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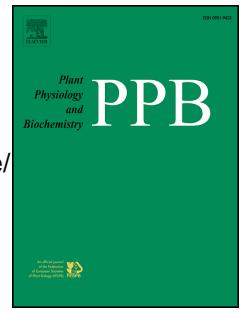

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Additional diterpenes from *Physcomitrella patens* synthesized by copalyl diphosphate/kaurene synthase (*Pp*CPS/KS)

Xin Zhan, Søren Spanner Bach, Nikolaj Lervad Hansen, Christina Lunde, and Henrik Toft Simonsen*
Plant Biochemistry Laboratory, Copenhagen Plant Science Centre, Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg, Denmark.

* Corresponding author: hts@plen.ku.dk

Abstract

The bifunctional diterpene synthase, copalyl diphosphate/kaurene synthase from the moss *Physcomitrella patens* (*Pp*CPS/KS), catalyzes the formation of at least four diterpenes, including *ent*-beyerene, *ent*-sandaracopimaradiene, *ent*-kaur-16-ene, and 16-hydroxy-*ent*-kaurene. The *in planta* enzymatic activity has been confirmed through generation of a targeted *Pp*CPS/KS knock-out mutant in *P. patens* via homologous recombination, transient expression of *Pp*CPS/KS in *N. benthamiana*, and expression in *E. coli*. GC-MS analysis of the knock-out mutant shows that it lacks the diterpenoids, supporting that all are products of *Pp*CPS/KS as observed in *N. benthamiana*. These results provide additional knowledge of the mechanism of this bifunctional diterpene synthase, and are in line with proposed reaction mechanisms in kaurene biosynthesis.

Keywords: diterpene, copalyl diphosphate/kaurene synthase, *Physcomitrella patens*, ent-beyerene, ent-sandaracopimaradiene.

1 Introduction

The bryophyte *Physcomitrella patens* is a non-vascular true land plant that for many years has been used as a model system to investigate plant metabolism and development, and as a green production system for commercially valuable small peptides or metabolites [1-3]. Mosses and flowering plant lineages diverged more than 400 million years ago, placing mosses such as *P. patens* as early progenitors of land-plants [4].

P. patens has a single functional diterpene synthase, the bifunctional copalyl diphosphate/kaurene synthase (*Pp*CPS/KS) that previously was shown to catalyse the biosynthesis of *ent*-kaur-16-ene (**4**) and 16-hydroxy-*ent*-kaurene (**5**), the precursors for gibberellic acid (GA) biosynthesis in vascular plants [5]. This catalytic activity was examined further demonstrating that Ala710 is a key amino acid involved in hydroxylation at the 16 position [6]. *P. patens* also encodes a cytochrome p450 known as kaurene oxidase (CYP701B1) that catalyse the oxidation of *ent*-kaur-16-ene (**4**) to kaurenoic acid. However, *P. patens* lacks any kaurenoic acid oxidase (CYP88-family), which catalyses the formation of GA_{12}, én route to bioactive gibberellins and accordingly no gibberellins are found in *P. patens* [4, 7, 8].

By characterizing *Pp*CPS/KS knockout lines devoid of kaurenoids and other diterpenoids, it was suggested that the function of kaurenoids in mosses is to enhance differentiation from chloronemal to caulonemal tissue and possibly spore formation [9]. Still, the *Pp*CPS/KS knock out lines were viable, had similar cell size, and spore germination as compared to wild type *P. patens,* suggesting a non-essential role of kauranoids in *P. patens* at laboratory conditions [9]. The phenotypically non-adverse consequences of abolishing kaurene biosynthesis in *P. paten* are possibly an indication that *P. patens* is generally not hypersensitive to diterpenes. Indeed overexpression of terpene synthases (patchoulol, santalene, sclareol and taxadiene) in *P. patens* is possible without adverse effects [10-12]. Thus, it has been suggested that *P. patens* is a suitable organism for manipulation of terpenoid metabolism that can be used for biotechnological production [13].

