Accepted Manuscript

Molecular cloning and functional characterization of three terpene synthases from unripe fruit of black pepper (*Piper nigrum*)

Zhehao Jin, Moonhyuk Kwon, Ah-Reum Lee, Dae-Kyun Ro, Juraithip Wungsintaweekul, Soo-Un Kim

PII: S0003-9861(17)30709-9

DOI: 10.1016/j.abb.2017.12.011

Reference: YABBI 7612

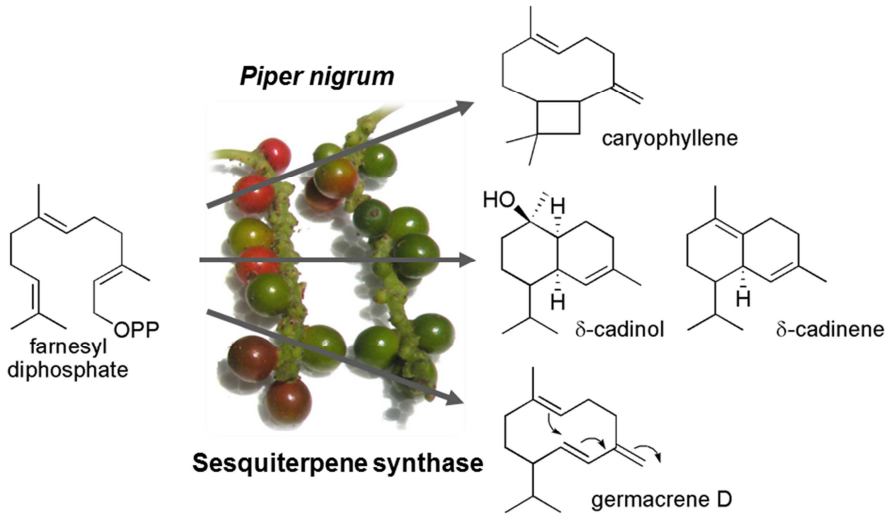
To appear in: *Archives of Biochemistry and Biophysics*

Received Date: 16 October 2017

Revised Date: 11 December 2017

Accepted Date: 13 December 2017

Please cite this article as: Z. Jin, M. Kwon, A.-R. Lee, D.-K. Ro, J. Wungsintaweekul, S.-U. Kim, Molecular cloning and functional characterization of three terpene synthases from unripe fruit of black pepper (*Piper nigrum*), *Archives of Biochemistry and Biophysics* (2018), doi: 10.1016/j.abb.2017.12.011.



Molecular cloning and functional characterization of three terpene synthases from unripe fruit of black pepper (*Piper nigrum*)

Zhehao Jin[a], Moonhyuk Kwon[a, b], Ah-Reum Lee[a], Dae-Kyun Ro[b], Juraithip Wungsintaweekul[3], Soo-Un Kim[a, d, *]

[a] Department of Agricultural Biotechnology and Research Institute for Agricultural Sciences, Seoul National University, Seoul 08826, Republic of Korea

[b] Department of Biological Sciences, University of Calgary, Calgary, Canada

[c] Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkla, Thailand

[d] College of Horticulture and Gardening, Yangtze University, Jingzhou, Hubei, China

*Corresponding author. Department of Agricultural Biotechnology and Research Institute for Agricultural Sciences, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Republic of Korea

E-mail address: soounkim@snu.ac.kr

ABSTRACT

To identify terpene synthases (TPS) responsible for the biosynthesis of the sesquiterpenes that contribute to the characteristic flavors of black pepper (*Piper nigrum*), unripe peppercorn was subjected to the Illumina transcriptome sequencing. The BLAST analysis using amorpha-4,11-diene synthase as a query identified 19 sesquiterpene synthases (sesqui-TPSs), of which three full-length cDNAs (*PnTPS1* through *3*) were cloned. These sesqui-TPS cDNAs were expressed in *E. coli* to produce recombinant enzymes for *in vitro* assays, and also expressed in the engineered yeast strain to assess their catalytic activities *in vivo*. PnTPS1 produced β-caryophyllene as a main product and humulene as a minor compound, and thus was named caryophyllene synthase (PnCPS). Likewise, PnTPS2 and PnTPS3 were, respectively, named cadinol/cadinene synthase (PnCO/CDS) and germacrene D synthase (PnGDS). PnGDS expression in yeast yielded β-cadinene and α-copaene, the rearrangement products of germacrene D. Their k_{cat}/K_m values (20-37.7 s^{-1} mM^{-1}) were comparable to those of other sesqui-TPSs. Among three PnTPSs, the transcript level of *PnCPS* was the highest, correlating with the predominant β-caryophyllene biosynthesis in the peppercorn. The products and rearranged products of three PnTPSs could account for about a half of the sesquiterpenes in number found in unripe peppercorn.

Keywords: Black pepper; Cadinol/cadinene synthase; Caryophyllene synthase; Germacrene D synthase; Next-generation sequencing; *Piper nigrum*

41

42

43 **Abbreviations**

CO/CDS	Cadinol/cadinene synthase
CPS	Caryophyllene synthase
GDS	Germacrene D synthase
GC-MS	Gas chromatography-mass spectrometry
NGS	Next-generation sequencing
Nt	Nucleotide
ORF	Open reading frame
PnTPS	*Piper nigrum* terpene synthase
QRT-PCR	Quantitative real-time polymerase chain reaction
SC	Synthetic complete
sesqui-TPS	Sesquiterpene synthase
TPS	Terpene synthase

44

45

1. Introduction

Black pepper, dried unripe fruit of *Piper nigrum* L. belonging to Piperaceae family, has been one of the most valued spices in the world from ancient times. It was found with the mummy of Ramesses II of ancient Egypt, confirming its ancient uses dating back at least as early as 13th century BCE [1]. According to Pliny the Elder, black pepper was already a wide-spread seasoning in the Roman Empire [2]. *P. nigrum* is native to India, but currently its leading producer is Vietnam, which produces 34% of the world-wide production of 470,000 metric ton [3]. Black pepper has been used in both cooking and traditional medicine to cure constipation, toothaches, oral abscesses, and sunburn, among others [4]. The spicy pungent principle of black pepper is alkaloid piperine and its structural analogs. Modern pharmacological studies support the beneficial health effect of black pepper [5]. Piperine has been shown to help digestion of food [6], to have antioxidant activity [7], and to enhance drug bioavailability [8].

The pepper fruit, *aka* peppercorn, contains various aroma-rendering terpenes, mostly mono- and sesquitepenes, including pinene, limonene, nopinene, caryophyllene, copaene, and cadinene (Fig. 1). Recently, rotundone, a sesquiterpene of guaiane skeleton, was identified to contribute unique peppery aroma to black pepper as well as Shiraz wine [9].

