Manoyl Oxide (13R), the Biosynthetic Precursor of Forskolin, Is Synthesized in Specialized Root Cork Cells in Coleus forskohlii

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Forskolin, a complex labdane diterpenoid found in the root of Coleus forskohlii (Lamiaceae), has received attention for its broad range of pharmacological activities, yet the biosynthesis has not been elucidated. We detected forskolin in the root cork of C. forskohlii in a specialized cell type containing characteristic structures with histochemical properties consistent with oil bodies. Organelle purification and chemical analysis confirmed the localization of forskolin and of its simplest diterpene precursor backbone, (13R) manoyl oxide, to the oil bodies. The labdane diterpene backbone is typically synthesized by two successive reactions catalyzed by two distinct classes of diterpene synthases. We have recently described the identification of a small gene family of diterpene synthase candidates (CiTPSs) in C. forskohlii. Here, we report the functional characterization of four CiTPSs using in vitro and in planta assays. CiTPS2, which synthesizes the intermediate copal-8-ol diphosphate, in combination with CiTPS3 resulted in the stereospecific formation of (13R) manoyl oxide, while the combination of CiTPS1 and CiTPS3 or CiTPS4 led to formation of miltiradiene, precursor of abietane diterpenoids in C. forskohlii. Expression profiling and phylogenetic analysis of the CiTPS family further support the functional diversification and distinct roles of the individual diterpene synthases and the involvement of CiTPS1 to CiTPS4 in specialized metabolism and of CiTPS14 and CiTPS15 in general metabolism. Our findings pave the way toward the discovery of the remaining components of the pathway to forskolin, likely localized in this specialized cell type, and support a role of oil bodies as storage organelles for lipophilic bioactive metabolites.

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Coleus forskohlii (synonym: Plectranthus barbatus) is a perennial medicinal shrub of the mint family (Lamiaceae) indigenous to the subtropical and temperate climate zones of India and southeast Asia (Kavitha et al., 2010). The plant has been used since ancient times in Hindu and Ayurvedic traditional medicine for treating a broad range of human health disorders (Valdés et al., 1987; Lukhoba et al., 2006). The main active compound of C. forskohlii is forskolin, a heterocyclic labdane-type diterpenoid found in the roots of the plant (Ammon and Müller, 1985). The diverse known and potential pharmaceutical applications of forskolin extend from alleviation of glaucoma (Wagh et al., 2012) and anti-HIV or antitumor activities (Li and Wang, 2006) to treatment of hypertension and heart failure (Yoneyama et al., 2002). The efficacy of forskolin relies on activation of the adenylate cyclase enzyme (Seamon et al., 1981; Daly, 1984), leading to a marked increase of the intracellular level of cAMP (3'-5'-cAMP) in mammalian in vitro and in vivo systems (Alasbahi and Melzig, 2010b). The semisynthetic forskolin derivative NKH477 has been approved for commercial use in Japan for treatment of cardiac surgery complications, heart failure, and cerebral vasospasm (Toya et al., 1998; Kikura et al., 2004), while a forskolin eye drop solution...
was recently approved as an effective treatment for glaucoma (Wagh et al., 2012). Despite much effort, the full stereospecific synthesis of forskolin has not yet been achieved with current methods yielding racemic mixtures (Ye et al., 2009).

The chemical complexity of *C. forskohlii* has been well studied, and a total of 68 different diterpenoids have been isolated and identified from different tissues of the plant, of which 25 belong to the class of abietanes and 43 to the class of labdanes (Alasbahi and Melzig, 2010a). While the tricyclic abietane diterpenes have been reported to accumulate predominantly in the aerial parts, labdane diterpenoids with a bicyclic decalin core were detected primarily in the roots. Forskolin is a representative of an unusual series of tricyclic (8,13)-epoxy-labdanes, characteristic for this plant. Given its importance as a pharmaceutical, we set out to discover genes involved in the biosynthesis of forskolin. Our general approach utilized and built on a recently established diterpene pathway gene discovery strategy in nonmodel systems (Zerbe et al., 2013). As guiding principles, we considered possible spatial separation of different diterpenoid pathways in *C. forskohlii*, the large diversity of diterpenoids found in *C. forskohlii*, and the particular heterocyclic structure of forskolin. Based on the identification of putative labdane intermediates en route to forskolin in hairy root cultures of *C. forskohlii*, a pathway from trans-geranylgeranyl diphosphate (GGPP) to forskolin has recently been hypothesized (Asada et al., 2012).

