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Microbial-type terpene synthase genes occur widely in nonseed land plants, but not in seed plants

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The vast abundance of terpene natural products in nature is due to enzymes known as terpene synthases (TPSs) that convert acyclic prenyl diphosphate precursors into a multitude of cyclic and acyclic carbon skeletons. Yet the evolution of TPSs is not well understood at higher levels of classification. Microbial TPSs from bacteria and fungi are only distantly related to typical plant TPSs, whereas genes similar to microbial TPS genes have been recently identified in the lycophyte *Selaginella moellendorffii*. The goal of this study was to investigate the distribution, evolution, and biochemical functions of microbial terpene synthase-like (MTPSL) genes in other plants. By analyzing the transcriptomes of 1,103 plant species ranging from green algae to flowering plants, putative MTPSL genes were identified predominantly from nonseed plants, including liverworts, mosses, hornworts, lycophytes, and monilophytes. Directed searching for MTPSL genes in the sequenced genomes of a wide range of seed plants confirmed their general absence in this group. Among themselves, MTPSL proteins from nonseed plants form four major groups, with two of these more closely related to bacterial TPSs and the other two to fungal TPSs. Two of the four groups contain a canonical aspartate-rich “DDxxD” motif. The third group has a “DDxxxD” motif, and the fourth group has only the first two “DD” conserved in this motif. Upon heterologous expression, representative members from each of the four groups displayed diverse catalytic functions as monoterpene and sesquiterpene synthases, suggesting these are important for terpene formation in nonseed plants.

terpene synthase | specialized metabolism | nonseed plant | gene evolution

Terpenoids are the largest class of land plant secondary metabolites, but they are not uniformly distributed in the plant kingdom (1). Many seed plants (angiosperms and gymnosperms) produce terpenoids of diverse types in large quantities. However, among nonseed plants, only liverworts are known as copious producers of terpenoids (2). Terpenoids have diverse biological and ecological functions with many serving as chemical defenses against herbivores and pathogens (3, 4). Some have lineage-specific functions, such as the volatile terpenoids in flowers that are involved in attracting pollinators (5). Characterizing the biosynthesis of terpenoids in all plant lineages is therefore an important avenue to understanding their roles in the adaptation of various lineages of terrestrial plants.

Terpene synthases (TPSs) are pivotal enzymes for terpenoid biosynthesis, forming a distinctive superfamily based on both sequence identity and structure classification. However, within this group, typical plant and microbial (bacterial and fungal) TPSs share very low sequence similarity and are therefore only distantly related (6). The typical plant TPSs form subfamilies with individual

subfamilies generally associated with specific biochemical functions, such as monoterpene, sesquiterpene, or diterpene biosynthesis (7, 8). Monoterpene synthases and sesquiterpene synthases have been proposed to have evolved independently in gymnosperms and angiosperms from diterpene synthase ancestors (7, 9). Interestingly, the typical plant TPSs in the moss *Physcomitrella patens* (10) and the lycophyte *Selaginella moellendorffii* (11–13), two nonseed plants, were found to be of the diterpene synthase type. Therefore, the molecular basis underlying the biosynthesis of monoterpenes and sesquiterpenes identified in nonseed plants has long been unclear.

Recently, microbial terpene synthase-like (MTPSL) genes were identified in *S. moellendorffii* that encode monoterpene and sesquiterpene synthases (13). Unlike typical plant TPSs, which are composed of either three domains ($\alpha\beta\gamma$) or two domains ($\alpha\beta$) (14, 15), MTPSLs contain only an α -domain. Phylogenetic analysis indicated that MTPSLs from *S. moellendorffii* are more closely related to microbial TPSs, in particular fungal TPSs, than to typical plant TPSs (13). So far, MTPSLs have only been identified in *S. moellendorffii* (13), raising intriguing questions

Significance

Terpenoids are ubiquitous products made by land plants with diverse biological functions. Their formation in seed plants is catalyzed by typical plant terpene synthases (TPSs), a well-characterized group of enzymes. In contrast, our knowledge of terpenoid biosynthesis in nonseed plants is very limited. By systematically analyzing the transcriptomes and/or genomes of more than 1000 plant species, we report that microbial terpene synthase-like genes, which are only distantly related to typical plant TPS genes, are widely distributed in nonseed plants, but virtually absent in seed plants. The study provides insights into the evolution of TPS genes in early land plants and opens the door to investigating the diversity and functions of terpenoids in nonseed plants.

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Data deposition: The sequences for the biochemically characterized MTPSLs reported in this paper have been deposited in the GenBank database (accession nos. [KX230835–KX230843](https://doi.org/10.1073/pnas.1607973113)).

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about the origin, evolution, and function of this type of plant terpene synthase genes. The goal of this study was to investigate the distribution of *MTPSL* genes in the green plants, infer their evolution, and determine their biochemical functions.