Terpenoids of plant origin are the largest and structurally most diverse group of natural products. Their carbon skeletons originate from the condensation of two simple isomeric five-carbon precursors, dimethylallyl diphosphate and isopentenyl diphosphate. For example, sesquiterpene with fifteen-carbon skeleton arises from the farnesyl diphosphate precursor

comprised of three C_5 units. To understand the make-up of volatile terpene components in black pepper, it is necessary to characterize the terpene synthases (TPSs) that biosynthesize the volatile terpenes in peppercorn.

The secondary (or specialized) metabolites and their respective enzymes and transcripts are often present in low quantities in specialized cell-types, making it difficult to elucidate their biosynthetic pathways. However, progresses in the next generation sequencings enabled us to unravel many important, but low abundant, enzymes involved in secondary metabolisms due to the deep sequencing depth. As of November 2017, RNA-sequencing and transcriptome assembly has been applied to more than 600 non-model plant species (https://www.ncbi.nlm.nih.gov/Traces/wgs/?page=1&view=TSA) [10].

In the present study, we aimed at identifying PnTPSs responsible for the biosynthesis of the flavor-imparting volatile sesquiterpenes in black pepper. In particular, from the transcriptome data of unripe pepper fruit, we isolated three full-length cDNAs encoding sesquiterpene TPSs (Sesqui-TPSs) and characterized their enzymatic products.

2. Materials and methods

2.1. Plant material and chemicals

Tissue samples, including immature fruit, leaves, stem and roots, of *P. nigrum* were harvested from garden of Prince of Songkla University, Thailand. Farnesyl diphosphate (FPP) was from Echelon Biosciences (USA), Yeast Dropout Medium Supplements from Takara Bio (Madison, WI), and Bacto Agar from Becton Dickinson. Adenine hemisulfate,

$MgCl_2$, MES monohydrate, and malachite green phosphate assay kit were from Sigma-Aldrich.

2.2. RNA isolation and cDNA library construction

Total RNA was extracted from unripe peppercorn, as shown in Table S4, by the CTAB method [11], treated with RNase-free DNase I (PhileKorea, Korea), and purified by phenol-chloroform method. Purified total RNA was evaluated for integrity on Bioanalyzer 2100 (Agilent, USA). Samples with RNA Integrity Number higher than 7.0 [12] was used in the ensuing experiment. Illumina cDNA library was prepared using total RNA by Beijing Genomics Institute Genomic Center in Hong Kong, and resulting cDNA library was sequenced on Illumina HiSeq 2000 platform.

2.3. Subcloning

With amino acid sequence of amorpha-4,11-dinene synthase (Genebank ABM88787) [13] as a query, homologous gene contigs were searched in the transcriptome database using tBLASTn (BioEdit, version 7.2.0). Three contigs, suggested to code full-length ORF with high homology against the query gene, were found. Primer pairs for these ORFs were designed (Table S1) to clone respective genes from cDNA library under the following conditions using Takara Ex Taq Polymerase (Takara, Japan): 98℃ for 5 min for initial, 98℃ for 15 s, 58℃ for 30 s, 72℃ for 2 min for 35 cycles and 72℃ for 5 min. PCR products were

purified by using Inclone Gel & PCR purification kit (Inclone Biotech, Korea) and finally TA cloned into pMD20 T vector (Takara, Japan).

2.4. Yeast transformation and fermentation

Yeast expression plasmid was constructed by inserting TPS ORF without stop codon into the yeast expression vector *pESC*-LEU2d at *Bam*HI and *Nhe*I double digestion site. The cloned vector was then transformed to engineered yeast EPY300 [14] by using LiAc/SS carrier DNA/PEG method [15]. All transformants were grown at 30℃ on SC medium [16] without histidine, methionine, and leucine (-HML) to select positive transformants. Single colony was inoculated to a test tube containing 3 ml of the SC medium (-HML) supplemented with 2% glucose and cultured for 16 h. Thirty milliliters of the SC medium (-HML) supplemented with 2% galactose, 0.2% glucose, and 1 mM methionine in a 150 mL flask was inoculated with 0.6 mL of the seed culture, and incubated for three days at 30℃ on a shaking incubator at 200 rpm. On completion of incubation, the medium was extracted with 3 ml of *n*-hexane for GC-MS analysis.

2.5. Heterologous expression and in vitro assay

ORF of each PnTPS was cloned into *pET21*a(+) vector at *Nde*I and *Xho*I double digestion site with C-terminal 6×His tag by using Gibson Assembly Cloning Kit (NEB, UK). The ORF-containing vector was then transformed into *E. coli* Rosetta 2(DE3). Single colony was taken to inoculate 5 ml LB medium supplemented with 100 μg/ml ampicillin and incubated at 37℃ overnight. The overnight culture was inoculated (1/100, v/v) to 50 ml

fresh LB medium with the same concentration of antibiotics. Incubation was continued at 37□ until OD_{600} reached 0.5, when the culture was added with IPTG to 0.1 mM and the incubating temperature was lowered to 20□. The culture was further incubated for 16 h, and the cells were collected by centrifugation at 5000 rpm (Beckman F1202 rotor) for 5 min at 4□. The cell pellet was resuspened in 5 ml of lysis buffer (20 mM Tris-HCl, pH 7.0, supplemented with 300 mM NaCl and 10 mM imidazole). The suspension was then sonicated (Sonic Dismembrator 550, Fisher Scientific) for total of 10 min with repeated 3 s on and 1 s interval. Soluble protein fraction was collected by centrifugation for 30 min at 7000 rpm and 4□. The supernatant was applied to 60 Ni Superflow Resin (Takara, Japan) and the TPS fraction was eluted by increasing concentration of imidazole. Finally, the eluted TPS was desalted by buffer exchange on a centrifugal filter (Amicon Ultracell-30k, Merck Millipore, Ireland). The proteins were subjected to SDS-PAGE (10% gel) and the purity of the protein was estimated by analyzing the image of the gel using ImageJ2x program version 2.1.4.7 (NIH ImageJ, USA).

The enzyme reaction was done in glass vial using 1 μg of the purified protein in a final volume of 1 ml of assay buffer (100 mM Tris-HCl, pH 7.0) supplemented with 1 mM dithiothreitol, 10 mM $MgCl_2$, and 100 μM FPP. The reaction mixture was gently overlaid with 0.5 ml of *n*-hexane and allowed to react for 2 h at 30□. The mixture was then vortexed and the organic phase was separated by centrifugation. The extraction was repeated once with the same volume of the solvent. The combined extract was concentrated to 0.3 ml under mild flow of nitrogen before direct analysis on GC-MS.