GGPP is synthesized in plant plastids by the plastidial methylethrythritol 4-P pathway (Rodríguez-Concejón and Boronat, 2002). For the formation of labdane-type diterpenoids in angiosperms, GGPP undergoes an initial protonation-initiated cationic cycloisomerization to a labadienyl/copalyl diphosphate intermediate, catalyzed by class II diterpene synthase (diTPS) enzymes (Peters, 2010; Chen et al., 2011). The class II diTPS reaction, which forms the bicyclic decalin core of labdane-type diterpenoids, is terminated either by deprotonation or by water capture of the diphosphate carbocation. Subsequently, class I diTPSs catalyze cleavage of the diphosphate group and often additional cyclization or rearrangement reactions on the resulting carbocation. As with the class II diTPSs, deprotonation or water capture terminate the class I diTPS reaction. Water capture as a mechanism of diTPSs that leads to oxygen functionalities in the diterpene products has been described for the class II active site of bifunctional class I/II gymnosperm diTPSs (Keeling et al., 2011; Zerbe et al., 2012, 2013), class II angiosperm diTPSs (Falara et al., 2010; Caniard et al., 2012; Zerbe et al., 2013), and class I angiosperm diTPS (Caniard et al., 2012). Further oxidative functionalization of diterpenoids is typically catalyzed by cytochrome P450-dependent monoxygenase enzymes (P450s; Ro et al., 2005; Swaminathan et al., 2009; Hamberger et al., 2011; Wang et al., 2011; Guo et al., 2013; Hamberger and Bak, 2013), which then provide molecular handles for addition of auxiliary functional groups, leading ultimately to highly complex and decorated products such as forskolin and its derivatives.

Recently, Zerbe et al. (2013) reported the sequencing and assembly of a root transcriptome of *C. forskohlii* using 454 and Illumina sequencing technologies. Mining of the transcriptome databases resulted in the identification of a panel of candidate *CfTPS* genes. Here, we describe the functional characterization of five of them. Specifically, we demonstrate the function of *CfTPS2* in combination with *CfTPS3* in the stereospecific biosynthesis of (13R) manoyl oxide, the putative precursor of forskolin. We show that forskolin accumulates within a specific cell type of the root, the cork cells. Oil body-like structures characteristic and unique for this type of cell are found in the cytosol. We propose that these oil body-like structures facilitate the accumulation of high amounts of lipophilic diterpene metabolites.

**RESULTS**

**Identification of Unique Lipophilic Organelles in *C. forskohlii* Root Cork Cells**

Although high-level accumulation of forskolin-related labdanes in the root of *C. forskohlii* is well established, the localization of the biosynthetic pathway and specialized anatomical structures for the storage of diterpenes have not been reported. When transverse sections of *C. forskohlii* root (Fig. 1A) were examined using light microscopy, we found that cells of the root cork contained oil body-like structures (hereafter termed oil bodies) with a typical distribution of one oil body per cell of the root cork (Fig. 1B). These oil bodies appeared to be highly characteristic of the cork tissue, because they were not found in other cell types of the root. In sections of younger parts of the root, the oil bodies appeared yellow and occurred as single compartments within the cytoplasm of the immature cork cells (Fig. 1B). The color and pigmentation of oil bodies was observed to change with tissue maturation, possibly indicating developmental changes in their metabolite profile (Fig. 1B). In addition, cells containing more than one oil body were occasionally seen in older tissue (Fig. 1C). Further microscopy of other tissues revealed only sporadic occurrence of oil bodies in stem cork tissue (Supplemental Fig. S1).