Here we show that under laboratory conditions *ent*-kaur-16-ene (**4**) and 16-hydroxy-*ent*-kaurene (**5**) are not the only products of *Pp*CPS/KS. Specifically, besides *ent*-kaur-16-ene (**4**) and 16-hydroxy-*ent*-kaurane (**5**) we report the detection of *ent*-beyerene (**1**), *ent*-sandaracopimaradiene (**2**) as a result of the enzymatic activity of PpCPS/KS in moss and in heterologous hosts, *N. benthamiana* and *E. coli*. We also observed formation of *ent*-kaur-15-ene (**3**), however, this could not be established as an enzyme product as it is also a breakdown product of 16-hydroxy-*ent*-kaurane (**5**).

The results described here show that the diterpenoid profile of *P. patens* is more complex than previously described with possible evolutionary implications for diterpenoid metabolism in higher plants.

2 Material and Methods

2.1 Plant material and growth conditions

P. patens (Gransden ecotype, International Moss Stock Center #40001) was grown as previously described [14] in a growth chamber with a 16 hour light/8 hour dark cycle at 25°C and with light intensity at 20-50 Wm-2 (700-800 µEinstein just above the plant).

2.2 Full length cDNA sequence confirmation

RNA was isolated from approximately 10-20 mg of fresh tissue using the RNaqueous Micro Kit (Ambion) following the manufacturer's instructions. DNA was digested by DNase treatment and RNA quality was confirmed using a Bioanalyzer 2100 (Agilent). Full length cDNA was prepared using oligo$(dT)_{18}$ primer and the First Strand cDNA Synthesis Kit (Thermo Scientific). The full length *Pp*CPS/KS was PCR amplified using the forward primer 5'-ATGGCTTCCAGCACCTTGATAC-3' (BK109) and the reverse primer 5'-TGAGGTAGTGGCTCGAAGAGC-3' (BK110) and cloned into pJET1.2 using the CloneJET PCR Cloning Kit (Thermo Scientific). Sequences were confirmed using primers binding in the plasmid backbone included in the kit, as well as the internal binding primers 5'-GATGGAAGATGCCAAGGCAC-3' (BK113) and 5'-CATGTTGAAGTCGGCTTTGG-3' (BK114) to get full sequence coverage. Sequence analysis of cDNA for *Pp*CPS/KS prepared from our wild-type moss showed complete sequence identity and exon splicing congruent with the annotation of locus *Pp*1s130_5V6.1 in the CosMoss database, and the GenBank locus XM_001770736. There is a single nucleotide polymorphism at residue 1905, which differs from the sequence (GenBank locus AB302933) described by Hayashi 2006 [5], however this SNP does not result in a change to the amino acid sequence.

2.3 Generation of *P. patens* lines with disrupted *Pp*CPS/KS functionality

Genomic DNA from *P. patens* was prepared as previously reported [15]. A 3 kb fragment of *PpCPS/KS* was amplified by PCR using the primers 5'-CTCTGTCTCTCCCACAATCCTCC-3' (oCL252) 5'-TGGTTTTCCTAACGAACAACTGA-3' (oCL254) and the nested primers 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCTCCAACATACTTGCTCTTCC-3' (oCL253) and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTGAGATCAATAACTGCAAGG-3' (oCL255) including attB1 and attB2 overhangs. The resulting PCR product was cloned into pDONR201 using Gateway technology according to the manufacturer's protocol (Invitrogen). This plasmid is referred to as pDONR201::CPS/KS. A cassette containing p35S-nptII-CamVter, expressing neomycin phosphotransferase II and conferring resistance to G418, was excised from pMBL6 (http://www.biology.wustl.edu/moss/pmbl6.jpg) using XhoI and inserted into the XhoI site in pDONR201::CPS/KS generating the knock out construct pDONR201::CPS/KS-nptII (referred to in this manuscript as pCL755). 30 µg of linearized pCL755 DNA was transformed into *P. patens* using

polyethylene glycol (PEG) mediated transformation and stable lines were recovered and analyzed by GC-MS as previously described [14, 16]. Genomic DNA from stable *P. patens* lines without detectible *ent*-kaurene was prepared as previously described [14]. Sequencing of PCR products using the primers 5'-AAGCCGACTTCAACATGTG-3' (oSSB94), 5'-AACCCGAAGCTCTTCCAC-3' (oSSB95), 5'-CCTGTGCAAGGTAAGAAGATGG-3' (oCL77) and 5'-CTCTGTCTCTCCCACAATCCTCC-3' (oCL252) was carried out to confirm insertion of the nptII cassette. One *P. patens* line, pCL755#29, with disrupted *Pp*CPS/KS functionality, was chosen for more detailed characterization.