152

153 *2.6. Steady-state kinetics*

154 Steady-state kinetic parameters of sesqui-TPS were determined by using malachite green
155 phosphate assay kit (Sigma-Aldrich, Cat. No. MAK307) [17]. One microgram of the
156 enzyme with varying concentration of FPP (1.56 μM to 50 μM) in 500 μl of the assay buffer
157 was reacted at 30□ for 10 min and the reaction was terminated by addition of 10 μl of 5 M
158 HCl. Released free phosphate was determined as described in the manual of the assay kit.
159 The data was fit to the Michaelis-Menten equation by using SigmaPlot 13 (Systat Software,
160 USA) to obtain V_{max} and K_m.

161

162 *2.7. GC-MS analysis of terpene*

163 GC System (Agilent model 6890, USA) equipped with a mass spectrometer was used to
164 analyze the terpenes. The GC column was ZB-5MSi (60 m × 0.25 mm × 0.25 μm) with
165 helium as carrier gas at a flow rate of 1 ml min^{-1}. For enzymatic reaction product analysis, 1
166 μl of *n*-hexane extract was directly injected into GC with the following temperature
167 program: injecton at 220℃, and initial temperature of 50℃ (5 min hold) raised to 110℃ at a
168 rate of 5℃/min and then at 10℃/min to 240℃ (10-min hold). The coupled mass spectrometer
169 was Agilent HP 5973 (USA) with transfer line temperature set at 320℃, source temperature
170 at 250℃, quadrupole temperature at 150℃, and ionization potential at 70 eV. Scan range
171 was 50 to 300 atomic mass units. Products were identified by using MS Search Program
172 v.2.0 (NIST Standard Reference Database, USA).

Peppercorn extract for GC-MS analysis was prepared as follows. Frozen peppercorn (5 g) was ground with liquid N_2 and extracted with *n*-hexane (20 ml) by gentle stirring overnight at room temperature. The hexane layer was separated by centrifugation at 4□ before concentrating to 4 ml under stream of nitrogen. The extract was analyzed by using temperature program of 50℃ (5 min hold) to 260℃ at 4℃ min^{-1} with other conditions same as above.

2.8. Quantitative real-time PCR

Transcript levels of *PnTPS*s were determined by QRT-PCR using primer pairs described on Table S1. The PCR in a total reaction volume of 20 μl containing 10 μl of 2× QuantiMix SYBR Kit PCR system (PhileKorea, Korea), 1 μl diluted cDNA, 0.25 μM of each primer, and double distilled H_2O. The PCR reaction was run on Rotor-Gene 2000 Real Time Cycler (Corbett Research, Australia) with a temperature program of 5 min at 95℃, 40 cycles of 15 s at 95℃, 20 s at 58℃, and 20 s at 72℃. The standard curve was made by running the PCR of each gene ranging from 10^2 to 10^8 copy/μl, and the copy numbers were calculated as described [18]. Each data point was obtained from five biological samples, each composed of four technical replicates.

2.9. Bioinformatics analyses

The amino acid sequences were aligned using the Vector NTI Advance 10 (Thermo Fisher Scientific, USA). The sequences were compared by using BioEdit (version 7.2.0) and

194 phylogenetic tree was constructed by MEGA6 software (www.megasoftware.net). Reverse
195 complementation sequences were searched by using Sequence Manipulation Suite
196 (http://www.bioinformatics.org/sms2/).
197
198 **3. Results and Discussion**
199 *3.1. Transcriptome analysis and sesqui-TPS mining*
200 The Illumina deep sequencing of the unripe peppercorn RNAs yielded total of 49,413,668
201 clean reads (Table S2.). All clean reads were *de novo* assembled into contigs by the Trinity
202 algorithm [19]. The clean read assembly represented a total of 66,787 unigenes, ranging
203 their length from 300 nucleotides (nt) to 3000 nt with a mean length of 647 nt. The number
204 of unigenes longer than 1 kb was 15,101 (22.6% of the total unigenes). This result was
205 comparable to the previously published transcriptome data from black pepper roots (SOLiD:
206 unigene number 10,338; mean length=168) [20], leaves (Illumina: unigene number 128,157;
207 mean length=449) [21], and fruits (Illumina: unigene number 44,061; mean length=1,345)
208 [22]. Hu et al. [22] discussed lysine/ornithine metabolic genes with respect to piperine
209 biosynthesis.
210 Computational search for the sesqui-TPSs from the database yielded nineteen candidate
211 cDNAs, including three full length *TPS*s (Fig. S1). One of the full length unigenes, named
212 *PnTPS1* (GenBank KU953957), was composed of 560 amino acids with a predicted
213 molecular mass of 64.9 kDa. The second (PnTPS2; KU953958) and third unigene (PnTPS3;
214 MF104556) encoded open reading frames of the identical lengths comprised of 563 amino

215 acids with ~64.6 kDa (Fig. S2). Amino acid identity between PnTPS1 and 2 was 55%, but
216 PnTPS3 had 39% identity with other two TPSs. All of the PnTPSs have well-conserved
217 regions characteristic for sesqui-TPS (Fig. S3).

218

219 *3.2. Analysis of sesquiterpenes in peppercorn*

220 To correlate the terpene profiles from peppercorn and the enzyme products, terpene
221 metabolites from the unripe peppercorn were profiled by GC-MS. Identification was based
222 on mass spectral data and literatures [23]. In the unripe fruit, thirteen sesquiterpenes
223 including β-caryophyllene, δ-elemene, α-copaene, cubebene, α-humulene, δ-cadinol, and γ-
224 and δ-cadinenes were found (Fig. 2 and Table S3). β-Caryophyllene was the most abundant
225 compound among the observed sesquitepenes (65.8% of the total), followed by copaene
226 (8.3%). Even though the reported composition of peppercorn varies in different literatures,
227 all literatures consistemtly indicated that β-caryophyllene is the most abundant
228 sesquiterpene in peppercorn [23-26]. Rotundone or its α-guaiene skeletal homologs could
229 not be detected in the present study nor described in most of the previous analysis reports
230 [24-26] except a few literatures [23].

231 As it is possible that rotundone and its related α-guaiene skeletal products could be
232 synthesized in different developmental stages of peppercorns, an independent experiment
233 was performed including early stage and fully ripened peppercorns. In these measurements,
234 changes in relative terpene content were observed as peppercorn was undergoing different
235 developmental stages: young, unripe, and ripe (Table S4). The contents of the

236 sesquiterpenes derived from germacrene D, such as cubebene, copaene, cubeol, and
237 germacrene D-4α-ol, were progressively declined, most likely due to diminished total
238 germacrene D synthase activity. However, accumulation of β-caryophyllene, the major
239 sesquiterpene in peppercorn, changed from 46% to 70% as fruits ripened. Here again, no α-
240 guaiene-type sesquiterpenes were detected regardless of fruit ages.

