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Diversity and Functional Evolution of Terpene Synthases in Dictyostelid Social Amoebae

Xinlu Chen¹, Tobias G. Köllner², Gad Shaulsky³, Qidong Jia¹, Jeroen S. Dickschat⁴, Jonathan Gershenzon² & Feng Chen¹

Dictyostelids, or social amoebae, have a unique life style in forming multicellular fruiting bodies from unicellular amoeboids upon starvation. Recently, dictyostelids were found to contain terpene synthase (TPS) genes, a gene type of secondary metabolism previously known to occur only in plants, fungi and bacteria. Here we report an evolutionary functional study of dictyostelid *TPS* genes. The number of *TPS* genes in six species of dictyostelids examined ranges from 1 to 19; and the model species *Dictyostelium purpureum* contains 12 genes. Using *in vitro* enzyme assays, the 12 *TPS* genes from *D. purpureum* were shown to encode functional enzymes with distinct product profiles. The expression of the 12 *TPS* genes in *D. purpureum* is developmentally regulated. During multicellular development, *D. purpureum* releases a mixture of volatile terpenes dominated by sesquiterpenes that are the *in vitro* products of a subset of the 12 *TPS* genes. The quality and quantity of the terpenes released from *D. purpureum*, however, bear little resemblance to those of *D. discoideum*, a closely related dictyostelid. Despite these variations, the conserved clade of dictyostelid TPSs, which have an evolutionary distance of more than 600 million years, has the same biochemical function, catalyzing the formation of a sesquiterpene protoillud-7-ene. Taken together, our results indicate that the dynamic evolution of dictyostelid *TPS* genes includes both purifying selection of an orthologous group and species-specific expansion with functional divergence. Consequently, the terpenes produced by these TPSs most likely have conserved as well as species-adaptive biological functions as chemical languages in dictyostelids.

Dictyostelids, known as social amoebae, are soil-dwelling eukaryotes that have a unique life style. When food (bacteria) is abundant, dictyostelids grow vegetatively as free-living, unicellular amoeboid cells through cell division. On the contrary, when food is depleted, dictyostelids form multicellular fruiting bodies by the aggregation of unicellular amoeboid cells. The transition from the unicellular form to fruiting bodies is a tightly regulated developmental process¹. With the aid of genome sequencing and comparative genomics, genes underlying conditional multicellularity in dictyostelids continue to be identified and characterized^{2–4}. Among the many features of the genome sequences of dictyostelids is the presence of notable types and numbers of genes of secondary metabolism^{5,6}, among which polyketide synthase genes are the best recognized⁷. Forming a large gene family in each species, polyketide synthase genes exhibit extensive species-specific expansion^{6,8} and have been suggested to synthesize various types of polyketides for signaling and defense^{6,9}. Along this line, an important recent finding is that dictyostelids also contain terpene synthase (TPS) genes¹⁰.

TPS genes encode enzymes that catalyze the conversion of oligoprenyl diphosphates of various chain lengths to terpene hydrocarbons or alcohols of enormous structural diversity^{11,12}. The finding that dictyostelids contain *TPS* genes is significant in a number of ways. First, it indicates a wider distribution of *TPS* genes, which were previously observed only in plants and fungi among the eukaryotes. Second, it raises an intriguing question about the function of *TPS* genes in dictyostelids. In plants, where *TPS* genes are best studied, they are responsible for making terpenes that are involved in many processes of plant interactions with the environment as a chemical language¹³. Our previous study showed that *TPS* genes are absent in several species of solitary amoeba that are closely related to dictyostelids¹⁰. It is therefore tempting to speculate that *TPS* genes contribute to the multicellular

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aspect of the dictyostelids' unique life style. *Dictyostelium discoideum* has been used as a model species for functional characterization of *TPS* genes. This species contains 9 *TPS* genes, all of which were shown to be expressed and active for the *in vitro* production of sesquiterpenes, while some of them also produce monoterpenes and diterpenes. Many of the identified *in vitro* terpene products were also released as volatile compounds from *D. discoideum* during multicellular development¹⁰.

The dictyostelids are a monophyletic clade that can be subdivided into four taxonomic groups using molecular phylogeny^{14,15}. These four groups display enormous diversity in morphology and development¹⁶. In our previous study, *TPS* genes were found in five species of dictyostelids and the number of *TPS* genes in these species ranges from 1 to 19¹⁰. Such variation in the number of *TPS* genes poses intriguing questions: do paralogs in each species have similar or different catalytic functions? do paralogs and orthologs have similar or diverging expression patterns? how do the *in vitro* enzyme activities and gene expression patterns determine the chemical diversity of volatile terpenes produced by each dictyostelid species? what biological functions do these *TPS* genes and their terpene products have? Towards answering these questions, in this report, we investigate the functional evolution of the *TPS* family in dictyostelids using *D. purpureum* as the main model species.