2.4 Cultivation and Sampling

For the SPME analysis of the wild type (WT) and *Pp*CPS/KS knockout (KO) *P. patens* lines, plants were cultured on 3 ml of solid Phy B agarose media in 20 ml glass vials with screw neck and magnetic caps with teflon coated silicone septa [14]. Cultivation conditions were 16 hour light/8 hour dark cycle at 25°C. After 2-3 weeks cultivation, the headspace volatiles of the WT and KO line were sampled by incubating a SPME fibre (DVB/CAR/PDMS; Supelco 57298-U) in the vials for 30 min at room temperature directly a rack on the CTC AOC-5000 auto sampler and analysed by GC-MS [17].

For the hexane extracts, protonemal tissue of both WT and KO lines were cultured for one week on cellophane covered Phy B plates, sealed with breathable 3M surgical tape. The moss tissue was then transferred into a 2 mL glass vial containing 1 mL n-hexane including a 1-eicosene internal standard (0.2 mg/L). The extraction was performed for 1 hour at 25°C, and the hexane fraction was transferred to a GC vial. Reference compounds of C7-C30 for calculation of the retention index values and 1-eicosene were obtained from Sigma-Aldrich, Denmark.

2.5 Construction of vectors pLife33_PpCPSKS and pGG_USER_ Δ43PpCPSKS

USER cloning [18, 19] was employed to insert the full or partial (bases 130-2642) CDS for *Pp*CPS/KS, into vectors pLife33 (for transient expression in *N. benthamiana*) and pGG_USER (for expression in *E. coli*), primers: GGCTTAAXatggcttccagcaccttgatacagaatc, GGCTTAAXATGaagaagcgtcgatgtttgcgacctac and GGTTTAAXtcaggtactggctcgaagagcacc (X = deoxy Uridine). pGG_USER was a modified version of the pACYCDuet(P15A) derived pGG [20] with a USER-Cassette replacing a Gateway-cassette in the multiple cloning site. Resulting expression vectors pLife33_PpCPSKS and pGG_USER_Δ43PpCPSKS were verified by sequencing before being further used.

2.6 Expression of *Pp*CPSKS in *N. benthamiana*

Transient expression of *Pp*CPSKS with the suppressor of silencing P19 [21] in leafs of 4-week old *N. benthamiana* was accomplished by co-infiltration of transformed *Agrobacterium tumefaciens*, strain AGL-1, as recently described [22]. 7 days post infiltration, infiltrated leafs were placed in 20 ml capped glass vials (teflon coated silicone septa) and volatiles were sampled with a conditioned SPME fiber (DVB/CAR/PDMS)

for 20 min at 60˚C [17]. In addition, liquid extractions of two leaf discs (ø=3cm) with 1 ml hexane were done in capped glass vials by gentle shaking for 30 min at room temperature. Samples were stored at -20˚C until further used.

2.7 Expression of *Pp*CPSEKS and *Ff*CPSKS in *E. coli*

E. coli strain C41 (Lucigen) was co-transformed with pGG_USER_Δ43PpCPSKS or pGG-*Ff*CPSKS (*Fusarium fujikuroi* CPS/KS) and pIRS (this facilitate overexpression of 1-deoxy-D-xylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductase, and isopentenyl diphosphate isomerase [23]) by electroporation. Recombinant strains were subsequently grown on LB agar plates with chloramphenicol and spectinomycin for selection (used for selection in all later steps). An overnight culture was started by inoculating 3 colonies in 5 ml LB media with selection and shaking at 200 rpm at 37˚C overnight. The following day expression cultures were started by diluting the overnight culture 1:50 in LB media with selection. Growth was continued until OD_{600} = 0.6 and the expression culture was cooled on ice and heterologous expression induced by addition of 1mM IPTG. The induced expression culture was grown for 24 hours at 20˚C with shaking at 180 rpm. Finally, volatile metabolites were sampled with a conditioned DVB/CAR/PDMS SPME fiber at 60˚C for 20 min from the headspace of 15 ml expression cultures in a capped 20 ml glass vial (teflon coated silicone septa).

