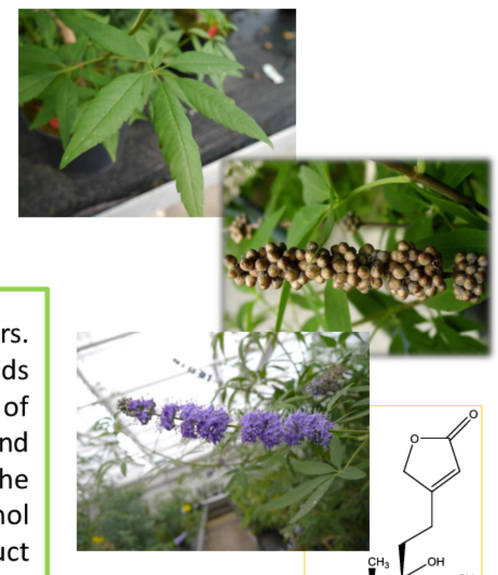
DEPARTMENT OF PLANT AND ENVIRONMENTAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF COPENHAGEN

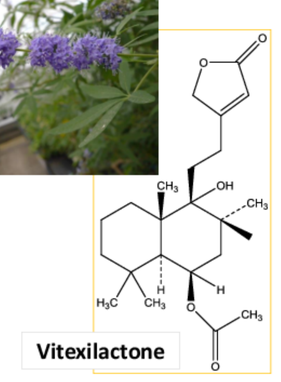
Diterpene synthases participating in the biosynthesis of *Vitex agnus-castus* diterpenoids

Tamil C.M. Sundram [1,2], Allison Maree Heskes[1], Irini Pateraki[1] and Björn Hamberger[1]

[1] Section for Plant Biochemistry, Department of Plant and Environmental Sciences, University of Copenhagen;
[2] Department of Plant Science, Kulliyyah of Science, International Islamic University Malaysia

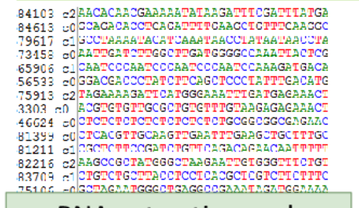


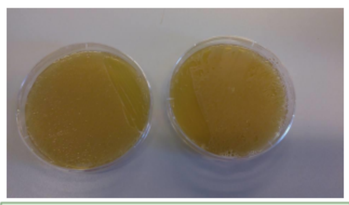
Vitex agnus-castus L. (VAC) is a medicinal plant belonging to the Lamiaceae family whose fruit extracts are used in the treatment of women's menstrual disorders. Additionally, VAC extracts are reported to have dopaminergic activities and anti-cancer properties. The bioactivity of VAC extracts is partly attributed to diterpenoids found in the plant, like vitexilactone[a] and rotundifuran[a]. Currently little is known about the biosynthesis of these diterpenoids. In our effort towards the elucidation of VAC diterpenoid biosynthetic pathways, we have identified six diterpene synthases (diTPSs), three corresponding to class II (VACTPS1, VACTPS3 and VACTPS5) and three from class I (VACTPS2, VACTPS4 and VACTPS6). Functional characterization through transient expression in *Nicotiana benthamiana*, and comparison with the already characterized diTPSs from the related species *Marrubium vulgare* (MvCPS1 and MvELS)[b] demonstrated that the major product of VACTPS1 is peregrinol diphosphate. In combination with VACTPS2 a number of structurally related labdane diterpenoids are formed e.g. 9,13-epoxy-labd-14-ene. Meanwhile, product formation was also detected for expression of VACTPS3 alone. Co-expression of VACTPS3 with VACTPS6 formed abietane-type diterpenoids. Through comparison with already characterized diTPSs, CfTPS1 and CfTPS3 from *Coleus forskohlii,* we identified one of the product as dehydroabietadiene[c]. Coupling of VACTPS3 with VACTPS2 has also resulted in the synthesis of a diterpenoid product. Discovery and characterization of these diTPSs is the critical first step towards understanding VAC diterpenoid biosynthesis.