Results and Discussion

Evolutionary relatedness of *TPS* genes from six species of dictyostelids. To conduct a comprehensive comparative analysis of the dictyostelid *TPS* gene family, six representative species were selected: *D. fasciculatum*, *Actyostelium subglobosum*, *Polysphondylium pallidum*, *D. lacteum*, *D. purpureum*, and *D. discoideum*. There were two main justifications for this selection. First, these six species cover all major taxonomic groups of dictyostelids, belonging to groups 1, 2, 2, 3, 4, and 4, respectively (Fig. 1A¹⁴). These species exhibit large or small variations in development and morphology. *D. fasciculatum*, *A. subglobosum*, and *P. pallidum* form fruiting bodies from the location of the aggregate and their fruiting bodies have branches, while *D. purpureum* and *D. discoideum* form slugs that migrate prior to culmination. Second, the genomes of the six species have been fully sequenced^{3,5,6,8,17}, allowing for an in-depth analysis of their *TPS* genes. As reported in our previous study, the genomes of the first five species contain 2 (*D. fasciculatum*), 1 (*A. subglobosum*), 19 (*P. pallidum*), 12 (*D. purpureum*), and 9 *TPS* genes (*D. discoideum*)¹⁰. The genome sequence of *D. lacteum* was recently reported³ and we identified 7 *TPS* genes there (Table S1).

To understand their evolutionary relatedness, *TPS*s from the six dictyostelids species were subjected to a phylogenetic analysis. Four clades (I to IV) could be recognized (Fig. 1B). The phylogeny of clade I is generally consistent with the phylogeny of the six species determined using other genes¹⁸. All species contain one gene in this clade except for *D. discoideum*, which contains two genes *DdTPS6* and *DdTPS2*. While *DdTPS6* appears to be the ortholog, *DdTPS2* most likely resulted from a relatively recent gene duplication of the *DdTPS6/2* ancestor that occurred only in *D. discoideum* after the split of *D. purpureum* and *D. discoideum* in group 4.

The split of the two branches of dictyostelids (groups 1 and 2 being one branch and groups 3 and 4 being the other branch) was estimated to have occurred between 600 and 400 million years ago (MYA)^{6,8}, indicating that dictyostelid *TPS*s are ancient. Clade II contains *TPS* genes from three taxonomic groups, group 2, group 3 and group 4, suggesting a possible loss of *TPS* genes in two species in branch I (*D. fasciculatum* and *A. subglobosum*). Clade III is specific to *P. pallidum*, implying extensive gene family expansion in this species after its divergence from the main branch. Clade IV is group 4-specific, containing only *TPS* genes from *D. purpureum* and *D. discoideum*, implying the emergence of the genes after the split of the *D. purpureum* and *D. discoideum* common ancestor from group 3. Between the 12 *TPS* genes from *D. purpureum* and 9 *TPS* genes from *D. discoideum*, two species in group 4, four putative orthologous groups could be identified (Fig. 1B).

Catalytic activities of *TPS*s from *D. purpureum*. Prior to this study, *D. discoideum* was the only species of dictyostelids in which *TPS* genes have been functionally characterized¹⁰. To gain insights into the functional evolution of dictyostelid *TPS*s through comparative analysis, in this study, *D. purpureum* was selected as a model species for *TPS* functional characterization. Both *D. purpureum* and *D. discoideum* belong to group 4 and are most closely related among the six species examined in this work (Fig. 1A), despite having a large evolutionary distance of 400–300 million years⁸. *D. purpureum* and *D. discoideum* have been used as a pair of model species for comparative genomics⁸, comparative transcriptome analyses¹⁹ and comparative biological studies²⁰ of dictyostelids.

D. purpureum contains 12 *TPS* genes (designated *DpTPS1–12*), in contrast to 9 *TPS* genes in *D. discoideum*. Full-length cDNAs for all 12 *TPS* genes from *D. purpureum* were cloned and heterologously expressed in *Escherichia coli*. Because most *DdTPS*s function as sesquiterpene synthases¹⁰, for comparison, individual recombinant *DpTPS*s were first tested with farnesyl diphosphate, the substrate for sesquiterpene synthases. All 12 *DpTPS*s were capable of producing sesquiterpene hydrocarbons or alcohols, indicating that the genes encode *bona fide* *TPS* enzymes (Fig. 2A). While *DpTPS1* produced a sole compound, protoillud-7-ene, all the other 11 *DpTPS*s catalyzed the formation of complex sesquiterpene mixtures. The product spectra of *DpTPS2* and *DpTPS6* were dominated by the acyclic compound (*E*)- β -farnesene. *DpTPS11* and *DpTPS12* produced (*E,E*)- α -farnesene and α -selinene, respectively, as main products. The other enzymes formed mixtures of mainly unidentified sesquiterpene hydrocarbons or oxygenated sesquiterpenes.

Beside their sesquiterpene synthase activity, *DpTPS*s except *DpTPS1* and *DpTPS10* also exhibited monoterpene synthase activity when provided with geranyl diphosphate as substrate. While *DpTPS3*, *DpTPS4*, *DpTPS6*, *DpTPS7*, *DpTPS8*, *DpTPS11*, and *DpTPS12* formed linalool as the major monoterpene product, *DpTPS2* and *DpTPS9* produced β -myrcene and *DpTPS5* produced (*Z*)- β -ocimene as major product (Fig. S1). Seven *DpTPS*s accepted also geranylgeranyl diphosphate as substrate and catalyzed the formation of different unidentified diterpene alcohols (Fig. S2).