2.8 GC-MS analysis

All GC-MS analyses were performed on a Shimadzu - GCMS-QP2010 plus (GC-2010) with a CTC autosampler AOC-5000, with cooled trays, agitation oven, and needle bake-out.

GCMS analysis utilizing SPME fibers was previously published [16, 17], but briefly the injection port temperature was set to 230°C, with a sampling time of 1 min. The flow control mode was pressure control with a total flow of 2.3 mL/min with H_2 as carrier gas, and a purge flow of 1.0 mL/min. The column was a 30 m HP-5MS column. The oven temperature program was 35°C for 3 min, 10°C/min to 230°C and a hold for 3 min. The MS settings were ion source temperature 260°C, interface temperature 280°C, and the scan range from m/z 50 to m/z 350 with 70eV electrical ionization.

GC-MS analysis of the hexane extract utilized previously published methods [24, 25]. Briefly, the injection volume was 1 µL, with split-less injection at 250°C. The column was a 30 m HP-5MS column, with a total flow of 2.2 mL/min H_2 as carrier gas, using pressure control as flow control. The oven temperature program was 50°C for 3 min, 15°C/min to 310°C and then a hold for 5 min. The MS settings were ion source temperature 260°C, interface temperature 280°C, solvent cut was set for 5 min, and the scan range was from m/z 50 to m/z 350 with 70eV electrical ionization.

All data were analysed using the Shimadzu software Lab Solutions, GCMS Solutions Version 2.70, using the libraries provided by NIST (NIST 08) and Wiley (Wiley 8.0). Obtained spectra were compared with the

spectra in the mass spectral libraries. Compounds were identified comparing the data with library information of MS and retention indices (*I*). All reference *I*s were taken from Adams book [26].

3 Results and Discussion

The two diterpene metabolites *ent*-kaur-16-ene (**4**) and 16-hydroxy-*ent*-kaurene (**5**) that were previously described as part of a hexane extract of wild type *P. patens* [5] were also observed in our volatile and liquid metabolite analyses of wild type *P. patens* along with three additional diterpenes detected by headspace solid phase micro-extraction (HS-SPME). The three additional diterpenes were identified as *ent*-beyerene (**1**), *ent*-sandaracopimaradiene (**2**) and *ent*-kaur-15-ene (**3**) (Fig. 1A). Identification was based on comparison of the MS-spectra with the libraries from Wiley and NIST and on the Kovats retention index of the diterpenoids [26]. The identification was also established with comparison to the products of the already characterized enzyme *Ff*CPS/KS [27]. The Kovats retention indices were determined on a HP5 column, with references from a DB5 column in parentheses **1**:1944 (1931), **2**:1960 (1969) **3**:2009 (1997), and **4**: 2057 (2043) (Fig. 1A-C).

We constructed a *Pp*CPS/KS knock out line of *P. patens* (Fig. 1D), in order to investigate whether the additional diterpenoids (**1-3)** are likely biosynthetic products of this enzyme. In our fully viable knockout line **1-5** were not detected, showing that *Pp*CPS/KS is needed for their biosynthesis. To further establish whether **1-3** are true enzymatic products of *Pp*CPS/KS we expressed the enzyme in *E. coli* together with *Abies grandis* GGPPS, as well as transiently in *N. benthamiana.* When *Pp*CPS/KS was expressed in these heterologous systems the same diterpenoids was produced as found in wild type *P. patens* (Fig. 1A and 1B). This suggests that **1**-**5** are all products of *Pp*CPS/KS. However, the presence of **3** could as well be a result of chemical rearrangement of **5**. In extracts stored for longer periods (months) it was observed that the levels of **3** increased with storage time in parallel with decreases in levels of **5**, showing that **5** was converted into **3**. Further supporting that non-enzymatic conversion of terpenoids can occur, a recent study has demonstrated instability of another hydroxylated terpene synthase product [17]. More studies are needed to determine whether **3** is a true enzymatic product of *Pp*CPS/KS and what the rate of conversion of **5** to **3** is.