To probe the nature of the oil bodies, histochemical staining was performed with Nile Red (9-diethylylamino-5H-benzof[a]-phenoxazine-5-one). Nile Red is a selective lipid-specific dye that is strongly fluorescent only in a hydrophobic environment. Its emission spectrum shifts depending on the polarity of its microenvironment, fluorescing magenta in the presence of polar lipids and green in the presence of neutral lipids (Diaz et al., 2008). Confocal laser scanning microscopy of *C. forskohlii* root cork stained with Nile Red indicated that the observed structures were oil bodies and that the composition of the lipophilic content was heterogeneous. Both neutral
(Fig. 1D) and polar (Fig. 1E) lipophilic compounds were observed to be nonuniformly distributed in the oil bodies with globules of neutral lipids dispersed in a predominantly polar lipid matrix (Fig. 1, D–F).

Localization of Forskolin and (13R) Manoyl Oxide in Root Cork Oil Bodies

As a first step to test whether the occurrence of forskolin is associated with the presence of oil bodies, we confirmed the localization of forskolin in the root cork. Separate methanol extracts of the root cork and the root cortex and stele were analyzed by HPLC using an evaporative light scattering detector (ELSD) and compared with flowers, leaves, and stems. Forskolin was primarily detected in the root cork and was not found in the root cortex and stele, leaves, or flowers (Fig. 2). Traces of forskolin were detected in the stem, consistent with the observed sporadic presence of a small number of oil bodies in stem cork tissue (Supplemental Fig. S1). To further examine if forskolin was present specifically in the oil bodies, methanol extracts of isolated oil bodies purified to apparent homogeneity (Fig. 3C) were subjected to HPLC–mass spectrometry (MS). Forskolin was detected in these structures, along with ions matching forskolin-related compounds (Fig. 3A; Table I). Ion peaks consistent with abietane-type diterpenes previously reported from C. forskohlii were also present in oil bodies (Table I).

To search for diterpenoid backbones that could serve as precursors to forskolin or to different abietane diterpenoids, isolated oil bodies as well as root cork, root cortex and stele, stem, leaves, and flowers were extracted with hexane, and the resulting extracts were analyzed by gas chromatography (GC)-MS (Figs. 3 and 4). (13R) Manoyl oxide was detected in root cork and, more specifically, in oil bodies isolated from this tissue (Fig. 3C), as well as in the root cortex and stele, stem, and flowers (Figs. 3, B and E, and 4). The localization of this compound to oil bodies is noteworthy given that it is considered the simplest diterpenoid backbone structure of forskolin and contains the correct stereochemical configuration (Fig. 3D). The abietane-type diterpenes miltiradiene, abietadiene, and dehydroabietadiene were also detected in various tissues (Fig. 4). Dehydroabietadiene was predominantly found in both types of root tissue, while abietadiene was mainly detected in the root cork tissue and miltiradiene in the stem and leaf tissue of C. forskohlii (Fig. 4).

C. forskohlii diTPSs Constitute a Small Gene Family

To investigate the molecular underpinnings of the diversity of diterpenoids found in the roots of C. forskohlii, we mined the root transcriptome of C. forskohlii for the

(Fig. 1) Localization of oil bodies within the root cork of C. forskohlii. A, Cross section of entire root with thick-tissue cork. Bottom right inset, the location of cork cells. B, Rows of cork cells each with one prominent oil body. C to F, Confocal imaging of Nile Red-labeled oil bodies. C, Transmitted light image of a cork cell with two oil bodies. Fluorescence images of the same oil bodies showing discrimination between neutral lipids (green fluorescence, D) and polar lipids (magenta fluorescence, E). F, Overlay of the two fluorescence images. Bars = 200 μm (A) and 10 μm (B–F).