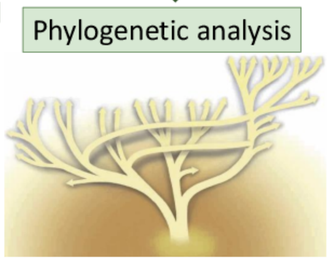
WORK-FLOW


Selection of VAC plant tissues


RNA extraction and transcriptome sequencing


Identification and cloning of candidate genes

Phylogenetic analysis


In planta characterization


Metabolite extraction and analysis

Figure 1: Work flow for identification and functional expression of diTPSs involved in production of *Vitex agnus-castus* diterpenoids.

GC-MS ANALYSIS

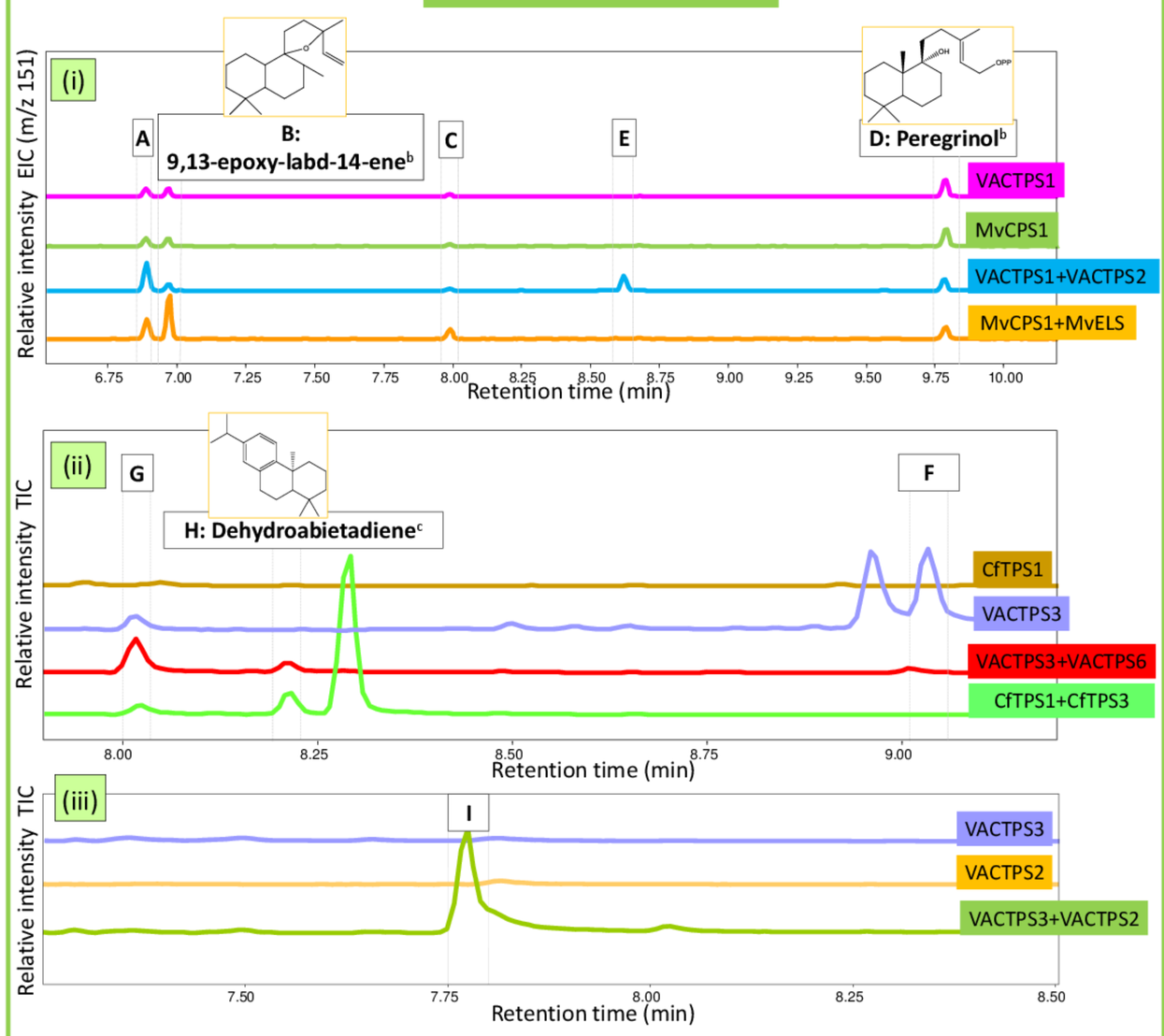


Figure 3: GCMS analysis of hexane extracts from *N. benthamiana* transiently expressing VAC diTPSs. (i) Comparison of product formation of VACTPS1 and VACTPS1+VACTPS2 with MvCPS1 and MvCPS1+MvELS. (ii) Comparison of product formation of VACTPS3 and VACTPS3+VACTPS6 with CfTPS1 and CfTPS1+CfTPS3. (iii) Product formation of VACTPS3+VACTPS2.