241

242 *3.3. Functional analyses of sesqui-TPSs*

243 The engineered yeast strain EPY300 that was previously used to assess sesqui-TPS
244 activities *in vivo* [16] was employed to characterize the catalytic activities of PnTPSs. In the
245 heterologous yeast system, a high level of farnesol (t_r = 28 min) appeared as a background
246 peak due to an endogenous dephosphorylation of farnesyl diphosphate (Fig. 3, left panel).
247 *PnTPS1*-transformed yeast produced caryophyllene (97%) and a trace of humulene (3%),
248 and *PnTPS2*-transformed yeast accumulated δ-cadinol as a major product (91%) and
249 smaller amounts of α-cadinene (2%) and δ-cadinene (7%) (Fig. 3 and Fig. S4). The products
250 from *PnTPS3*-transformed yeast were copaene (59%), β-cadinene (38%), and a trace of
251 cubebene (3%).

252 Because an acidic condition prevailed (pH 4.6) at the end of yeast fermentation, we
253 conducted *in vitro* enzyme assay using partially purified enzymes with at least 80% purity to
254 avoid the possible acid-catalyzed rearrangement of initial enzymatic product(s) (Fig. S2). For
255 PnTPS1 and PnTPS2, the products were the same as those from yeast fermentation. Based on
256 these findings, we named PnTPS1 as PnCPS and PnTPS2 as PnCO/CDS. CPSs have been

commonly found from plant [27] to microorganism [28]. PnTPS2 showed higher levels of α-cadinene (12%) and δ-cadinene (10%), comparing to the *in vivo* product composition, and thus was promiscuous in its reaction products. δ-Cadinol synthase has been previously described from the fungus *Boreostereum vibrans* [29]. In the case of PnTPS3, however, the *in vitro* reaction in yeast yielded germacrene D as a major product (91%) and small amounts of copaene (6%) and cubebene (3%). Therefore, PnTPS3 was named PnGDS (Fig. 3). GDS have been cloned from various sources such as *Streptomyces* [30], goldenrod [31], and grape [32].

Germacrene D is a labile compound that easily undergoes rearrangements [33,34]. In particular, in the presence of acid, germacrene D rearranges into copaene and various cadinene-type products [34], which explains the appearance of prominent copaene and β-cadinene peaks from the fermentation of the yeast expressing *PnTPS3*. Therefore, the copaene and cadinene found in PnTPS3, either *in vivo* or *in vitro*, are likely the rearranged products of germacrene D. To confirm the possible rearrangement of germacrene D under the acidic condition, we conducted *in vivo* enzyme reaction under at pH 6.0 (Fig. S5). Reaction at a lower pH clearly favored the formation of copaene and β-cadinene. Thus, large copaene and β-cadinene peaks in the yeast experiment (Fig. 3, Lower panel) could be justified by acidification of broth during fermentation. The moderate copaene content and the lack of β-cadinene in unripe peppercorn (Fig. 2 and Table S3) reflect relatively neutral cellular environment for PnGDS *in planta*. These observations for the PnTPS3 activity demonstrates that heterologous *in vivo* product in yeast does not necessarily advocate the identities of true

enzymatic reaction product(s) [35,36].

We could not identify the gene for guaiene skeleton, a direct precursor to rotundone. The low concentration of rotundone at 1.2 ppm [9] and δ-guaiene (<0.1%) [24] in black pepper may suggest extremely low abundance of the transcript for α-guaiene (rotundone backbone) biosynthesis in peppercorn. Alternatively, only some black pepper cultivars may encode α-guaiene synthases, and the black pepper cultivars subjected to metabolite and transcript profiling in this work could innately not produce α-guaiene and rotindone. Nevertheless, the importance of rotundone in flavor industry warrants further search for the gene in black pepper.

3.4. Kinetic parameters

Steady-state kinetic parameters of sesqui-TPSs were determined by malachite green assay to measure the release of diphosphate from FPP on cyclization of the substrate into terpene products. Vardaku et al. [17] validated the assay method that the measured kinetic efficiency (k_{cat}/K_m) was consistent with radioactive assay, although the phosphate assay gave slightly higher k_{cat} and K_m values compared to radioactive assay. The PnTPSs had k_{cat}/K_m values ranging from 20.2-37.7 s^{-1} mM^{-1} (Table 1 and Fig. S6) comparable to other sesqui-TPSs [17, 37].

3.5. Catalytic mechanism

Mechanistic pathways for three PnTPSs to explain the formation of various products are

proposed in Fig. 4. Caryophyllene synthases that co-produce humulene have previously been described [38]. The cyclization of FPP to form caryophyllene by PnCPS apparently calls for so-called 1,10-ring closure. Here we depicted the cyclization process catalyzed by PnTPSs follows 1,10-closure mechanism assuming that the present PnTPSs have evolved from a common ancestor (section 3.6) to share a common mechanistic feature. Nevertheless, 1,6-closure mechanism is also possible for PnCO/CDS [39].

3.6. Phylogenetic analysis

To construct phylogenetic tree, sesqui-TPSs with catalytic function similar to PnTPSs were selected. The phylogenetic tree, constructed using neighbor-joining method (MEGA 6), placed all three PnTPSs studied in this work in a small sub-clade within the angiosperm sesqui-TPS family (Fig. S7). This result suggests that the present PnTPSs are paralogs evolved from common ancestor after speciation.

3.7. PnTPS transcript levels among pepper organs

PnTPS transcript levels were relatively low in roots and fruits compared to stem and leaves (Fig. 5). Stem exhibited high levels of comparable *PnCPS* and *PnGDS* transcripts with low *PnCO/CDS* level, whereas leaves were characterized by 9-fold higher level of *PnGDS* transcripts than *PnCPS* and *PnCO/CDS* transcripts. The abundant *PnGDS* transcript levels in leaves explains α-cubebene content as high as 20.2% among terpenes in the leaf extract [40]. Among *PnTPS*s in the peppercorn, *PnCPS* level was the highest, correlating well with

the high amount of combined caryophyllene and humulene content (68.4%) in the peppercorn (Table S3). However, about 4-fold higher *PnCO/CDS* transcript level in comparison to *PnGDS* level in peppercorn (Fig. 4) could not explain the comparable enzymatic product contents from PnCO/CDS (9.1% for γ- and δ-cadiene and δ-cadinol) and PnGDS (9.5% for copaene and cubebene) (Table S3). The preferential loss of initial sesquiterpene products by unknown secondary enzymatic modifications or sample preparation (i.e., evaporation) could explain the discrepancy between sesqui-TPS transcript levels and the accumulation of their enzymatic product.