Figure 2. Forskolin content (mg g⁻¹ dry weight [DW]) as determined by HPLC-ELSD analysis from different tissues of C. forskohlii. Data are the mean ± se of three independent biological replicates. Ck, Root cork; CS, root cortex and stele; Fl, flowers; St, stems; Lv, leaves.
identification of CfTPS candidates (Zerbe et al., 2013). A panel of six diterpene synthases was identified, CfTPS1, CfTPS2, CfTPS3, CfTPS4, CfTPS14, and CfTPS15, which, with the exception of CfTPS15, represented full-length complementary DNAs (cDNAs) with predicted N-terminal plastidial transit peptides. CfTPS1, CfTPS2, and CfTPS15 contained the Asp-rich conserved motif DxDD characteristic of class II diTPS, while CfTPS3, CfTPS4, and CfTPS14 carried the DDxxD signature motif of class I diTPS (Supplemental Fig. S2). To further substantiate the classification, relationships, and evolution of the CfTPSs, we performed separate phylogenetic analyses of class II and class I CfTPSs, including functionally characterized representatives from the Lamiaceae and other angiosperm species. Included in the phylogenies were representative gymnosperm class II and class I diTPSs from white spruce (Picea glauca) (PgCPS and PgEKS; Keeling et al., 2010) and the bifunctional diTPS from the moss Physcomitrella patens (PpCPS/EKS; Hayashi et al., 2010), as it is considered an ancestral archetype of plant diTPSs (Fig. 5).

Among the class II diTPSs, two distinct clades were apparent for dicotyledon species. One clade includes the single-copy Arabidopsis (Arabidopsis thaliana) AtCPS involved in general metabolism of gibberellic acid formation and orthologous from other species with characterized CPS functions. Also in this clade are paralogous gene pairs from pumpkin (Cucurbita maxima; CmCPS1 and CmCPS2) and Isodon eriocalyx (IeCPS1 and IeCPS2), which resulted from gene duplications potentially allowing for neofunctionalization of diTPS in the biosynthesis of specialized metabolites (Li et al., 2012a). In the second and apparently more divergent clade, enzymes of rock rose (Cistus creticus) copal-8-ol diphosphate synthase [CcCLS], tobacco (Nicotiana tabacum; labda-13-en-8-ol diphosphate synthase [LPPS]; NtLPPS), and two closely related Lamiaceae (clary sage [Salvia sclarea; S. scl] and Salvia miltiorrhiza, SmCPS1) are involved in specialized metabolism and have been shown to produce copal-8-ol diphosphate and (+)-copalyl diphosphate, respectively (Gao et al., 2009; Caniard et al., 2012). In C. forskohlii, we identified CfTPS15, a single-copy class II diTPS grouping with the bona fide CPS (general metabolism), and CfTPS1 and CfTPS2, a pair of diTPSs grouping with the class II enzymes involved in specialized metabolism.

The overall topology of the class I diTPS phylogeny resembles the class II phylogeny. One clade includes the EKS of general metabolism of gibberellic acid formation together with a set of rice (Oryza sativa) class I diTPS that evolved through species-specific duplications associated with neofunctionalization in rice-specialized metabolism (Morrone et al., 2011). CfTPS14 falls into this clade. A separate clade contains members involved in specialized metabolism, including tobacco NtCAS, converting copal-8-ol diphosphate to the specialized metabolite cis-abienol (Sallaud et al., 2012). The enzymes from S. sclarea and S. miltiorrhiza, producing the labdane-specialized metabolites sclareol (SsSCS) and the abietane miltiradiene, respectively (Gao et al., 2009; Caniard et al., 2012), are members of a subgroup consisting of Lamiaceae enzymes. A second pair of C. forskohlii diTPS, CfTPS3 and CfTPS4, is clustered with this clade. All members of this group of Lamiaceae sequences represent bidomain (βα) class I diTPS, lacking the γ-domain.
Transcript Levels of *C. forskohlii* diTPSs in Various Tissues