Results and Discussion

Terpene Synthase Genes of the Microbial Type Are Widespread in the Transcriptomes of Nonseed Land Plants, but Not in Green Algae and Seed Plants. To determine the distribution of *MTPSL* genes in the green plants, the transcriptomes of 1,103 species (*SI Appendix, Table S1*) of green plants (779 species of seed plants, 166 species of nonseed land plants, 47 species of charophytes, and 111 species of chlorophytes) generated from the 1,000 Plant (OneKP) initiative (<https://sites.google.com/a/ualberta.ca/onekp/>) were searched for microbial type terpene synthase genes using a HMMER method as previously described (13). A total of 712 *MTPSL* genes were identified from the transcriptomes of 146 species. Strikingly, the vast majority of *MTPSL* genes (706 of the 712 *MTPSL* genes or 99.2%) were found in the transcriptomes of nonseed land plants (Fig. 1).

Bryophytes consist of three lineages: hornworts, mosses, and liverworts, which have 7, 41, and 26 species in the OneKP dataset, respectively. The number of hornwort, moss, and liverwort species whose transcriptomes contain *MTPSL* genes was 3, 30, and 24, respectively. Among the 22 lycophyte species, 21 possessed *MTPSL* genes in their transcriptomes. For monilophytes, 65 of the 70 species were found to contain *MTPSL* genes in their transcriptomes. The median number of *MTPSL* genes from the transcriptome of each species for hornworts, mosses, liverworts, lycophytes, and monilophytes was 0, 1, 8, 3, and 4.5, respectively (Fig. 1 and *SI Appendix, Table S2*). Among all species, the monilophyte *Cystopteris utahensis* (a tetraploid) was found to contain the most *MTPSL* genes with 20 members (*SI Appendix, Table S1*).

On the other hand, extremely low occurrences of *MTPSL* genes were found in the transcriptomes of seed plants and

charophytes. Among the 779 species of seed plants, only two species, *Phytolacca bogotensis* and *Opuntia* sp., both members of the Caryophyllales, were found to contain *MTPSL* genes in their transcriptomes with one and four members, respectively. Among the 47 species of charophytes, only one species, *Micrasterias fimbriata*, contained a *MTPSL* gene (one member) in its transcriptome. No *MTPSL* genes were found in the transcriptomes of 111 species of chlorophytes.

The Majority of *MTPSL* Genes Identified in Plant Transcriptomes Belong to Four Groups Clustered with Either Fungal or Bacterial Terpene Synthases. Phylogenetic analysis was performed for the 712 *MTPSL* genes identified from plant transcriptomes together with the 48 known *MTPSL* genes from *S. moellendorffii* (13) and selected terpene synthase genes from bacteria and fungi. The resulting phylogenetic tree indicates that the distribution of *MTPSL* genes in nonseed plants exhibits lineage-specific characteristics and that the majority of them (690 of 712) were clustered into four major groups with either similarity to bacterial TPS (groups I and II) or fungal TPS (groups III and IV) (Fig. 2).

Group I, the second largest *MTPSL* gene group, contains about 86% of *MTPSL* genes (152 of 177) from 23 species of liverworts, 34% of *MTPSL* genes (27 of 79) from 10 species of mosses, and 28% of *MTPSL* genes (23 of 83) from 9 species of lycophytes. Group II was composed of *MTPSL* genes primarily from mosses (about 66% of *MTPSL* genes from 24 species) and hornworts (50% of *MTPSL* genes from all three species in which *MTPSL* genes have been found). There was also one group II *MTPSL* gene found in a liverwort species, *Scapania nemorea*. Members of *MTPSL* genes in this species were also present in groups I and III. Group III, the smallest group, contains 4 *MTPSL* genes from 3 species of hornworts and 14 *MTPSL* genes from 7 species of liverworts. These 18 genes were clustered with the fungal trichodiene synthase (Tri5) genes. Group IV contains almost all *MTPSL* genes found from 65 species of monilophytes (352 of 353) and about 70% of *MTPSL* genes in lycophytes (58 of 83). The known *MTPSL* genes from *S. moellendorffii* were closely related to *MTPSL* genes from the transcriptomes of other lycophyte species.

Twenty-two *MTPSL* genes that lie outside of the four major groups were designated as “unclassified” (*SI Appendix, Table S3*). For example, the *MTPSL* genes found in two seed plant species, *Opuntia* sp. (four members) and *P. bogotensis* (one member), and one green alga, *M. fimbriata* (one member), were included in this list (Fig. 2 and *SI Appendix, Table S3*).

The Majority of *MTPSL* Genes Identified from Plant Transcriptomes Are Genuine Plant Genes. The putative *MTPSL* genes identified in the plant transcriptomes could have one of two possible origins: from plants or from plant-associated microbes. Three lines of evidence were used to judge that the vast majority, if not all, of these genes in the four major *MTPSL* clades are plant genes.