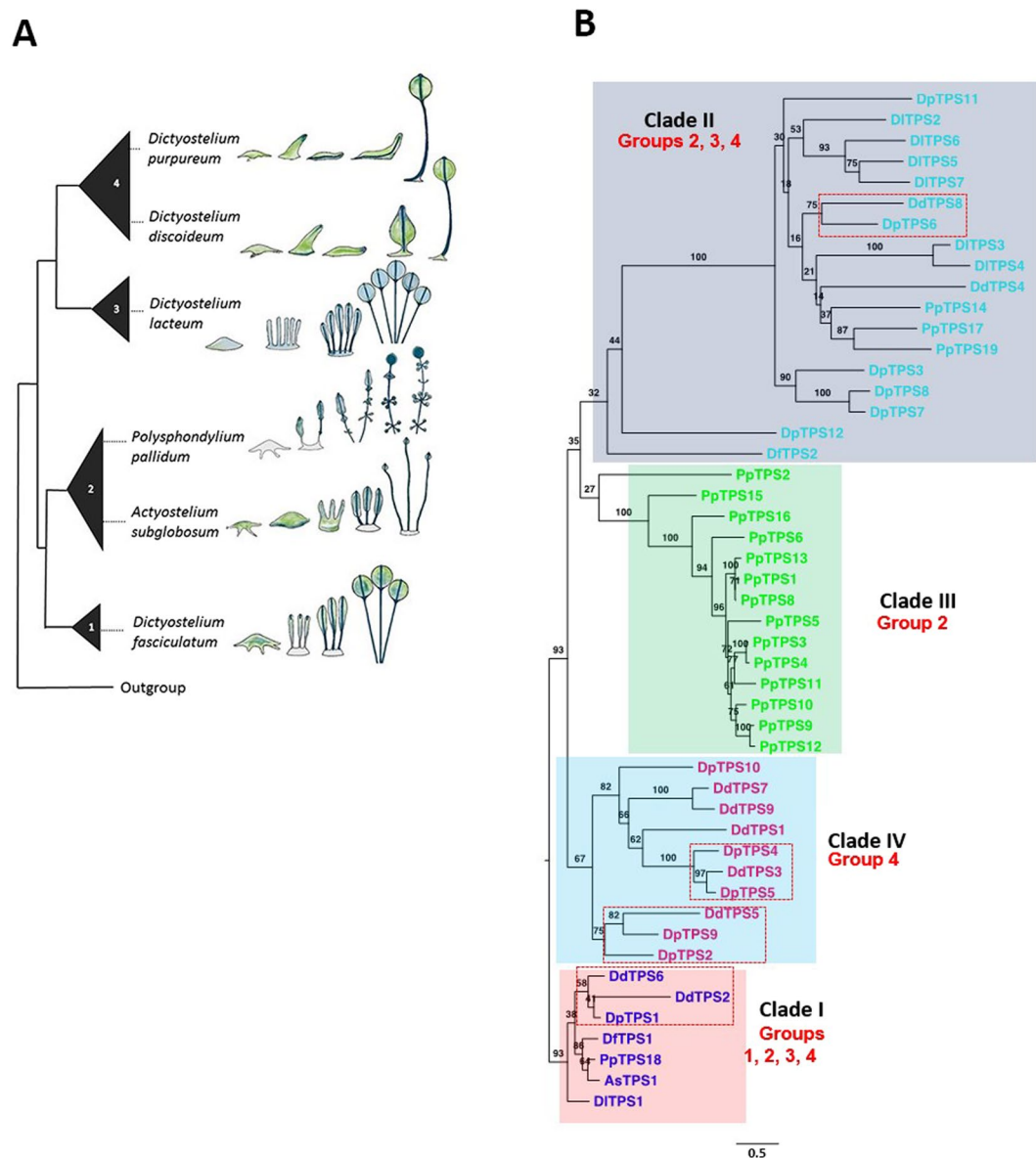


Figure 1. Phylogeny of six species of dictyostelid social amoeba and their terpene synthase genes. **(A)** Phylogeny of six species of dictyostelid social amoeba representing the four taxonomic groups adapted from³. Cartoons show the development patterns of the six species. **(B)** Phylogenetic tree of dictyostelid TPSs reconstructed using the maximum likelihood method. Protein sequences of 50 TPS genes include 19, 12, 9, 7, 2 and, 1 from *P. pallidum*, *D. purpureum*, *D. discoideum*, *D. lacteum*, *D. fasciculatum* and, *A. subglobosum*, respectively. These genes formed four groups, highlighted with skyblue, light pink, light green, and grey. Ten genes from *D. discoideum* and *D. purpureum* occurred in the skyblue group. Representative genes from each species *PpTPS18*, *DdTPS6*, *DpTPS1*, *DfTPS1*, *DITPS1*, and *AsTPS1* were found in the light pink group. Fourteen genes from *P. pallidum* made up the light green group. Five genes from *D. purpureum* and six genes from *D. lacteum* were present in the grey group.