To correlate the transcript levels of CfTPS genes with accumulation of forskolin-related labdane diterpenoids and abietane diterpenoids in *C. forskohlii* tissues, quantitative reverse transcription-PCR analysis was performed using cDNA templates derived from total RNA extracted from root cork, root cortex and stele (root without cork), leaves, stems, and flowers. CfTPS1, CfTPS2, and CfTPS3 shared similar transcript profiles across all tissues, with high transcript accumulation in root cork cells, up to 1,000-fold compared with all other tissues tested (Fig. 6). These data provide support for the involvement of CfTPS1, CfTPS2, and CfTPS3 in the formation of specialized metabolites in the root cork. By contrast, the transcript levels of CfTPS4, CfTPS14, and CfTPS15 were relatively low across all tissues tested. Despite the close phylogenetic relation of CfTPS3 and CfTPS4 (Fig. 5), they show surprisingly different expression patterns. In contrast to CfTPS3, CfTPS4 transcripts were mostly detected in the aerial parts of the plant, especially in the leaves, while its transcripts accumulate only to very low levels in the root (Fig. 6).

Transcript profiles of the individual CfTPS genes, including both spatial patterns of distribution across tissues and relative transcript abundance, support a role for CfTPS14 and CfTPS15 in general metabolism and the involvement of CfTPS1, CfTPS2, and CfTPS3 in localized, active biosynthesis of specialized metabolites. These patterns are also in agreement with the results of the phylogenetic analysis (Fig. 5). Transcript profiles did not provide indications for a role of CfTPS4.

In Vitro Functional Characterization of *C. forskohlii* diTPSs

For the functional characterization of the CfTPSs described here (except for CfTPS15, for which no full-length sequence could be retrieved), cDNAs were heterologously expressed in *Escherichia coli* with a C-terminal 6×His epitope tag. Purified recombinant proteins were tested individually in single or coupled in vitro assays and supplied with appropriate substrates, and the reaction products were analyzed by GC-MS. Products of the in vitro single assays with the class II diTPSs, CfTPS1 and CfTPS2, were treated with alkaline phosphatase before GC-MS analysis.

Enzyme assays with CfTPS1 yielded a diterpene with a mass spectrum matching copal-15-ol, indicating that the primary product before dephosphorylation is copalyl diphosphate (Fig. 7A). Assays of CfTPS2 resulted in the formation of 13(16)-14-labdien-8-ol and labd-13-en-8,15-diol as major products (Fig. 7B), supporting a function as labda-13-en-8-ol (or copal-8-ol) diphosphate.

Table I. Abietane- and labdane-type diterpenoids detected by LC-MS analysis of oil bodies isolated from *C. forskohlii* root cork
Compounds were identified based on accurate mass measurements with the exception of forskolin, which was also identified based on coelution with an authentic standard.

<table>
<thead>
<tr>
<th>Retention time</th>
<th>m/z</th>
<th>Adduct</th>
<th>Predicted Formula</th>
<th>Mass Accuracy</th>
<th>Compound</th>
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<tr>
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<td>[M + H]⁺</td>
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<td>[M + Na]⁺</td>
<td>C₂₀H₂₈O₄</td>
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<td>Deoxy-deacetyl-forskolin</td>
<td>Asada et al., 2012</td>
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<tr>
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<td>C₂₀H₂₈O₄</td>
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<td>Asada et al., 2012</td>
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Figure 4. GC-MS analysis of hexane extracts from *C. forskohlii* tissues. The letter "b" indicates (13R) manoyl oxide, "h" indicates dehydroabietadiene, "f" indicates miltiadiene, and "i" indicates abietadiene. IS, Internal standard (1 mg L⁻¹ 1-eicosene).
synthase, similar to the functions of previously reported similar enzymes (Falara et al., 2010; Caniard et al., 2012; Sallaud et al., 2012; Zerbe et al., 2013). We also detected the nonstereoselective formation of the (13R) and (13S) epimers of manoyl oxide, which were previously observed in vitro reactions of similar class II diTPSs and were suggested to be the result of a nonenzymatic reaction (Caniard et al., 2012; Zerbe et al., 2013). These results indicate that CfTPS1 and CfTPS2 represent functionally distinct class II diTPSs, both necessary and sufficient to form the diphosphate intermediates required for the abietane and labdane classes of diterpenoids detected in the root of *C. forskohlii*.