Conflicts of interest statement

The authors declare no conflict of interest.

Acknowledgements

This work was supported by Cooperative Research Program for Agriculture Science & Technology Development (Next Generation Biogreen 21 Program; PJ0110552017) administered by Rural Development Administration, Republic of Korea (S.U.K.). This work was also supported by National Science and Engineering Research Council of Canada (NSERC) (D.K.R.), and Basic Science Research Program through the National Research Foundation (NRF) of Republic of Korea funded by the Ministry of Education

(2017R1A6A3A03003409) (M.H.K.). We thank Professor Jeong Han Kim at Department of Agricultural Biotechnology, SNU, for free access to GC-MS.

References

[1] S. Fitzgerald, Ramses II, Egyptian Pharaoh, Warrior, and Builder, Compass Point Books, Minneapolis, MN, 2008, pp. 88-90.
[2] Pliny the Elder, Natural History XII. transl. Eichholz D, Jones WHS, and Rackham H (1949-1954), Harvard University Press, Cambridge, MA, 1979, pp. 310-313.
[3] Nedspice, Pepper crop report 2015. http://www.nedspice.com, (2015), accessed Aug. 16, 2017.
[4] J. Turner, Spice: The history of a temptation, Vintage Books, New York, NY, 2008, pp. 160-165.
[5] K. Srinivasan, Black pepper and its pungent principle-piperine: a review of diverse physiological effects, Crit. Rev. Food Sci., 47 (2007) 735-748.
[6] K. Platel, K. Srinivasan, Influence of dietary spices and their active principles on pancreatic digestive enzymes in albino rats, Food/Nahrung, 44 (2000) 42-46.
[7] F.M. Rauscher, R.A. Sanders, J.B. Watkins, Effects of piperine on antioxidant pathways in tissues from normal and streptozotocin-induced diabetic rats, J. Biochem. Mol. Toxic., 14 (2000) 329-334.
[8] J.D. Lambert, J. Hong, D.H. Kim, V.M. Mishin, C.S. Yang, Piperine enhances the bioavailability of the tea polyphenol (−)-epigallocatechin-3-gallate in mice, J. Nutr., 134 (2004) 1948-1952.
[9] T.E. Siebert, C. Wood, G.M. Elsey, A.P. Pollnitz, Determination of rotundone, the pepper aroma impact compound, in grapes and wine, J. Agr. Food Chem., 56 (2008) 3745-3748.
[10] M.E. Bolger, B. Arsova, B. Usadel, Plant genome and transcriptome annotations: from misconceptions to simple solutions, Brief. Bioinform., (2017) 1-13.
[11] S. Chang, J. Puryear, J. Cairney, A simple and efficient method for isolating RNA from pine trees, Plant Mol. Biol. Rep., 11 (1993) 113-116.
[12] A. Schroeder, O. Mueller, S. Stocker, R. Salowsky, M. Leiber, M. Gassmann, S. Lightfoot, W. Menzel, M. Granzow, T. Ragg, The RIN: an RNA integrity number for assigning integrity values to RNA measurements, BMC Mol. Biol., 7 (2006) 1-14.
[13] Y.-J. Chang, S.-H. Song, S.-H. Park, S.-U. Kim, Amorpha-4, 11-diene synthase of *Artemisia annua*: cDNA isolation and bacterial expression of a terpene synthase involved in artemisinin biosynthesis, Arch. Biochem. Biophys., 383 (2000) 178-184.
[14] D.-K. Ro, E.M. Paradise, M. Ouellet, K.J. Fisher, K.L. Newman, J.M. Ndungu, K.A. Ho, R.A. Eachus, T.S. Ham, J. Kirby, Production of the antimalarial drug precursor artemisinic acid in engineered yeast, Nature, 440 (2006) 940-943.
[15] R.D. Gietz, R.H. Schiestl, High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method, Nat. Protoc., 2 (2007) 31-34.
[16] T.-D. Nguyen, G. MacNevin, D.-K. Ro, *De novo* synthesis of high-value plant sesquiterpenoids in yeast, Method. Enzymol., 517 (2012) 261-278.

[17] M. Vardakou, M. Salmon, J.A. Faraldos, P.E. O'Maille, Comparative analysis and validation of the malachite green assay for the high throughput biochemical characterization of terpene synthases, MethodsX, 1 (2014) 187-196.

[18] J.L. Yin, N.A. Shackel, A. Zekry, P.H. McGuinness, C. Richards, K. Van Der Putten, G.W. McCaughan, J.M. Eris, G.A. Bishop, Real-time reverse transcriptase–polymerase chain reaction (RT–PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I, Immunol. Cell Biol., 79 (2001) 213-221.

[19] M.G. Grabherr, B.J. Haas, M. Yassour, J.Z. Levin, D.A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Full-length transcriptome assembly from RNA-Seq data without a reference genome, Nat. Biotechnol., 29 (2011) 644-652.

[20] S.M. Gordo, D.G. Pinheiro, E.C. Moreira, S.M. Rodrigues, M.C. Poltronieri, O.F. de Lemos, I.T. da Silva, R.T. Ramos, A. Silva, H. Schneider, High-throughput sequencing of black pepper root transcriptome, BMC Plant Biol., 12 (2012) 168.

[21] N. Joy, S. Asha, V. Mallika, E.V. Soniya, De novo transcriptome sequencing reveals a considerable bias in the incidence of simple sequence repeats towards the downstream of 'pre-miRNAs' of black pepper, PLoS One, 8 (2013) e56694.

[22] L. Hu, C. Hao, R. Fan, B. Wu, L. Tan, H. Wu, *De novo* assembly and characterization of fruit transcriptome in black pepper (*Piper nigrum*), PloS One, 10 (2015) e0129822.

[23] H.H. Jeleń, A. Gracka, Analysis of black pepper volatiles by solid phase microextraction–gas chromatography: A comparison of terpenes profiles with hydrodistillation, J. Chromatogr. A, 1418 (2015) 200-209.

[24] H. Kollmannsberger, S. Nitz, F. Drawert, über die Aromastoffzusammensetzung von Hochdruckextrakten, Z. Lebensm. Unters. Forsch. A, 194 (1992) 545-551.

[25] L. Liu, G. Song, Y. Hu, GC–MS analysis of the essential oils of *Piper nigrum* L. and *Piper longum* L, Chromatographia, 66 (2007) 785-790.

[26] A. Gupta, M. Gupta, S. Gupta, Isolation of piperine and few sesquiterpenes from the cold petroleum ether extract of *Piper nigrum* (black pepper) and its antibacterial activity, Inter. J. Pharmacol. Phytochem., 5 (2013) 101-105.