The first line of evidence comes from the analysis of putative *MTPSL* genes from axenic culture. The liverwort *S. nemorea* was selected for this purpose. A total of eight putative *MTPSL* genes were identified in the transcriptome of *S. nemorea* (*SI Appendix, Table S4*), belonging to group I (five genes), group II (one gene), group III (one gene), and unclassified (one gene). An axenic culture of *S. nemorea* was initiated by germinating isolated spores, using sterile culture methods. This culture was therefore free of contamination of endophytic microbes. We extracted genomic DNA from axenically cultured *S. nemorea* and used PCR to amplify DNA fragments for each of the eight putative *MTPSL* genes, and the results were compared with those obtained from the transcriptome analysis. Six of the eight *MTPSL* genes (five from group I and one from group III) were amplified and confirmed by sequencing. However, the amplification of the members from group II and the unclassified group failed. The

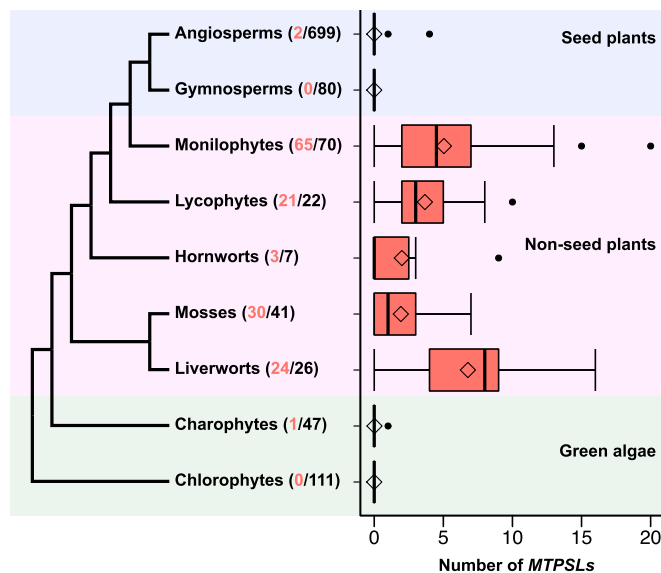


Fig. 1. Distribution of *MTPSL* genes identified from the transcriptomes of 1,103 plant species. The numbers in parentheses represent the number of transcriptomes containing putative *MTPSL*s (in red) and total transcriptomes analyzed in each lineage (in black). The phylogeny of green plants presented was modified from refs. 25 and 26. Each boxplot represents the number of *MTPSL*s found for individual species in each plant lineage. The solid black lines denote the median number of *MTPSL*s from each species. Whiskers represent 1.5 times the quartile of the data. Points outside of the range of the whiskers are outliers.