Based on the phylogenetic analysis, *D. purpureum* DpTPS1, DpTPS6, DpTPS2/9, and DpTPS4/5 were inferred to be orthologous to *D. discoideum* DdTPS6, DdTPS8, DdTPS5, and DdTPS3, respectively (Fig. 1B). The complete biochemical characterization of the DdTPS family (Fig. 2) and the DpTPS family^{10,21} enabled the functional comparison of these putative orthologs (Fig. S3). The first orthologous pair, DpTPS1 and DdTPS6, exhibited identical activities, producing a single sesquiterpene protoillud-7-ene, whereas the second orthologous pair, DpTPS6 and DdTPS8, exhibited distinct activities. The major product of DpTPS6 is (*E*)- β -farnesene, whereas the major product of DdTPS8 is an unidentified sesquiterpene. (*E,E*)- α -Farnesene is a common product of DpTPS7 and DdTPS8. The third putative pair of orthologs contains two genes from *D. purpureum*, DpTPS2 and DpTPS9. These two have completely distinct catalytic activities. The product profile of DdTPS5 is more similar to that of DpTPS2, with (*E*)- β -farnesene being the most abundant product for both enzymes. The fourth putative ortholog pair also contains two genes from *D. purpureum*, DpTPS4 and DpTPS5. While the catalytic activities of DpTPS4