PHYLOGENETIC ANALYSIS

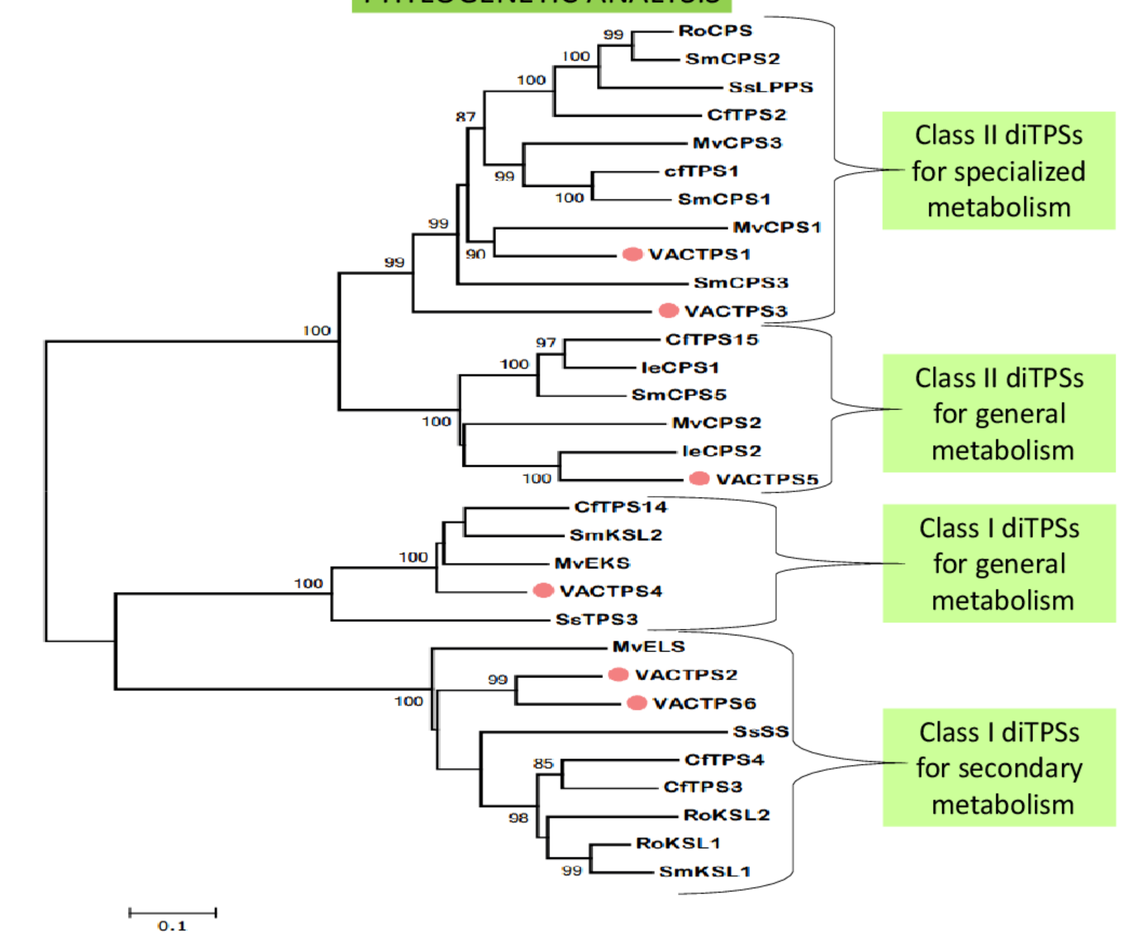


Figure 2: Phylogenetic classification of *Vitex agnus-castus* diTPSs with known class II and class I sequences from Lamiaceae species. Numbers at the branches are bootstrap values that support more than 80% bootstrap confidence.