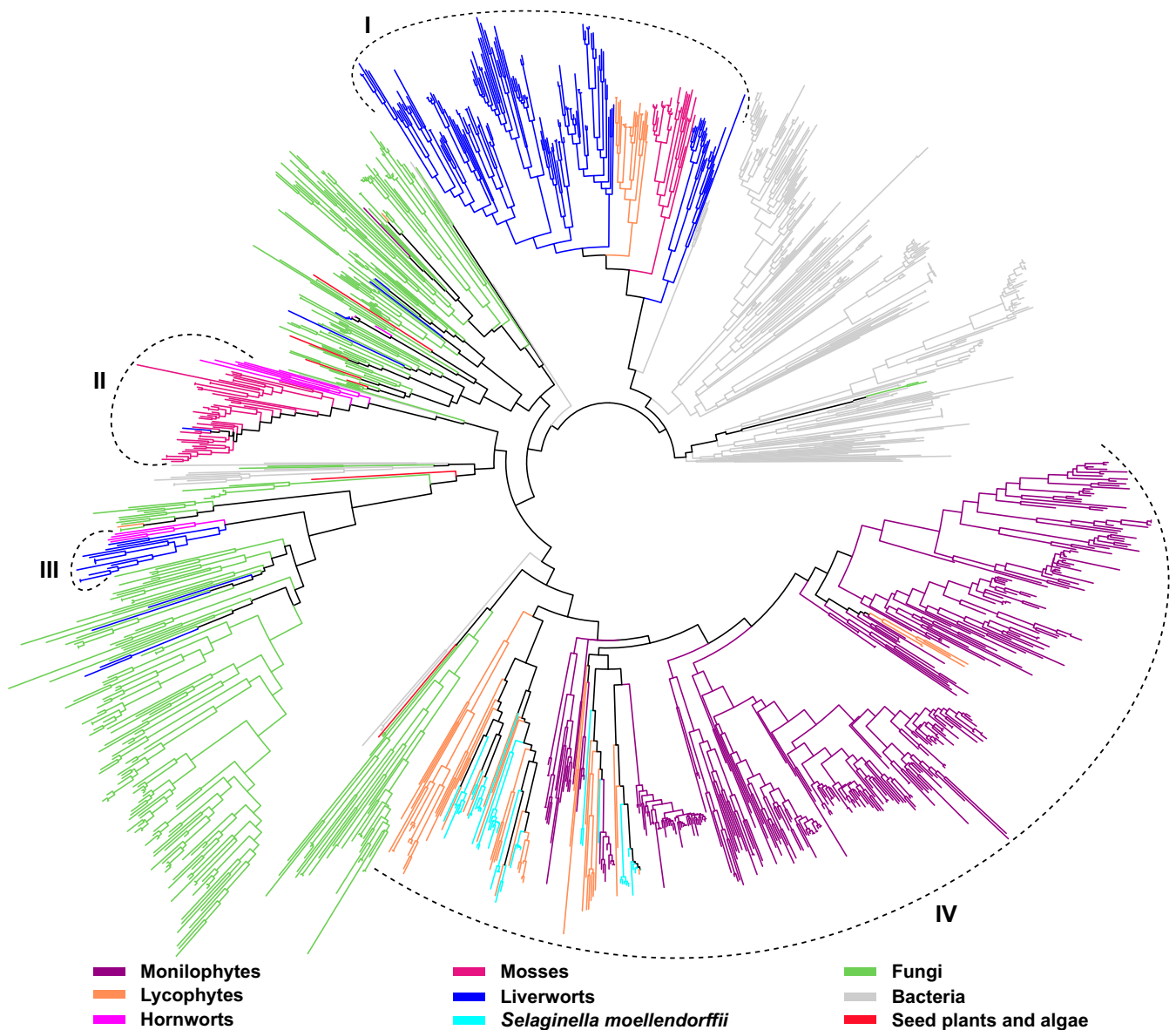


Fig. 2. Phylogeny of MTPSLs identified from OneKP with known MTPSLs from *S. moellendorffii*, bacterial TPSs, and fungal TPSs. Genes are color-coded based on their source. The majority of the newly identified MTPSLs are clustered into four major groups (I–IV).

member from the unclassified group was suggested to be a contaminant from endophytic fungi (*SI Appendix*, Fig. S1). Analysis of the OneKP transcriptomes has suggested that *S. nemorea* was contaminated with an unknown source of plant material (<https://pods.iplantcollaborative.org/wiki/display/iptol/Sample+source+and+purity>). The putative MTPSL from *S. nemorea* in group II is the only MTPSL from liverworts to be assigned to this group, suggesting that it was also derived from contamination by other plant material. Overall, this experimental study confirms that the group I and group III MTPSLs from the *S. nemorea* transcriptome are endogenous *S. nemorea* genes.

The second line of evidence for the plant origin of the majority of MTPSL genes reported is the identification of their immediate genomic neighbors as bona fide plant genes. We obtained such evidence for group II and III genes of the hornwort *Anthoceros punctatus*, whose genome has been recently sequenced (13). Assembling the raw genome sequence and then searching for MTPSL genes using the HMMER search, seven putative full-length MTPSL genes were identified; two of the seven genes

belong to group II and the remaining five to group III (*SI Appendix*, Table S5). *ApMTPSL1* from group II and *ApMTPSL2* from group III were then selected as representatives for the following study. In the assembled genome of *A. punctatus*, *ApMTPSL1* is the immediate neighbor to a cytochrome P450 gene, for which the most similar gene is from the plant *Oryza brachyantha* (Fig. 3A). Similarly, *ApMTPSL2* is the immediate neighbor to a leucine-rich receptor-like kinase gene, for which the most similar gene is from the plant *Theobroma cacao* (Fig. 3B). We extracted genomic DNA from *A. punctatus* grown in axenic culture and performed PCR to amplify the coding sequence of *ApMTPSL1* and -2 and their respective neighbors. The amplified DNA fragment was cloned and fully sequenced confirming that both *ApMTPSL1* (Fig. 3A) and *ApMTPSL2* genes (Fig. 3B) reside in the *A. punctatus* genome and are neighbors to a plant gene. Similar evidence for a group I gene was obtained from the moss *Sphagnum fallax*. The sequenced *S. fallax* genome contains 21 MTPSL genes, all of which belong to group I (*SI Appendix*, Table S6). A representative MTPSL gene (*SfMTPSL1*)