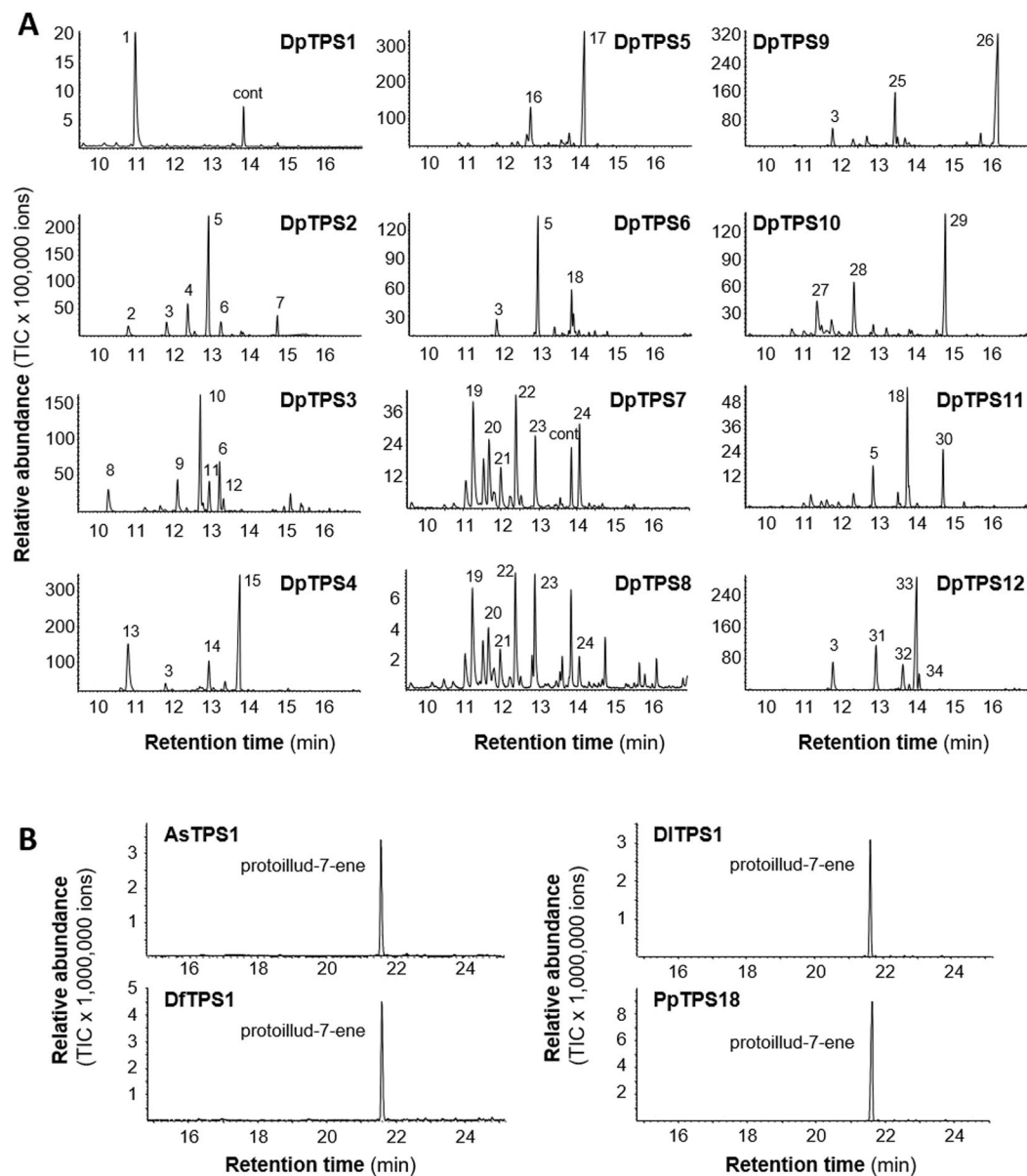


Figure 2. Sesquiterpene synthase activities of recombinant terpene synthases from Dictyostelids. **(A)** Sesquiterpene synthase activities of 12 TPSs from *D. purpureum*. *DpTPS* genes were expressed in *E. coli* individually, and crude proteins were isolated and their activities were analyzed with farnesyl diphosphate as substrate. 1, protoillud-7-ene; 2, δ -elemene; 3, β -elemene; 4, (*E*)- β -caryophyllene; 5, (*E*)- β -farnesene; 6, 9-*epi*-(*E*)-caryophyllene; 7–9, unidentified sesquiterpenes (STs); 10, CAS 137235-51-9; 11; α -neoclovene; 12, β -neoclovene; 13, bicycloelemene; 14–17; unidentified STs; 18, (*E,E*)- α -farnesene; 19–24, unidentified STs; 25, germacrene D; 26, unidentified oxygenated ST; 27–31, unidentified STs; 32, valencene; 33, α -selinene; 34, 7-*epi*- α -selinene; cont, contamination. **(B)** Sesquiterpene synthase activities of recombinant TPSs in the cluster of *DpTPS1* orthologs identified by phylogenetic analysis (Fig. 1). *AsTPS1* from *A. subglobosum*, *DfTPS1* from *D. fasciculatum*, *PpTPS18* from *P. pallidum* and *DITPS1* from *D. lacteum*. The sesquiterpene products from all four enzymes were identified to be protoillud-7-ene.

and *DpTPS5* are completely different, the two major products of *DpTPS5* (unidentified sesquiterpenes) have the same mass spectra as the two unidentified major products of *DdTPS3*.

Orthologous TPSs of clade I across the six dictyostelid species have the same catalytic activities. The orthologous pair of *DpTPS1* and *DdTPS6* belongs to clade I, and these enzymes have apparent orthologs in the other four species: *DfTPS1* from *D. fasciculatum*, *AsTPS1* from *A. subglobosum*, *PpTPS18* from *P. pallidum*, and *DITPS1* from *D. lacteum* (Fig. 1B). The observation that *DpTPS1* and *DdTPS6* have the same catalytic activity prompted us to ask whether the other orthologs have the same catalytic activities as well. To answer this question, the full-length cDNAs for *DfTPS1*, *AsTPS1*, *PpTPS18* and *DITPS1* were cloned from the respective

species and expressed in *E. coli* to produce recombinant proteins. Testing with farnesyl diphosphate, all four TPSs catalyzed the formation of the same sesquiterpene protoillud-7-ene, exhibiting the same catalytic activity as DpTPS1 and DdTPS6 (Fig. 2B). This indicates that the sole orthologous group among all the six species has an identical biochemical function despite more than 600 million years of divergence⁶. Such functional conservation is rare among TPSs from plants, fungi and bacteria.

Expression patterns of TPS genes during development of *D. purpureum* and comparative analysis. The multicellular development program in *D. purpureum* is initiated upon starvation. The 24-hour developmental process can be broadly divided into several stages: streaming, loose aggregate, mound, Mexican hat, and fruiting body (Fig. 1A). The expression of the DpTPS genes at seven time points during the 24 h development with 4 h intervals was extracted from a previous transcriptome analysis¹⁹ and presented in Fig. 3A.

All twelve TPS genes exhibited moderate levels of mRNA abundance and vast changes during the 24-hour course of the developmental process. Based on the mRNA abundance at the time of peak expression, the 12 genes can be categorized into three groups (Fig. 3A): high-abundance genes included DpTPS1, DpTPS8, DpTPS9 and DpTPS12, while medium-abundance genes were represented by DpTPS3, DpTPS5, DpTPS6 and DpTPS7, and low-abundance genes were DpTPS2, DpTPS4, DpTPS10 and DpTSP11. Furthermore, different time points for the highest expression levels of the DpTPS genes were observed. DpTPS2 was the only gene whose highest abundance levels occurred during vegetative growth (0 h). For all other genes, peak abundance occurred at various times during development. DpTPS1 and DpTPS7 were the two genes whose peak abundance happened at a time point during the first half of development. The highest expression of DpTPS9 and DpTPS12 occurred at the half point of development (12 h), while the peak abundance of the rest of the DpTPS genes was recorded during the second half of development (Fig. 3A).

The expression of orthologous pairs/groups between *D. purpureum* and *D. discoideum* was also compared (Fig. S4). It appeared that DpTPS9 and DdTPS5 had similar patterns of expression, and the same applied to DpTPS1 and DdTPS6. There were similarities between the expression patterns of DpTPS5 and DdTPS3, although the abundance of DdTPS5 was much higher.

During multicellular development, cells of *Dictyostelium* amoebae differentiate into two types: prestalk cells and prespore cells, which will eventually develop into the stalk and spores of the fruiting body²². All 12 DpTPS genes in *D. purpureum* were enriched in prestalk cells (Fig. 3B). This pattern is different from that of DdTPS genes in *D. discoideum*. While the majority of DdTPS genes also showed preferential expression in prestalk cells, two of them, DdTPS1 and DdTPS8, have higher expression in prespore cells (Fig. 3C). This is an intriguing observation, but its significance cannot be evaluated until we understand the biological functions of the terpenes produced by these gene products.

Emission of volatile terpenes during development of *D. purpureum* and comparative analysis. Most DpTPS genes showed dynamic expression during multicellular development, suggesting that they may have a function during this process. In addition, all of the DpTPSs were active in producing sesquiterpenes and seven of them were active in producing monoterpenes in the *in vitro* enzyme assays (Fig. 2A). Since sesquiterpenes and monoterpenes are volatiles, we performed volatile profiling of *D. purpureum* culture during 24 h development. Volatile collections were performed in every four hours.