FUTURE DIRECTION

- Further experiments are planned to fully characterize the diTPSs products.
- Ongoing: Identification and characterization of cythochrome P450 candidate genes that are involved in next steps of VAC diterpenoid biosynthesis
- More advanced biotechnological platforms (i.e. microbial hosts) for the production of clean pharmacological bioactive VAC diterpenoids are being considered.

REFERENCES

a) Heskes, AM et al. *MALDI-MS imaging of diterpenoids in Vitex agnus-castus and characterisation of the enzymes involved in their biosynthesis.* Terpnet 2015
b) Zerbe, P et al. *Diterpene synthases of the biosynthetic system of medicinally active diterpenoids in Marrubium vulgare*. Plant J., 2014, 79(6), pp 914-27, DOI: 10.1111/tpj.12589
c) Pateraki, I. et al. *Manoyl Oxide (13R), the Biosynthetic Precursor of Forskolin, Is Synthesized in Specialized Root Cork Cells in Coleus forskohlii*. Plant Physiol., 2014, 164, pp 1222–1236, DOI: 10.1104/pp.113.228429

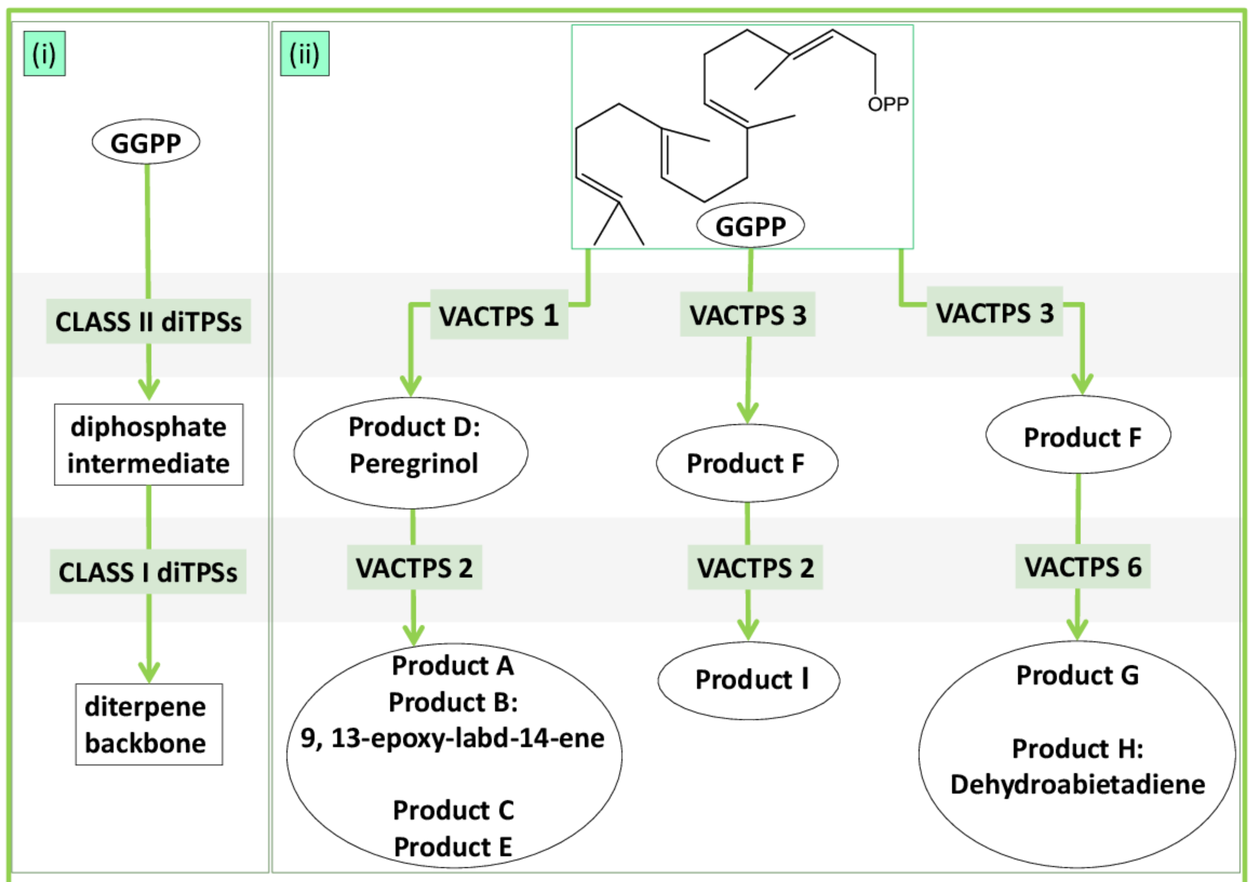


Figure 4: (i) Schematic overview of biosynthesis of diterpene backbone. (ii) Summary on enzymatic reactions catalyzed by class I and class II diTPSs from *Vitex agnus-castus*

The 9th

Conference Book

SCANDINAVIAN PLANT PHYSIOLOGY SOCIETY

PhD STUDENT CONFERENCE

June 20-23rd 2016, Oslo, Norway

SPPS

TOPIC: RESPONSES TO THE ENVIRONMENT

P5: Discovery and characterization of diterpene synthases participating in the biosynthesis of *Vitex agnus-castus* diterpenoids

Tamil C.M. Sundram[1,2], Allison Maree Heskes [1], Birger Lindberg Møller[1], Sylvia Hansen[1] and Paraskevi Pateraki

[1]Section for Plant Biochemistry, Department of Plant and Environmental Sciences, University of Copenhagen;
[2]Department of Plant Science, Kulliyyah of Science, International Islamic University Malaysia,
[3]Department of Biochemistry and Molecular Biology, Michigan State University

Vitex agnus-castus L. (VAC) is a medicinal plant belonging to the Lamiaceae family whose fruit extracts are used in the treatment of women's menstrual disorders. Additionally, VAC extracts are reported to have dopaminergic activities and anti-cancer properties. The bioactivity of VAC extracts is partly attributed to diterpenoids found in the plant, like vitexilactone and rotundifuran. Currently little