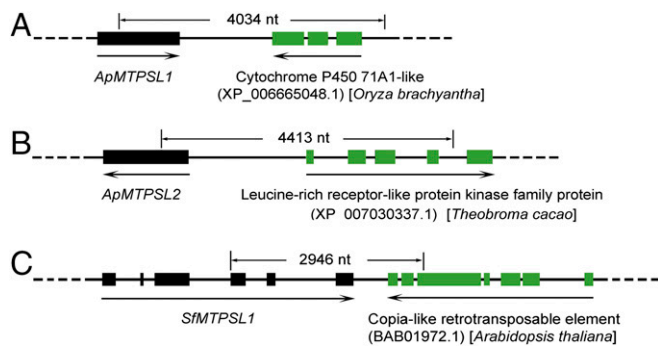


Fig. 3. Validation that representative *MTPSL* genes are of plant origin. Schematic genomic organization of representative *MTPSL* genes with their neighboring genes is shown. The genomic region spanning each *MTPSL* gene, its neighboring gene, and the intergenic region were amplified using PCR from genomic DNA and confirmed by sequencing. (A) *ApMTPSL1* with its neighboring gene annotated as a cytochrome P450 71A1-like from the hornwort *A. punctatus*. (B) *ApMTPSL2* and its neighboring gene annotated as a leucine-rich receptor-like kinase family protein from the hornwort *A. punctatus*. (C) *SmMTPSL1* with its neighboring gene annotated as a Copia-like retrotransposon element from the moss *S. fallax*. In each schematic figure, underneath the neighboring gene, the ID in parentheses represents the accession number of the best hit of this gene by searching the nonredundant database of the National Center for Biotechnology Information. The plant species indicates from which species the best hit was identified.

from *S. fallax* was confirmed to reside in its genome and neighbor with a Copia-like retrotransposable element, to which the most similar sequence is from the plant *Arabidopsis thaliana* (Fig. 3C). The genes in group IV form a clade with *SmMTPSL* genes from *S. moellendorffii*, which have already been determined to be plant genes (13). This evidence that representative *MTPSL* genes of groups I–IV originated from plant genomes supports that their apparent orthologs/homologs in each group are also of plant origin.

The third line of evidence for plant origin is that the overall evolutionary relationships of *MTPSLs* are largely congruent with the relationships of the land plant species from which the *MTPSL* genes are identified. The *MTPSL* genes in each of the four groups are inferred to share a common evolutionary origin (Fig. 2). Within each group, the *MTPSLs* from the same plant lineage showed higher sequence similarity than with *MTPSLs* from a different plant lineage. For instance, in group I the *MTPSLs* from three plant groups, liverworts, mosses, and lycophytes, form three distinct subclades (Fig. 2). The *MTPSLs* from closely related species are often also most closely related. For instance, the analyzed mosses included three species from the same genus *Sphagnum*: *Sphagnum lescurii*, *Sphagnum palustre*, and *Sphagnum recurvatum*. In group I, the *MTPSLs* from these three species reside in a clade (Fig. 2). Such fine-scale correlations of *MTPSL* sequence similarity and plant phylogeny also support a plant origin for most of these genes.

On the other hand, the 22 unclassified *MTPSL* genes (*SI Appendix*, Table S3) have a high probability of being derived from plant-associated microbes because their similarities with microbial TPS genes are extremely high (*SI Appendix*, Table S3), as in the example of the *S. nemorea* gene mentioned above, and so were not considered further in this study. Nevertheless, some of these genes may have been obtained from microbes very recently through horizontal gene transfer (HGT), which will be a subject of future investigation.

***MTPSL* Genes Are Patchily Distributed in Green Plants: Evolutionary Implications.** The confirmation that the vast majority of *MTPSL* genes identified from the OneKP transcriptomes are plant genes indicates that *MTPSL* genes occur widely in nonseed plants. Group I contains *MTPSL* genes from liverworts, mosses, and

lycophytes (Fig. 2), which implies the presence of *MTPSL* genes in the common ancestor of land plants. However, our survey found that *MTPSL* genes are generally absent from the transcriptomes of green algae (Fig. 1). To provide further evidence on the presence/absence of *MTPSL* genes in green algae, we conducted a focused search on sequenced genomes for six species of chlorophytes and one species of charophyte (*SI Appendix*, Table S7). No *MTPSL* genes were detected in these sequenced green algae. The absence of *MTPSL* genes in the transcriptomes of a wide range of chlorophytes and the genome of the charophyte, *Klebsormidium flaccidum* (16) suggests that *MTPSL* genes may have their origin in an ancestral land plant rather than an algal ancestor. Broader genome sampling from green algae, especially charophytes, is needed to test this hypothesis. Nonetheless, the evolution of *MTPSL* genes may have been associated with the transition of plants from aquatic to terrestrial habitats. The pioneer land plants faced a harsh environment replete with many new biotic and abiotic stresses. Many products of TPSs are volatile hydrocarbons that may be more useful in a terrestrial environment than in an aquatic one.