During this time course experiment a mixture of eight volatile sesquiterpenes was detected (Fig. 4), most of them were identified as the products of DpTPS1, DpTPS9 and DpTPS12. The predominant sesquiterpene was α -selinene, the major product of DpTPS12. Besides α -selinene, β -elemene, an unidentified sesquiterpene (peak#31) and valencene were minor products produced by DpTPS12. From time point 4 to 24 hour, emission of these four sesquiterpenes produced by DpTPS12 increased with time, and reached the highest levels at 24 h. Protoillud-7-ene and germacrene D were produced by DpTPS1 and DpTPS9, respectively.

D. discoideum also released a mixture of volatile terpenes, including nine sesquiterpenes, one monoterpene and one diterpene¹⁰. In contrast to *D. purpureum* in which three DpTPS genes are main contributors of volatile production, the terpene products of all DdTPSs except DdTPS8 could be validated by the detection of DdTPS products in the volatile bouquet of *D. discoideum*¹⁰. Among all the volatile terpenes emitted from the two species, only one terpene is common: protoillud-7-ene, which is the product of the ortholog pair DpTPS1 and DdTPS6 in the clade 1 (Fig. 1B). In *D. purpureum*, α -selinene is the most abundant terpene at any time throughout multicellular development. In contrast, in *D. discoideum* the most abundant terpene before 24 hours is different from that at 24 h during development.

Concluding Remarks

This study has led to new insights into the diversity and functional evolution of the TPS gene family in dictyostelid social amoebae. Through phylogenetic analysis, a conserved clade of TPS genes from the six species of social amoebae was identified (Fig. 1B), implying a common evolutionary origin of dictyostelid TPS genes. The variation in the number of TPS genes among the six species suggests group- or species-specific expansion of TPS genes through gene duplication. At the biochemical level, individual orthologs in this conserved clade encode enzymes of the same catalytic activity (Fig. 2B), while the paralogs in *D. purpureum* (Fig. 2A) as well as those in *D. discoideum*¹⁰ exhibit divergent catalytic functions. While essentially nothing is known about the biological function of terpenoids in dictyostelids, this family of metabolites is involved in diverse biological processes in other organisms. In plants, for example, some terpenoids function as phytohormones critical for regulating growth and development and many others are involved in mediating plant-environment interactions¹³. In analogy, protoillud-7-ene, the product of DpTPS6 and its orthologs, may play a conserved, critical role, such as in regulating multicellular development, while the products of other TPS genes may have group- or species-adaptive functions, such as in chemotaxis²³, defense²⁴, or attracting beneficial organisms²⁵. Such functions have been

