RACE techniques. The open reading frame of each clone was obtained by Nested PCR. The obtained OSCs are EJ01 from *Eriobotrya japonica*, CJNEW11 from *Camelia japonica* and CAK from *Centella asiatica*.

In case of CAK, it has been cloned from *Centella asiatica* leaves using the DNA sequence data of CabAS gene (GenBank accession number AY520818) reported previously (Kim et al., 2005). The DNA sequences of CAK revealed 3 different nucleotides from those of CabAS. They are t1892g, t1934c and t2074c which resulted in the different amino acid residues, V631G, V645A and F692L, respectively.

Each OSC was digested with restriction enzymes and ligated into the same restriction sites of pYES2 (Invitrogen) to construct 3 plasmids, pYES2-EJ01, pYES2-CJNew11 and pYES2-CAK. The information of these 3 plasmids, together with 3 known plasmids, (pYES2-PSM, pYES2-OEA, and pYES2-TRH) was shown in Table 1 and 2.

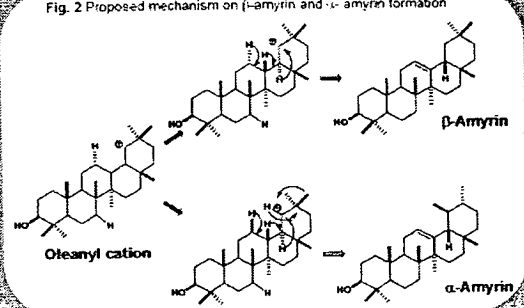
3. Among 6 MTSs, PSM has the lowest amino acid identity to others (53-81%). PSM amino acid sequence are higher similar to the group of β-amyrin synthase than MTS (70% amino acid identity to *Pisum sativum* b-amyrin synthase). The OEA, CAK and TRH share high amino acid identity at (74-82%), while EJ01 shows low amino acid identity to other MTS and also β-amyrin synthase. At least 3 groups of MTS was revealed.

4. Functions of OSCs were analysed by expression in lanosterol synthase auxotroph *Saccharomyces cerevisiae* strain GIL77. The mutant yeast was transformed with each plasmid. The OSC proteins were induced by galactose and the yeast cells were collected and extracted with 20% KOH/50% EtOH. After extraction with the same volume of hexane, the extract was concentrated and analyzed by TLC and GC-MS. The GC chromatograms were shown in Fig. 1.

5. All three OSC clones showed their functions as multi functional triterpene synthases. Major product was α-amyrin while the β-amyrin was second major product. The triterpene profiles are nearly the same pattern in all 6 MTSs although amino acid identities of among MTSs are different (Table 2).

The biosynthesis of α-amyrin is proposed to be branched from β-amyrin at the pentacyclic C-19 cation stage. Migration of methyl group takes place at C-20 leads to α-amyrin while hydride migration takes place from C-18 leads to β-amyrin. It seems easier to regulate migration of methyl group by blocking them through steric hindrance than that of hydride. This might be the reason why β-amyrin is always produced together with α-amyrin. And sole α-amyrin synthase may not exist in the nature. The further mutation study will lead to the understand of the production of α- and β-amyrins.

Fig. 2 Proposed mechanism on β-amyrin and α- amyrin formation



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บทความสำหรับการเผยแพร่โดยย่อ

-เนื่องจากการทดลองในโครงการนี้ ยังไม่เสร็จสมบูรณ์แม้จะปิดโครงการไปก็ตาม จึงยังไม่สมควรเผยแพร่ข้อมูลสู่สาธารณะ -