The overall expression level of CfTPS14 in *E. coli* cells was low, with little activity in coupled assays with CfTPS1 and no detectable product formation in combination with CfTPS2 (data not shown). However, we have previously suggested a function based on coexpression with a diTPS from *Euphorbia peplus* (Zerbe et al., 2013), which supports a function of CfTPS14 in the general metabolism of gibberellin phytohormones. Assays of CfTPS1 coupled to either CfTPS3 or CfTPS4 resulted in formation of miltiradiene (Fig. 7A), an abietane diterpene formed from copalyl diphosphate (Gao et al., 2009), and is consistent with the results of the single enzyme assay of CfTPS1. Coupled assays with CfTPS2 and CfTPS3 showed the formation of the pure (13R) enantiomer of manoyl oxide (Fig. 7B), supporting a role of CfTPS2 in formation of the copal-8-ol diphosphate intermediate as detected in the single enzyme assay. In coupled assays of CfTPS2 with CfTPS4, both (13R) and (13S) manoyl oxide epimers were detected, albeit at a ratio different from the dephosphorylation product of CfTPS2 alone (Fig. 7B). The stereospecific production of the (13R) manoyl oxide epimer when CfTPS2 (class II)
and CfTPS3 (class I) enzymes were combined, along with their transcriptional coexpression in root cork tissue, where (13R) manoyl oxide and forskolin were detected, supports their involvement in the formation of the intermediate of forskolin in plant. Single enzyme assays using CfTPS3 and CfTPS4 with geranyl diphosphate (GPP) as substrate did not result in any product formation (Supplemental Fig. S3).

In Planta Heterologous Expression and Functional Characterization of C. forskohlii diTPSs

To validate the results obtained with CfTPS produced as recombinant proteins in E. coli, we investigated the in planta function of the CfTPSs by transient heterologous expression in Nicotiana benthamiana leaves after agroinfiltration. GC-MS analyses of extracts from N. benthamiana leaves transiently expressing the individual class I CfTPS3, CfTPS4, and CfTPS14 did not result in detectable accumulation of additional metabolites compared with control plants (data not shown). Extracts from N. benthamiana expressing the class II CfTPS2 alone showed only trace amounts of additional diterpenes compared with the controls, none of which could be accurately identified (Fig. 8B). Consistent with the in vitro enzyme assays, both (13R) and (13S) epimers of manoyl oxide were identified in the extracts from N. benthamiana expressing the class II CfTPS2 (Fig. 8A). Coexpression of CfTPS2 and CfTPS14 did not change the product profile compared with expression of CfTPS2 alone, suggesting that CfTPS14 does not accept the copal-8-ol diphosphate as substrate (Fig. 8A). In extracts of plants coexpressing CfTPS1 with CfTPS3 or CfTPS4, miltiradiene was observed as the main product together with minor traces of dehydroabietadiene and abietadiene (Fig. 8B).

In extracts from N. benthamiana coexpressing CfTPS2 with CfTPS3 or CfTPS4, only the (13R) epimer of manoyl oxide was identified (Fig. 8A), consistent with the stereochemical conformation of forskolin and the related series of labdane-type diterpenoids. This result suggests that the class I CfTPS3 and CfTPS4 can accept the copal-8-ol diphosphate synthesized by CfTPS2 and catalyze the stereospecific formation of (13R) manoyl oxide.

DISCUSSION

Intracellular Accumulation of Diterpenes in the C. forskohlii Root Cork May Be Facilitated by Unique Hydrophobic Oil Body Structures: A Suitable Means for Near-Surface Sequestration of Terpenoids in Roots