MTPSLs from nonseed land plants exhibited different degrees of relatedness to bacterial TPSs and fungal TPSs. Group I is most closely related to bacterial TPSs, whereas groups III and IV are most closely related to fungal TPSs. Group II is most closely related to a number of bacterial TPSs, which, however, reside within a fungal clade (Fig. 2). These patterns suggest a complex evolutionary history of microbial type TPSs. Whereas it is possible that microbial-type TPS genes are ancestral in all kingdoms of life and took different evolutionary trajectories, their confinement to bacteria, fungi, and plants implies HGT. Assuming that bacterial and fungal TPS genes are ancestral to *MTPSLs*, the distribution pattern of *MTPSLs* can be explained by multiple HGT events from bacteria and fungi. However, it is premature to make strong claims about the donors and recipients of such transfer events because our understanding of phylogenetic relationships of TPSs in bacteria and fungi is still very limited. A better understanding of relationships among TPS genes in bacteria and fungi will allow testing of this hypothesis.

The absence of *MTPSL* genes in seed plants is also notable (Fig. 1). To gain additional evidence about the presence/absence of *MTPSL* genes in seed plants, we analyzed the genomic sequences of 48 species of seed plants (*SI Appendix*, Table S7): no *MTPSL* genes were identified in them. As mentioned previously, land plants contain typical plant TPSs, which catalyze similar biochemical reactions for the production of terpenoids as do *MTPSLs*, but typical plant TPSs are only distantly related to *MTPSLs* (13). However, in the nonseed plants that have been studied, the typical plant TPSs function as diterpene synthases (7) rather than the full range of monoterpene synthases, sesquiterpene synthases, and diterpene synthases found in seed plants. Several *MTPSL* genes from *S. moellendorffii* have been demonstrated to encode monoterpene synthases and sesquiterpene synthases (13), and we hypothesized that most *MTPSL* genes in nonseed plants function in this way.

Representative *MTPSL* Genes Encode Active TPSs with Diverse Catalytic Activities. The presence of *MTPSL* genes only in nonseed land plants poses an intriguing question about their functions. In seed plants, TPSs are responsible for the production of a diversity of terpenoids important for ecological interactions, especially as defenses against herbivores and pathogens. Some nonseed plants, such as liverworts (17) and mosses (18), also produce a vast diversity of terpenoids. However, little is known about how such terpenoids are synthesized or about their biological functions.

In general, TPSs contain two highly conserved motifs: the DDxxD and NSD/DTE motifs, which are both involved in substrate binding (14, 15, 19). Whereas the NSD/DTE motif is