While we exclusively found **5** in hexane extract of *P. patens*, we only detected **1** and **2** by HS-SPME analysis. On the other hand, **4** and **3** were found in both analysis methods (Fig. 1A and 1B). These detection patterns may reflect a volatile nature of **1**, **2** and a less volatile nature **5** at 60°C (temperature used during fiber exposure). Our results highlight the importance of using complementary methods to reveal metabolites with different volatility and further supports the use of HS-SPME as a valuable tool for discovery and characterisation of volatile metabolites [16, 17]. A lack of head-space sampling in former studies of *Pp*CPS/KS may explain why **1**, **2** have not previously been found [5, 6].

Our findings are in support of the previously reported biosynthesis of *ent*-kaurene and 16-hydroxy-*ent*-kaurene by *Pp*CPS/KS [5], and further extend the product profile for this multiproduct enzyme under the described conditions. In addition, our findings support quantum chemical calculations of the carbocation intermediates in biosynthesis of *ent*-copalyl diphosphate derived diterpenes [[5, 28]. It was previously discussed whether the secondary carbocations exists during the triple shift reaction that leads to *ent*-kaurene formation [28, 29]. Our findings show that the secondary carbocation at C13 can be stabilized in *Pp*CPS/KS for **1** to be formed through proton abstraction (Fig. 2). Similarly **2** can be formed by proton abstraction.
The results show that *Pp*CPS/KS is a multiproduct enzyme. *Pp*CPS/KS is one of the evolutionary earliest occurring plant diterpene synthases. Thus, *Pp*CPS/KS may have been a multiproduct platform for later evolved diterpene synthases in plants.

4 Conclusions

The PpCPS/KS enzyme in *Physcomitrella patens* catalyzes the *in planta* formation of *ent*-beyerene (**1**), *ent*-sandaracopimaradiene (**2**), *ent*-kaur-16-ene (**4**), and 16-hydroxy-*ent*-kaurene (**5**), from the common diterpenoid precursor geranylgeranyldiphosphate (GGPP). The activity has been confirmed through generation of a knockout mutant of *Pp*CPS/KS, transient expression in *N. benthamiana* and *E.coli*. These results extent the product profile of *Pp*CPS/KS and supports previous quantum calculations of carbocation intermediates in biosynthesis of *ent*-copalyl diphosphate derived diterpenes.

5 Acknowledgements

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6 Contributions

Christina Lunde established the first knockout mutant of *Pp*CPS/KS in our lab, and commenced the work. Søren Spanner Bach performed the first chemical and genomic characterization, Xin Zhan identified the new diterpenoids during the chemical characterization and in parts wrote the manuscript. Nikolaj L. Hansen performed the characterization in *N. benthamiana* and *E. coli* along with the expression and characterization

of the *Ff*CPS/KS. Henrik T. Simonsen assisted the chemical identification, manuscript writing and sustained the project.

7 References

[1] A. Anterola, et al., Gibberellin precursor is involved in spore germination in the moss *Physcomitrella patens*, Planta, 229 (2009) 1003-1007.

[2] E.L. Decker, R. Reski, Current achievements in the production of complex biopharmaceuticals with moss bioreactors, Bioprocess Biosyst Eng, 31 (2008) 3-9.

[3] H.T. Simonsen, et al., Perspectives on Using *Physcomitrella Patens* as an Alternative Production Platform for Thapsigargin and Other Terpenoid Drug Candidates, Perspect Medicin Chem, 3 (2009) 1-6.

[4] S.A. Rensing, et al., The Physcomitrella Genome Reveals Evolutionary Insights into the Conquest of Land by Plants, Science, 319 (2008) 64-69.

[5] K. Hayashi, et al., Identification and functional analysis of bifunctional ent-kaurene synthase from the moss *Physcomitrella patens*, FEBS Lett, 580 (2006) 6175-6181.