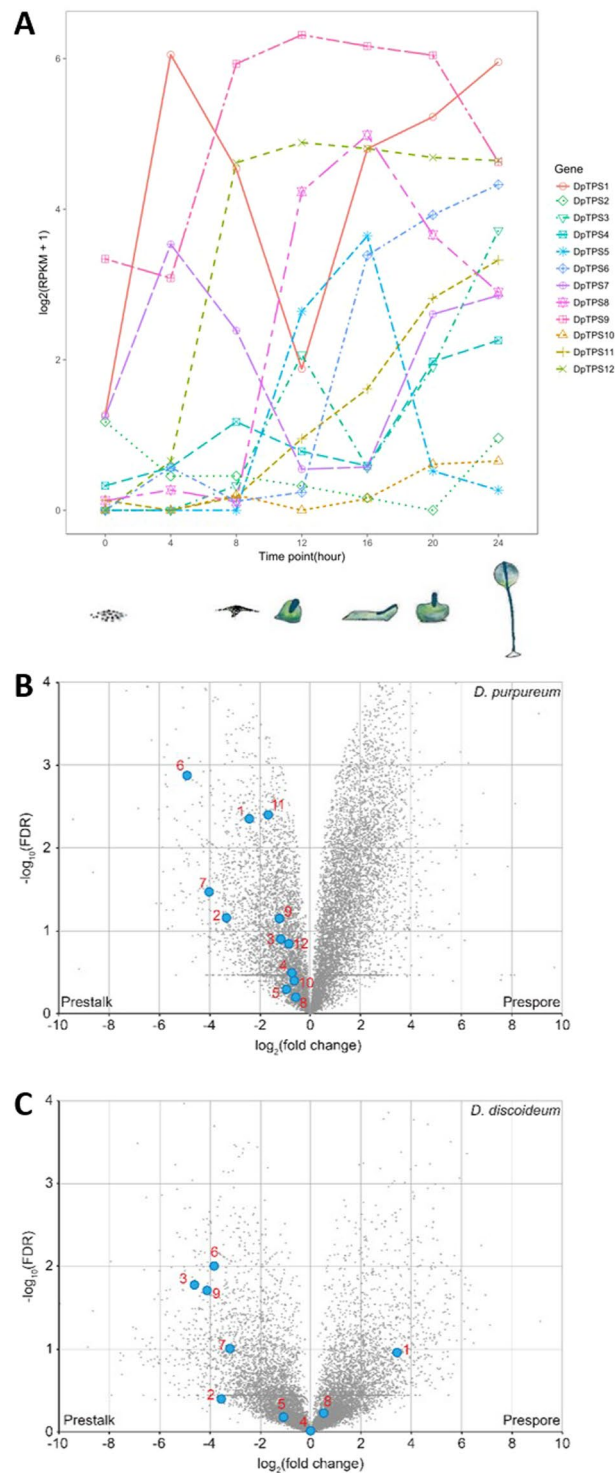


Figure 3. Expression of terpene synthase genes during dictyostelid development. **(A)** Expression of *DpTPS* genes during 24 hour development. Expression of *DpTPS* genes was measured by RPKM (reads per kilobase per million sequenced reads) based on RNA-Seq data produced from web-based interface program (<http://dictyexpress.biolab.si/>) and then displayed on a $\log_2(\text{RPKM}+1)$ scale in this line plot. The cartoons depict six stages of multicellular development: vegetative, individual cells (0 h), streaming (8 h), loose aggregate (10 h), slug (16 h), Mexican hat (20 h), and fruiting bodies (24 h). **(B)** Expression of *D. purpureum* TPS genes in prestalk and prespore cells. The numbers 1 to 12 correspond to *DpTPS1* to *DpTPS12*. **(C)** Expression of *DdTPS* genes from *D. discoideum* in prestalk and prespore cells. The numbers 1 to 9 correspond to *DdTPS1* to *DdTPS9*.

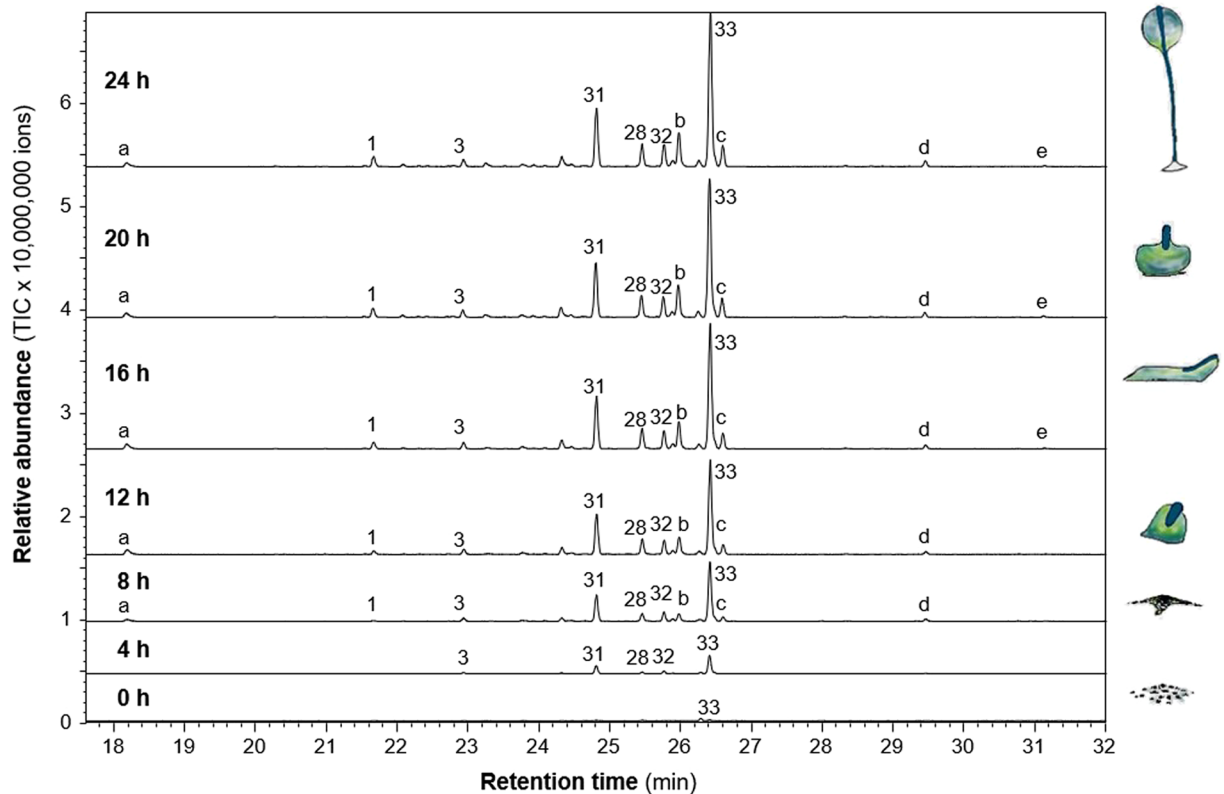


Figure 4. Volatile profiles of *Dictyostelium purpureum* culture during development. Volatile emissions during development of *D. purpureum* were collected by SPME and analyzed using GC-MS. All peaks labeled with a number are sesquiterpenes and the numbers correspond to the numbers of peaks in Figs 2 and 3. 1, protoillud-7-ene*; 3, β -elemene*; 28, germacrene D; 31, unidentified sesquiterpene hydrocarbon; 32, valencene; 33, α -selinene. Letters indicate compounds that are not found in the product spectra of DpTPS. “a”, unidentified compound; “b”, widdrol; “c”, unidentified oxygenated sesquiterpene; “d”, allo-hedycaryol; “e”, unidentified oxygenated terpene. Compounds marked with asterisks (*) were identified using authentic standards. The cartoons depict six stages of multicellular development: vegetative, individual cells (0 h), streaming (8 h), loose aggregate (10 h), slug (16 h), Mexican hat (20 h) and fruiting bodies (24 h).

proposed for terpenoids as well as other types of secondary metabolites made by dictyostelid social amoebae²⁶. Knowledge of the catalytic activities of individual TPSs and their contribution to the biosynthesis of volatile terpenoids during multicellular development in *D. purpureum* and *D. discoideum* now enables future researchers to elucidate the specific role of individual TPS genes and their terpenoid products. For this endeavor, mutant strains with disrupted TPS genes that could be produced using restriction enzyme-mediated integration (REMI) mutagenesis²⁷ or recently reported CRISPR/Cas9 technology for *Dictyostelium*²⁸ will be particularly useful.