[27] Y. Cai, J.-W. Jia, J. Crock, Z.-X. Lin, X.-Y. Chen, R. Croteau, A cDNA clone for β-caryophyllene synthase from *Artemisia annua*, Phytochemistry, 61 (2002) 523-529.

[28] C. Nakano, S. Horinouchi, Y. Ohnishi, Characterization of a novel sesquiterpene cyclase involved in (+)-caryolan-1-ol biosynthesis in *Streptomyces griseus*, J. Biol. Chem., 286 (2011) 27980-27987.

[29] H. Zhou, Y.-L. Yang, J. Zeng, L. Zhang, Z.-H. Ding, Y. Zeng, Identification and characterization of a δ-cadinol synthase potentially involved in the formation of boreovibrins in *Boreostereum vibrans* of basidiomycota, Nat. Prod. Bioprospect., 6 (2016) 167-171.

[30] X. He, D.E. Cane, Mechanism and stereochemistry of the germacradienol/germacrene D synthase of *Streptomyces c oelicolor* A3 (2), J. Am. Chem. Soc., 126 (2004) 2678-2679.

[31] I. Prosser, I.G. Altug, A.L. Phillips, W.A. König, H.J. Bouwmeester, M.H. Beale, Enantiospecific (+)-and (−)-germacrene D synthases, cloned from goldenrod, reveal a functionally active variant of the universal isoprenoid-biosynthesis aspartate-rich motif, Arch. Biochem. Biophys., 432 (2004) 136-144.

[32] J. Lücker, P. Bowen, J. Bohlmann, *Vitis vinifera* terpenoid cyclases: functional identification of two sesquiterpene synthase cDNAs encoding (+)-valencene synthase and (−)-germacrene D synthase and expression of mono-and sesquiterpene synthases in grapevine flowers and berries, Phytochemistry, 65 (2004) 2649-2659.

[33] N. Bülow, W.A. König, The role of germacrene D as a precursor in sesquiterpene biosynthesis: investigations of acid catalyzed, photochemically and thermally induced rearrangements, Phytochemistry, 55 (2000) 141-168.

[34] A.M. Adio, Germacrenes A–E and related compounds: thermal, photochemical and acid induced transannular cyclizations, Tetrahedron, 65 (2009) 1533-1552.

[35] D.T. Nguyen, J.C. Göpfert, N. Ikezawa, G. MacNevin, M. Kathiresan, J. Conrad, O. Spring, D.-K. Ro, Biochemical conservation and evolution of germacrene A oxidase in Asteraceae, J. Biol. Chem., 285 (2010) 16588-16598.

[36] B. Pickel, D.P. Drew, T. Manczak, C. Weitzel, H.T. Simonsen, D.-K. Ro, Identification and characterization of a kunzeaol synthase from *Thapsia garganica*: implications for the biosynthesis of the pharmaceutical thapsigargin, Biochem. J., 448 (2012) 261-271.

[37] T. Manczak, H.T. Simonsen, Insight into biochemical characterization of plant sesquiterpene synthases, Anal. Chem. Insights, 11 (2016) 1-6.

[38] G. Wang, L. Tian, N. Aziz, P. Broun, X. Dai, J. He, A. King, P.X. Zhao, R.A. Dixon, Terpene biosynthesis in glandular trichomes of hop, Plant Physiol., 148 (2008) 1254-1266.

[39] J.A. Faraldos, D.J. Miller, V.n. González, Z. Yoosuf-Aly, O. Cascón, A. Li, R.K. Allemann, A 1, 6-ring closure mechanism for (+)-δ-cadinene synthase?, J. Am. Chem. Soc., 134 (2012) 5900-5908.

[40] T.K. Lim, Edible medicinal andnon-medicinal plants. Vol. 4, Springer, New York, NY, 2012, pp. 322-350.

List of Table and Figures

Table 1 Kinetic properties of three PnTPSs. Lineweaver-Burk plots to derive the parameters are shown in Fig. S6.

Fig. 1. Volatile mono- and sesqui-terpenes found in dried green peppercorn. Only major representative terpenes are shown.

Fig. 2. GC/MS profiling of terpenes from immature black pepper fruit.

Fig. 3. Total ion chromatogram of fermentation broth extract (left side column) and in-vitro enzyme assays (right side column). Fermentation was done by yeast EPY300 expressing PnTPSs. PnTPS1: peak **1**, caryophyllene; peak **2**, humulene. PnTPS2: peak **3**, α-cadinene (cadina-4,9-diene); peak **4**, δ-cadinene (cadina-1(10),4-diene); peak **5**, δ-cadinol. PnTPS3: peak **6**, copaene; peak **7**, cubebene; peak **8**, β-cadinene (cadina-3,9-diene). PnTPS3: peak **9**, germacrene D. Peak at 28 min in the chromatograms of the left side column was farnesol.

Fig. 4. Possible mechanism of sesquiterpene synthases from *P. nigrum*. The reaction pathway was based on 1,10-ring closure pathway.

468 **Fig. 5.** Absolute transcript copy numbers of *PnTPS*s estimated by QRT-PCR in four

469 different organs of *P. nigrum.* Each data point was determined from five biological samples

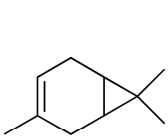
470 each consisted of four technical replicates.

471

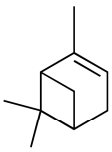
Table 1

Kinetic properties of three PnTPSs. Data were from three replicate assays.

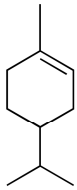
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
PnTPS1	21.06 ± 4.71	0.455 ± 0.003	0.0216
PnTPS2	18.40 ± 4.94	0.694 ± 0.066	0.0377
PnTPS3	9.152 ± 1.813	0.185 ± 0.012	0.0202