[6] H. Kawaide, et al., Identification of the single amino acid involved in quenching the ent-kauranyl cation by a water molecule in ent-kaurene synthase of *Physcomitrella patens*, FEBS J, 278 (2011) 123-133.

[7] A. Anterola, E. Shanle, Genomic insights in moss gibberellin biosynthesis, Bryologist, 111 (2008) 218-230.

[8] S. Miyazaki, et al., The CYP701B1 of *Physcomitrella patens* is an ent-kaurene oxidase that resists inhibition by uniconazole-P, FEBS Lett, 585 (2011) 1879-1883.

[9] K.-i. Hayashi, et al., Endogenous Diterpenes Derived from ent-Kaurene, a Common Gibberellin Precursor, Regulate Protonema Differentiation of the Moss *Physcomitrella patens*, Plant Physiol, 153 (2010) 1085-1097.

[10] A. Anterola, et al., Production of taxa-4(5),11(12)-diene by transgenic *Physcomitrella patens*, Transgenic Res, 18 (2009) 655-660.

[11] X. Zhan, et al., Metabolic engineering of the moss *Physcomitrella patens* to produce the sesquiterpenoids patchoulol and α/β-santalene, Front Plant Sci, 5 (2014) 636.

[12] X.-W. Pan, et al., Sclareol production in the moss Physcomitrella patens and observations on growth and terpenoid biosynthesis, Plant Biotechnol Rep, (2015) 1-11.

[13] N.K.B.K. Ikram, et al., Stable Heterologous Expression of Biologically Active Terpenoids in Green Plant Cells, Front Plant Sci, 6 (2015).

[14] S.S. Bach, et al., Heterologous stable expression of terpenoid biosynthetic genes using the moss *Physcomitrella patens*, in: M. Rodríguez-Concepción (Ed.) Plant Isoprenoids Springer New York, New York, USA, 2014, pp. 257-271.

[15] K. Edwards, et al., A Simple and Rapid Method for the Preparation of Plant Genomic DNA for PCR Analysis, Nucleic Acids Res, 19 (1991) 1349-1349.

[16] D.P. Drew, et al., A Comparison of Headspace Solid-phase Microextraction and Classic Hydrodistillation for the Identification of Volatile Constituents from *Thapsia* spp. Provides Insights into Guaianolide Biosynthesis in Apiaceae, Phytochem Anal, 23 (2012) 44-51.

[17] T.B. Andersen, et al., Optimization of biochemical screening methods for volatile and unstable sesquiterpenoids using HS-SPME-GC-MS, Chromatography, 2 (2015) 277-292.

[18] F. Geu-Flores, et al., USER fusion: a rapid and efficient method for simultaneous fusion and cloning of multiple PCR products, Nucleic Acids Res, 35 (2007) e55.

[19] H.H. Nour-Eldin, et al., Advancing uracil-excision based cloning towards an ideal technique for cloning PCR fragments, Nucleic Acids Res, 34 (2006) e122.

[20] A. Cyr, et al., A modular approach for facile biosynthesis of labdane-related diterpenes, J Am Chem Soc, 129 (2007) 6684-6685.

[21] O. Voinnet, et al., An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus, Plant J, 33 (2003) 949-956.

[22] S.S. Bach, et al., High throughput testing of terpenoid biosynthesis candidate genes using transient expression in *Nicotiana benthamiana*, in: M. Rodríguez-Concepción (Ed.) Plant Isoprenoids Springer New York, New York, USA, 2014, pp. 245-255.

[23] D. Morrone, et al., Increasing diterpene yield with a modular metabolic engineering system in *E. coli*: comparison of MEV and MEP isoprenoid precursor pathway engineering, Appl Microbiol Biotechnol, 85 (2010) 1893-1906.

[24] H.T. Simonsen, et al., Chemical Composition and Antibacterial Activity of the Leaf Essential Oil of *Baccharis magellanica* (Lam.) Pers. and *Baccharis elaeoides* Remy From Chile, J Essent Oil Res, 21 (2009) 377-380.

[25] H.T. Simonsen, et al., Ethnopharmacological evaluation of radal (leaves of *Lomatia hirsuta*) and isolation of 2-methoxyjuglone, BMC Comp Alt Med, 6 (2006) 29.