Materials and Methods

Experimental organisms. *D. purpureum* DpAX1 (DBS0308472), *Acytostelium subglobosum* LB1 (DBS0235452), *D. fasciculatum* SH3 (DBS0235810), *D. lacteum* (DBS0235831) and *Polysphondylium pallidum* PN500 (DBS0236808) were obtained from Dicty Stock Center (<http://dictybase.org/>). Five fruiting bodies of *D. purpureum* DpAX1 were added into 300 μ l liquid culture of *Klebsiella pneumoniae*, mixed well, and spread onto SM agar plate (<http://dictybase.org/>). Five fruiting bodies of *A. subglobosum* LB1, *D. fasciculatum* SH3, *D. lacteum* and *P. pallidum* PN500 were mixed with *E. coli* B/r stain, and spread onto LP agar plates (<http://dictybase.org/>). The plates were incubated at 22 °C in the dark.

Sequence and phylogenetic analysis. Amino acid sequence dataset of *D. lacteum* (<http://sacgb.leibniz-fl.de>) was downloaded and searched against Pfam-A database locally using HMMER 3.0. Putative terpene synthase genes were identified using a HMM profile Terpene_synth_C (PF03936) with an e-value of $1e^{-2}$. Multiple sequences were aligned using MAFFT(L-INS-i). The maximum likelihood phylogenetic trees were built using RAxML under the LG + G + F model with 1000 bootstrap replicates.

Full-length cDNA cloning. Social amoeba tissues at the stage of fruiting body were collected and homogenized using TissueLyser II according to manufacturer’s manual (<https://www.qiagen.com>). Total RNA was isolated using RNeasy Mini kit following manufacturer’s protocol (<https://www.qiagen.com>). cDNA was synthesized using First Strand Synthesis Kit according to manufacturer’s protocol (<http://www.gelifsciences.com>).

Full-length cDNA of individual *TPS* genes was amplified with gene specific primers (Table S2), cloned into vector pEXP5-CT/TOPO (<https://www.thermofisher.com>), and fully sequenced.

Terpene synthase enzyme assays. Individual social amoeba *TPS* genes in the pEXP5-CT/TOPO protein expression vector were transformed into *E. coli* BL21(DE3) for heterologous protein expression. Crude protein extracts were desalted into assay buffer (10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 10% (v/v) glycerol) before enzyme assays. The catalytic activity of each social amoeba *TPS* was determined in assays containing 50 µl of the crude protein and 50 µl of assay buffer with 10 µM substrate (geranyl diphosphate, farnesyl diphosphate or geranylgeranyl diphosphate), 10 mM MgCl₂ and 0.05 mM MnCl₂. A solid phase microextraction (SPME) fiber consisting of 100 µm polydimethylsiloxane (SUPELCO, Bellefonte, PA, USA) was inserted into the headspace of the assay vial. After incubation at 30 °C for 1 h, the SPME fiber was withdrawn from the vial and then inserted directly into the injector of the gas chromatograph. For the substrate geranylgeranyl diphosphate, assays were overlaid with 100 µl hexane. After extraction by vortexing, 2 µl of the organic phase was analyzed by GC/MS. A Hewlett-Packard model 6890 gas chromatograph coupled with a Hewlett-Packard model 5973 mass spectrometer with a quadrupole mass selective detector was employed for product identification. Each assay was repeated at least three times and the results were found to be consistent.

Expression analysis of terpene synthase genes in *D. purpureum* and *D. discoideum*. The expression data of individual *TPS* genes in *D. purpureum* and *D. discoideum* was obtained from the dataset reported previously²⁹ and profiled using the dictyExpress web interface (<https://dictyexpressresearch.bcm.edu/landing/>).

Volatile profiling. *D. purpureum* spores and freshly grown *K. pneumonia* were mixed and spread onto SM agar plates to initiate culture. Volatiles of *D. purpureum* cultures during 24-hour multicellular development were collected at seven time points (every 4 hours) using SPME and analyzed using GC-MS as previously reported¹⁰.

Data Availability

Sequences of functionally characterized terpenes synthase genes from Dictyostelid social amoebae are available on GenBank: MG262459-MG262475.

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Author Contributions

F.C., X.C., T.G.K., G.S., J.S.D., and J.G. designed the experiments. X.C., T.G.K., and Q.J. performed experiments. X.C., T.G.K., Q.J., G.S., J.S.D., J.G., and F.C. performed data analysis and interpretation. F.C., X.C., T.G.K. and G.S. wrote the manuscript. F.C. supervised the project.

Additional Information

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