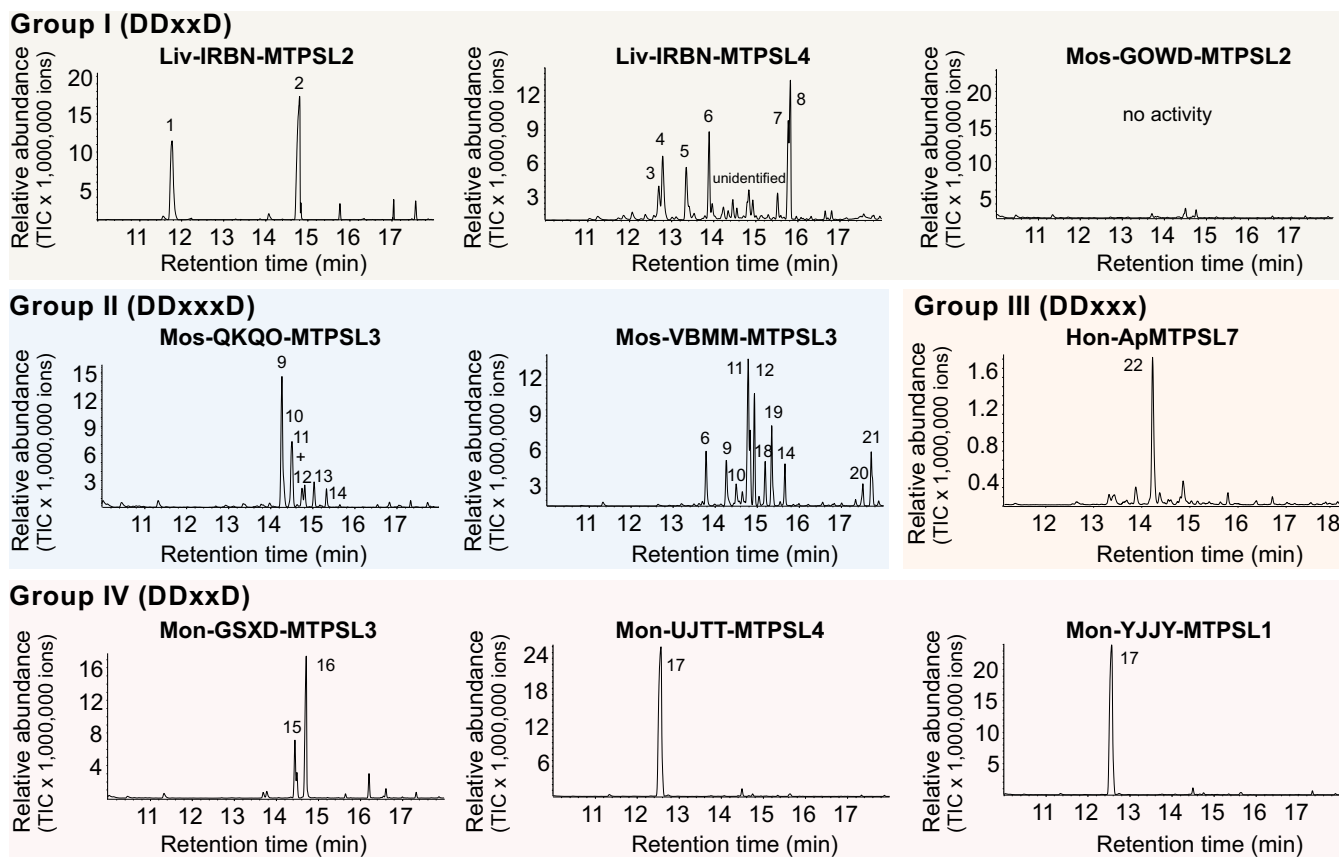


Fig. 4. Representative *MTPSL* genes from four groups (I–IV) encode active TPSs. The aspartate-rich motif associated with each group is indicated. *MTPSL* genes were heterologously expressed in *E. coli* and crude protein extracts were incubated with the potential substrate farnesyl diphosphate [(*E,E*)-FPP]. Enzyme products were collected using solid-phase microextraction and analyzed with gas chromatography/mass spectrometry. 1, bicycloelemene; 2, bicyclogermacrene; 3, α -isocomene; 4, β -elemene*; 5, (*E*)- β -caryophyllene*; 6, (*E*)- β -farnesene*; 7, nerolidol*; 8, dactylo; 9, γ -curcumene; 10, α -zingiberene; 11, β -bisabolene*; 12, β -curcumene; 13, sesquiphellandrene*; 14, (*E*)- α -bisabolene; 15, (*Z,E*)- α -farnesene; 16, (*E,E*)- α -farnesene; 17, protoillud-6-ene*; 18, (*Z*)- γ -bisabolene; 19, (*E*)- γ -bisabolene; 20, β -bisabolol; 21, α -bisabolol; and 22, β -acordiadiene. Compounds marked with an asterisk (*) were identified using authentic standards. The origin of each *MTPSL* gene is listed in *SI Appendix, Table S8*. Ap, *A. punctatus*; GOWD, *S. lescurii*; GSXD, *M. eatonii*; Hon, hornwort; IRBN, *S. nemorea*; Liv, liverwort; Mon, monilophyte; Mos, moss; QKQO, *Pseudotaxiphyllum elegans*; UJTT, *Pityrogramma trifoliata*; VBMM, *Anomodon rostratus*; and YJJY, *Woodsia scopulina*.

highly conserved in the *MTPSL*s, the aspartate-rich DDxxD motif exhibits variations (*SI Appendix, Fig. S2*). Group I and IV proteins contain the canonical DDxxD motif, but group II proteins displayed a conserved DDxxxD motif. In the group III proteins, only the first two aspartates (DD) are conserved.

To gain an initial assessment of the biochemical functions of *MTPSL*s, a total of nine genes representing the four *MTPSL* groups (*SI Appendix, Table S8*) were selected for experimental work. Recombinant *MTPSL*s produced from *Escherichia coli* were tested for TPS activity with geranyl diphosphate [(*E*)-GPP], farnesyl diphosphate [(*E,E*)-FPP], and geranylgeranyl diphosphate [(*E,E,E*)-GGPP], the substrates for monoterpenes, sesquiterpenes, and diterpenes, respectively. With the exception of Mos-GOWD-MTPSL2, all tested enzymes were able to convert (*E,E*)-FPP into individual sesquiterpenes or complex sesquiterpene mixtures (Fig. 4). Whereas most of the *MTPSL* sesquiterpene products are also known as products from typical plant TPSs, Mon-UJTT-MTPSL4 and Mon-YJJY-MTPSL1, which are closely related to fungal TPS, produced protoillud-6-ene, a sesquiterpene that has only been reported from a fungus so far (20). Mos-GOWD-MTPSL2 showed exclusively monoterpene synthase activity and converted (*E*)-GPP into (*Z*)- β -ocimene and some minor monoterpene products (*SI Appendix, Fig. S3*). In addition to sesquiterpene synthase activity, Liv-IRBN-MTPSL2, Mon-UJTT-MTPSL4, and Hon-Ap-MTPSL7 were able to produce monoterpenes from (*E*)-GPP and Liv-IRBN-