[26] R.P. Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed., Allured Pub Corp, Carol Stream, IL, USA, 2007.

[27] T. Toyomasu, et al., Cloning of a Full-length cDNA Encoding ent-Kaurene Synthase from *Gibberella fujikuroi*: Functional Analysis of a Bifunctional Diterpene Cyclase, Biosci Biotechnol Biochem, 64 (2000) 660-664.

[28] Y.J. Hong, D.J. Tantillo, Formation of Beyerene, Kaurene, Trachylobane, and Atiserene Diterpenes by Rearrangements That Avoid Secondary Carbocations, J Am Chem Soc, 132 (2010) 5375-5386.

[29] Y.J. Hong, et al., Changes in Charge Distribution, Molecular Volume, Accessible Surface Area and Electronic Structure along the Reaction Coordinate for a Carbocationic Triple Shift Rearrangement of Relevance to Diterpene Biosynthesis, J Phys Chem A, 116 (2012) 8902-8909.

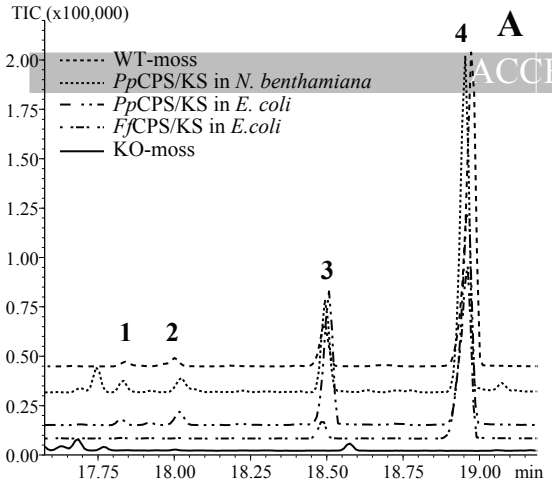
Figure legends:

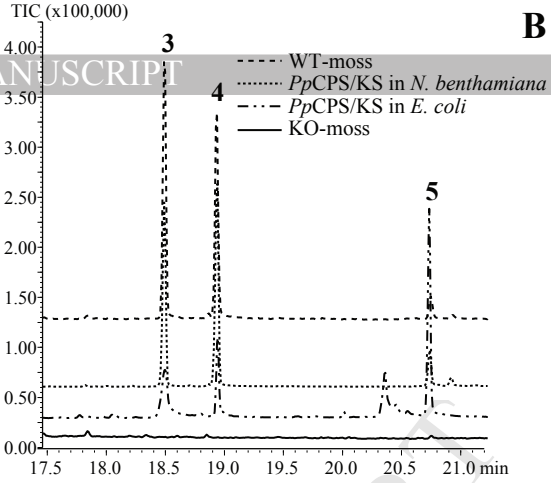
Figure 1:

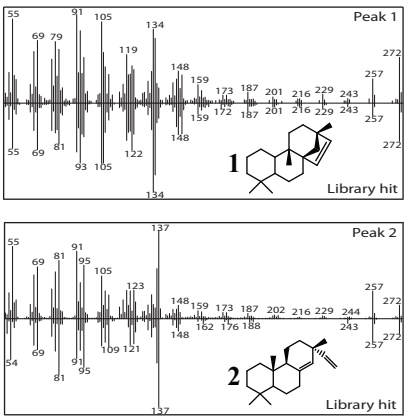
Gas chromatography chromatograms illustrating the presences of *ent*-beyerene (**1**), *ent*-sandaracopimaradiene (**2**), *ent*-kaur-15-ene (**3**), *ent*-kaur-16-ene (**4**), and 16-hydroxy-*ent*-kaurene (**5**). A: HS-SPME analysis of wild type *P. patens*, *Pp*CPS/KS knock-out *P. patens*, *Pp*CPS/KS expressed in *N. benthamiana* and *E. coli*, and *Ff*CPS/KS expressed in *E. coli*. B: hexane extract analysis of wild type *P. patens*, *Pp*CPS/KS knock-out *P. patens*, and *Pp*CPS/KS expressed in *N. benthamiana* and *E. coli*. C: shows the mass spectrum of the detected compounds. D: shows the insertion of NptII cassette into *Pp*CPS/KS gene that provided the knock-out of *Pp*CPS/KS through disruption of the gene transcription.