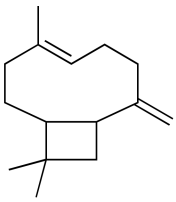
3-carene



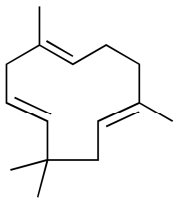
α-pinene



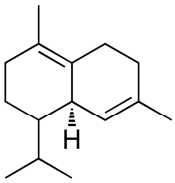
limonene



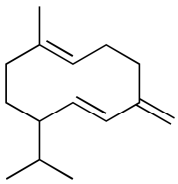
β-caryophyllene



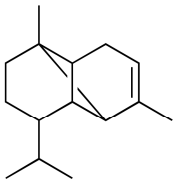
humulene



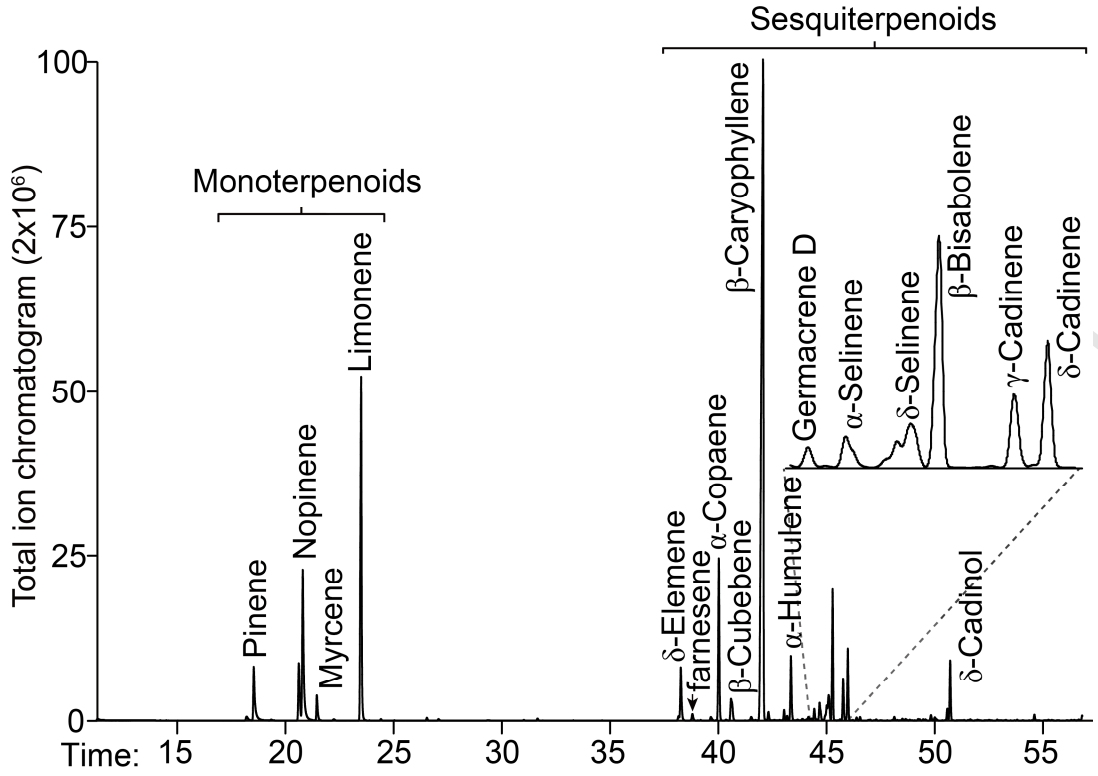
δ-cadinene

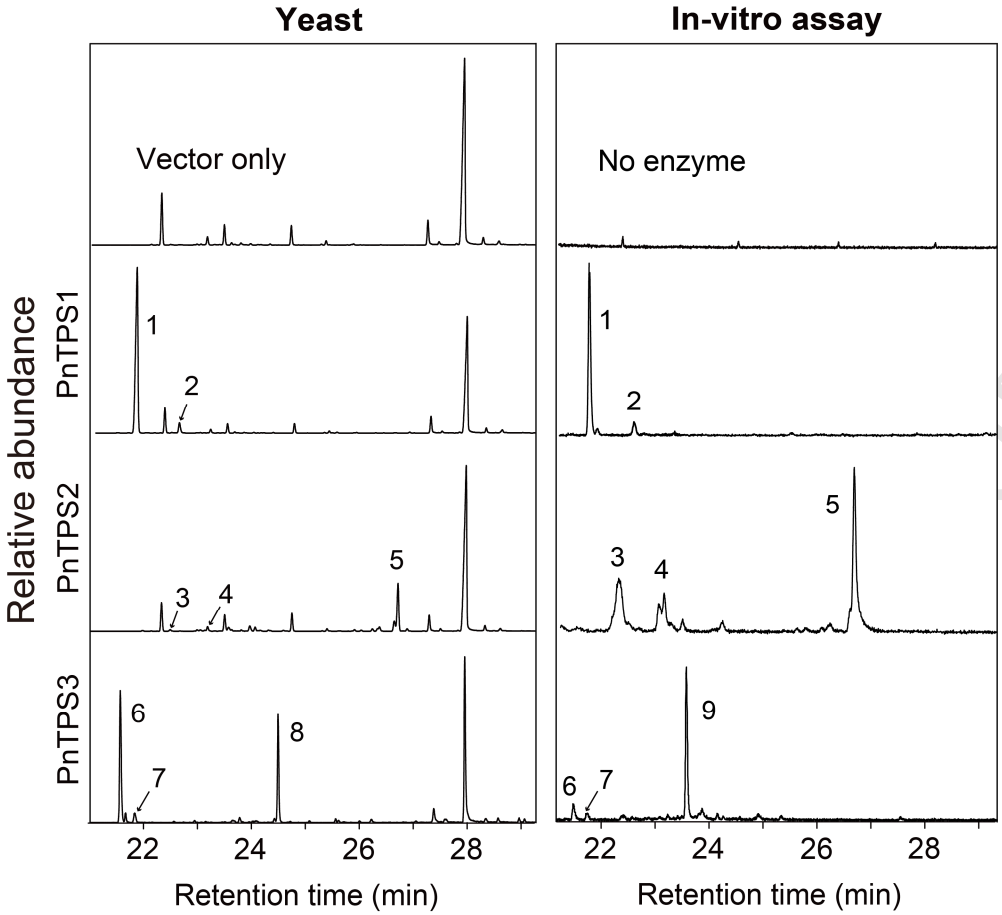


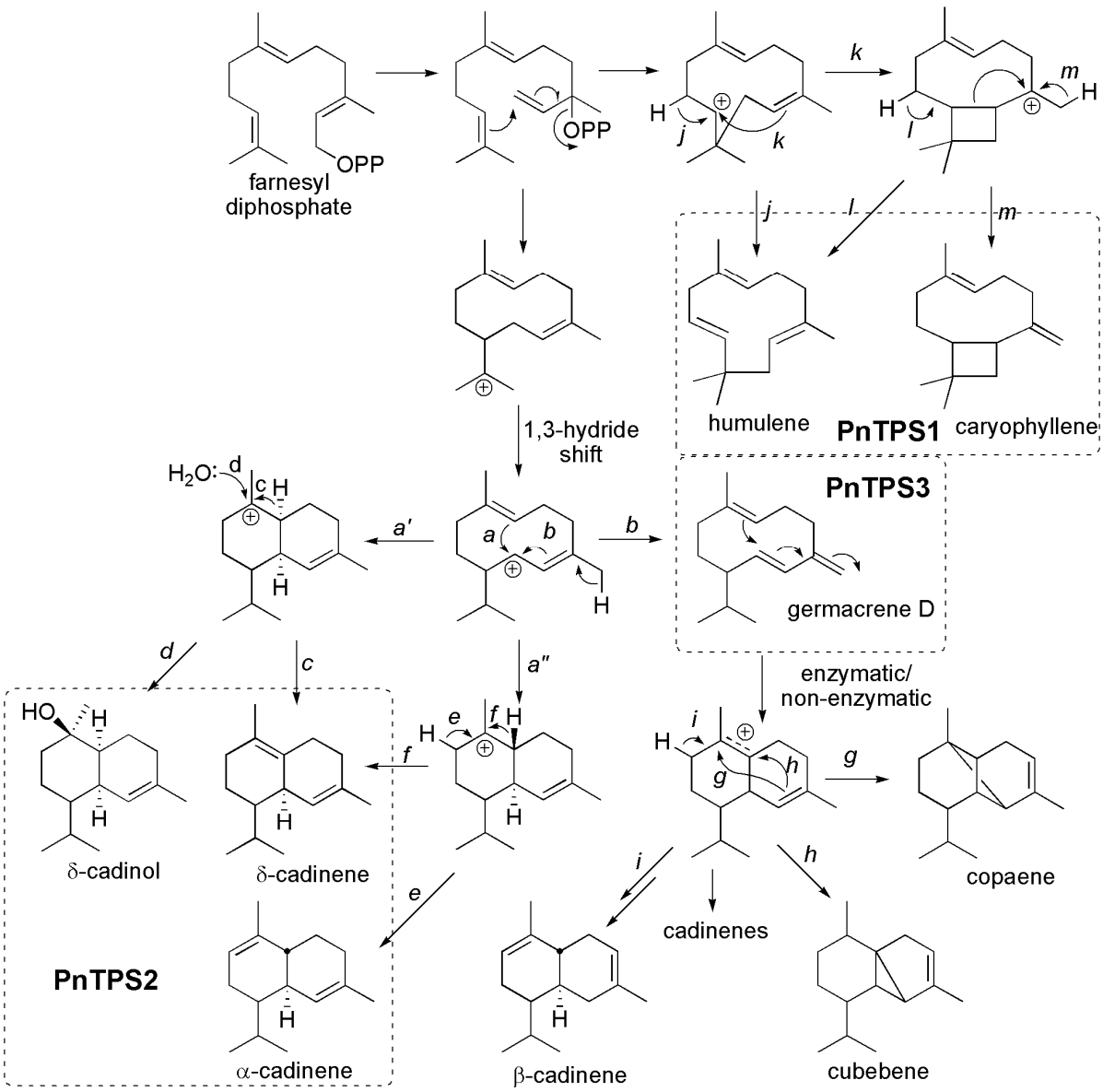
germacrene D

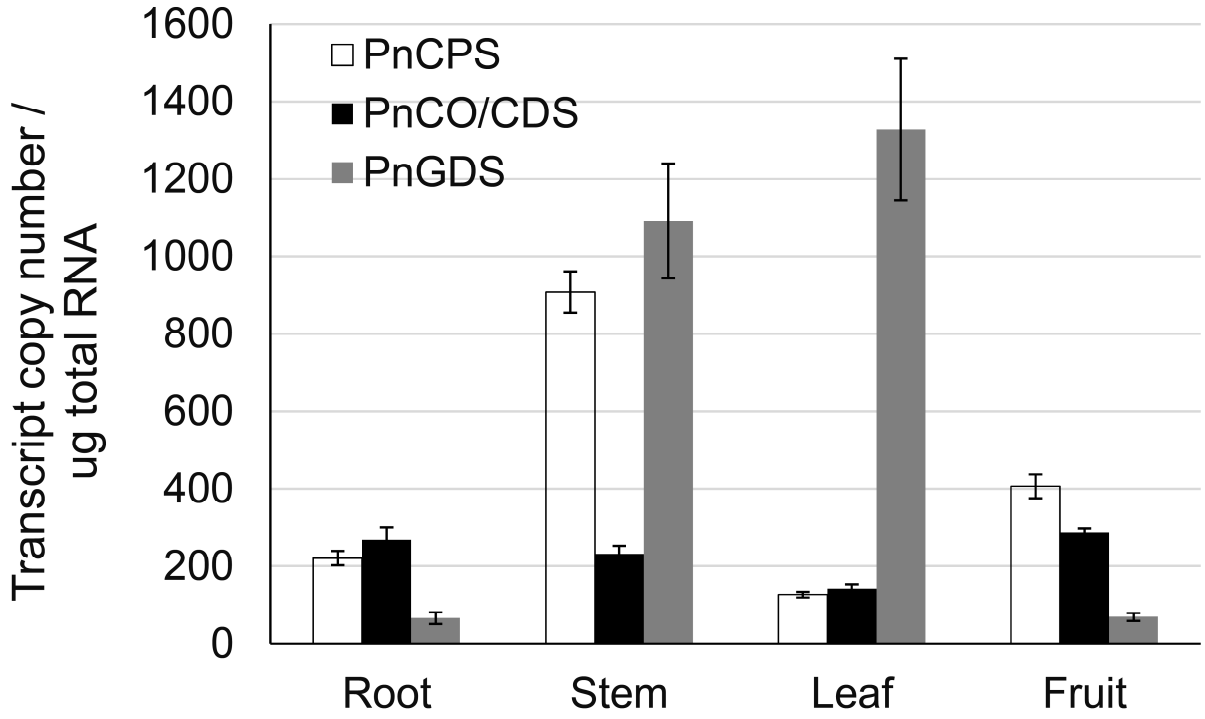


copaene






farnesyl diphosphate
OPP
OPP
H
j
k
k
H
l
m
H
j
l
m
humulene
PnTPS1
caryophyllene
1,3-hydride shift
PnTPS3
germacrene D
enzymatic/ non-enzymatic
H2O: d
c H
a′
a
b
b
H
d
c
a″
HO
H
δ-cadinol
δ-cadinene
e
f H
H
f
i
H
g
h
g
copaene
i
h
e
cadinenes
PnTPS2
α-cadinene
β-cadinene
cubebene



Highlights

- Transcriptome of unripe fruit of *Piper nigrum*, black pepper plant, was analyzed.
- Three sesquiterpene synthases were predicted from transcriptome database and cloned.
- They were identified as caryophyllene synthase, cadinol/cadinene synthase, and germacrene D synthase.
- Their kinetic parameters and tissue distribution were determined.