MTPSL4 and Mos-VBMM-MTPSL3 were able to convert (*E,E,E*)-GGPP into diterpenes (*SI Appendix, Fig. S4*). Recently, it was shown that certain typical plant TPSs naturally use *cis*-prenyl diphosphates as substrates for terpene production (21). Because most of the representative *MTPSL*s tested showed sesquiterpene synthase activity using (*E,E*)-FPP (Fig. 4), we performed additional assays to determine whether (*Z,E*)-FPP and (*Z,Z*)-FPP, the *cis*-isomers of FPP, could serve as substrates for these enzymes. Mos-GOWD-MTPSL2 was inactive with either substrate, whereas all of the other eight characterized *MTPSL*s showed activity with either (*Z,E*)-FPP or (*Z,Z*)-FPP or both (*SI Appendix, Fig. S5*). It is interesting to observe that some enzymes, such as Mos-QKQO-MTPSL3, produced the same products using the *cis*-FPP isomers as with (*E,E*)-FPP, whereas others such as Mon-UJTT-MTPSL4 produced different products (Fig. 4 and *SI Appendix, Fig. S5*). Nevertheless, the fact that the *Myriopteris eatonii* UJTT-MTPSL4 forms a single distinctive product from (*E,E*)-FPP and broad mixtures of over 10 products from each of the *cis*-FPP isomers suggests that (*E,E*)-FPP is the natural substrate. However, more information about the actual occurrence of these assay products in *M. eatonii* is needed before the natural substrates of these enzymes can be determined with certainty.

The substrates actually used by seed plant TPSs depend on their subcellular locations because the various prenyl diphosphate substrates are restricted to different subcellular compartments

(GPP and GGPP to plastids and FPP to the cytosol) (22). Thus, in seed plants, mono- and diterpene synthases are present in plastids and sesquiterpene synthases in the cytosol. However, information on TPS enzyme and substrate localization is not yet available for nonseed plants. To learn more, we used an in silico protein-targeting program (Target P, www.cbs.dtu.dk/services/TargetP) for Mon-UJTT-MTPSL4, an enzyme that produced a single sesquiterpene from (*E,E*)-FPP but also formed monoterpenes from (*E*)-GPP. Because the program suggests a cytosolic, nonplastid location for Mon-UJTT-MTPSL4, and FPP is known to be cytosolic in seed plants, this enzyme is likely to act as a sesquiterpene synthase in planta. Under steady-state conditions, the apparent K_M and k_{cat} values of Mon-UJTT-MTPSL4 using (*E,E*)-FPP as substrate were determined to be $2.13 \pm 0.23 \mu\text{M}$ and 0.15-s^{-1} , respectively. Such kinetic parameters are very comparable to those of typical plant TPSs (23), suggesting that MTPSL enzymes function in almost the same way as typical plant TPSs.

Based on the in vitro biochemical activities of representative MTPSLs, one could speculate that the MTPSLs have been the primary enzymes to make mono- and sesquiterpenes in early land plants and that the evolution of monoterpene and sesquiterpene synthases among the typical plant TPS family allowed the eventual loss of MTPSL genes in seed plants.

Conclusions

In this study, microbial-type terpene synthase genes, once thought to be confined to bacteria and fungi, were systematically mined from large-scale plant transcriptomes. Of 779 seed plant species, only 5 MTPSL genes were found in 2 species, whereas 706 MTPSL genes were found in 143 nonseed land plant species. So, in addition to the previous report on *S. moellendorffii* (13), MTPSL genes are widely distributed in nonseed land plants, but generally absent from seed plants and green algae. Although these genes are also found in fungi and bacteria, their occurrence in plants is in most cases not due to microbial contamination of the plant samples used for sequencing, based on experiments with axenic cultures,

phylogenetic analyses, and their embedment in plant genomes with bona fide plant genes as neighbors. MTPSL genes form four lineage-specific groups that exhibit diverse structural features, which implies multiple evolutionary origins. Biochemical studies of selected MTPSL genes showed that they encode sesquiterpene and monoterpene synthases. However, much more remains to be done to investigate the biological functions of their products and how they have influenced the evolution of the MTPSL gene family in nonseed land plants.

Materials and Methods

MTPSL genes were searched against the assembled transcriptomes for 1,103 nonmodel plant species derived from the OneKP (sites.google.com/ualberta.ca/onekp/) (24). Fresh materials of three axenically cultured plants, *A. punctatus*, *S. nemorea*, and *S. fallax*, were used for the extraction of genomic DNA that served as template for PCR analysis. For TPS activity assays, crude proteins extracted from *E. coli* expressing individual representative MTPSL genes were assayed with individual prenyl diphosphates. The kinetic properties of Mon-UJTT-MTPSL4 were measured with its purified recombinant enzyme following a radiochemical protocol as previously described (23). Details on transcriptome and genome assembly, sequence searches, phylogenetic reconstruction, plant cultures, and biochemical analysis of MTPSLs are provided in *SI Appendix*.

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