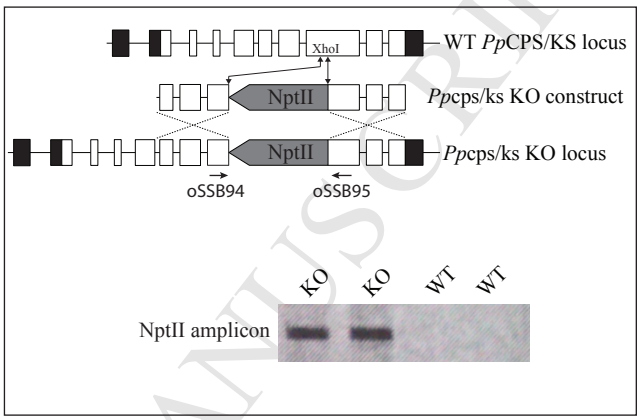
Figure 2:

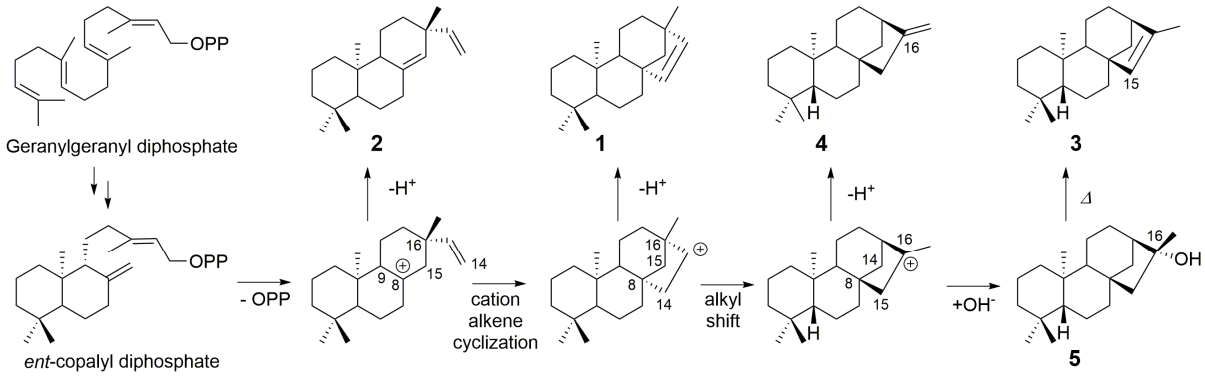
The biosynthetic and heat induced rearrangement route to *ent*-beyerene (**1**), *ent*-sandaracopimaradiene (**2**), *ent*-kaur-15-ene (**3**), *ent*-kaur-16-ene (**4**), and 16-hydroxy-*ent*-kaurene (**5**), via three intermediate carbocations. The mechanisms shown is based on previous publications [5, 6, 28].











Additional diterpenes from *Physcomitrella patens* synthesized by copalyl diphosphate/kaurene synthase (*Pp*CPS/KS)

- We found additional diterpenes produced by the copalyl diphosphate/kaurene synthase (PpCPS/KS)
- We made a knock-out mutant of PpCPS/KS in *Physcomitrella patens*
- The findings support the suggested biochemical route
- The findings confirm that *Pp*CPS/KS is a multiproduct enzyme that may have provide the evolutionary platform for later evolved diterpene synthases in plants.

Christina Lunde established the first knockout mutant of *Pp*CPS/KS in our lab, and commenced the work. Søren Spanner Bach performed the first chemical and genomic characterization.

Xin Zhan identified the new diterpenoids during the chemical characterization and in parts wrote the manuscript.

Nikolaj L. Hansen performed the characterization in *N. benthamiana* and *E. coli* along with the expression and characterization of the *Ff*CPS/KS.

Henrik T. Simonsen assisted the chemical identification, manuscript writing and sustained the project.