is known about the biosynthesis of these diterpenoids. In our effort towards the elucidation of VAC diterpenoids biosynthetic pathways, we have identified, isolated and cloned six diterpene synthases (diTPSs) from fruit cDNAs. Three diTPSs corresponds to class II (VACTPS1, VACTPS3 and VACTPS5) and three to class I (VACTPS2, VACTPS4 and VACTPS6). These enzymes are responsible for the cyclization and dephosphorylation of GGPP (geranylgeranyl diphosphate), the general precursor of all diterpenoids. In order to functionally characterize them, they have been heterologously expressed *in vivo* (in *Nicotiana benthamiana* leaves) and *in vitro* (in *E. coli*). The results obtained from the two different characterization methods verify/complement each other. In both cases, diterpenoids synthesized by the selected VAC diTPSs were extracted and analyzed by GC-MS. These results showed that VACTPS1 seems to have a function similar to *Marrubium vulgare* copalyl diphosphate synthase 1 (*Mv*CPS1)[a]. Meanwhile, when compared with the previously characterized OsCPSsyn from *Oryza sativa*, VACTPS3 showed to function as syn-copalyl diphosphaste synthase[b]. Co-expression of VACTPS3 with VACTPS6 formed two structurally related diterpenes. After comparing the products synthesized from this enzyme combination with previously known diTPSs (OsCPSsyn from *Oryza sativa* paired with CfTPS3 from *Coleus forskohlii*), we identified one of the products as dehydroabietadiene[c] while the other is unknown. Discovery and characterization of these diTPSs is the critical first step towards understanding VAC diterpenoid biosynthesis.

REFERENCES: [a]Zerbe, P et al. Diterpene synthases of the biosynthetic system of medicinally active diterpenoids in *Marrubium vulgare*. Plant J., 2014, 79(6), pp 914-27, DOI: 10.1111/tpj.12589 [b] Xu, M et al. Functional identification of rice syn-copalyl diphosphate synthase and its role in initiating biosynthesis of diterpenoid phytoalexin/allelopathic natural products. Plant J., 2004, 39(3), pp 309-318, DOI: 10.1111/j.1365-313X.2004.02137.x [c] Andersen-Ranberg J et al. Expanding the landscape of diterpene structural diversity through stereochemically controlled combinatorial biosynthesis. Angew. Chem.Int. Ed. Engl., 2016, 55, pp 1-6, DOI: 10.1002/anie.201510650