Plants have evolved both specialized mechanisms and specialized anatomical structures for the secretion, sequestration, and accumulation of defense-related and potentially toxic molecules (Morant et al., 2008; Schilmiller et al., 2008; Sirikantaramas et al., 2008). These metabolites may otherwise display adverse activities for the producing plant cell. Intracellular storage of such biologically active metabolites in the vacuole is well established for water-soluble compounds and for compounds that become water soluble through conjugation (Marinova et al., 2007; Ferreres et al., 2011; Zhao et al., 2011; Li et al., 2012b). Similarly, for the large class of often-lipophilic terpenoids, it has been suggested that their intracellular accumulation may be limited by nonspecific interference with cellular
processes and structures, such as interaction with membrane integrity (Uribe et al., 1985; Gershenzon and Dudareva, 2007; Sirikantaramas et al., 2008; Zore et al., 2011). Plant anatomical features and cellular structures typically associated with the biosynthesis and storage of large amounts of terpenoids are well studied and include glandular trichomes (Gershenzon et al., 2000; Iijima et al., 2004; Siebert, 2004; Schilmiller et al., 2008; Xie et al., 2008; Chatzopoulou et al., 2010; Lane et al., 2010), laticifer cells (Mahlberg, 1993; Post et al., 2012), resin cells, resin blisters, or resin ducts (Martin et al., 2002; Zulak and Bohlmann, 2010), and glandular cavities lined by epithelial cells (Heskes et al., 2012; Voo et al., 2012).

Figure 7. GC-MS analysis of in vitro assays with C. forskohlii diTPS. A, In vitro assays with CfTPS1 alone and coupled assays with CfTPS1 and CfTPS3 and CfTPS4. Extracts of CfTPS1 assays were treated with calf intestinal alkaline phosphatase (CIP). B, In vitro assays with CfTPS2 and coupled with CfTPS3 and CfTPS4. Extracts of CfTPS2 were treated with calf intestinal alkaline phosphatase. The letter “b” indicates (13R) manoyl oxide, “c” indicates (13S) manoyl oxide, “d” indicates labd-13-en-8,15-diol, “e” indicates labden-8-ol, “f” indicates miltiradiene, and “g” indicates copal-15-ol. IS, Internal standard (1 mg L⁻¹ 1-eicosene). C, Mass spectra of compounds identified from assays. Structures tentatively identified as described in “Materials and Methods.”
Here, we showed accumulation of the partly lipophilic diterpenoid forskolin and the presence of its precursor, (13R) manoyl oxide, together with a series of other labdane- and abietane-type diterpenoids, in root cork cells of \textit{C. forskohlii}. In these unique cell types, large oil bodies represent dominant and highly characteristic intracellular compartments that are not found in other tissues of the plant. Interestingly, the presence of both forskolin and (13R) manoyl oxide in these oil bodies indicates that the biosynthetic route to forskolin is active in this specific cell type. Accumulation of forskolin specifically in these oil bodies may facilitate the effective storage of terpenoids in a localized intracellular and lipophilic environment that is compartmentalized from the rest of the cytosol. Our designation of these oil bodies as terpenoid accumulation compartments in the cork cells of \textit{C. forskohlii} roots extends the known anatomical repertoire of specialized cells and cell compartments for producing and accumulating biologically active terpenoids in plants.

Given the biological activities of diterpenoids such as forskolin and their high level of accumulation in oil bodies of root cork cells, the diterpenoid-enriched root cork could provide a barrier against soil-born pests or pathogens. For the roots of \textit{C. forskohlii}, the near-to-the-surface sequestration of terpenoids in a cork layer may serve a similar protective function below ground, as is attributed to glandular trichomes on surfaces of the above-ground parts of plants. Glandular trichomes exist on the above-ground parts of \textit{C. forskohlii} but are generally not known to exist on root surfaces. The exclusive accumulation of forskolin, and detection of manoyl oxide and a series of related labdane diterpenoids in the cells of the root cork and the fact that labdane-type diterpenoids have been implied before in plant defense against microbes (Fragoso-Serrano et al., 1999; Habibi et al., 2000; Peters, 2006; da Silva et al., 2008), suggests a specific role of these compounds in the interaction of the plant root with the soil ecosystem and defense.

A New Role for Oil Bodies

Oil bodies are intracellular organelles mainly consisting of neutral lipids such as triacylglycerides, which have been suggested to function as carbon and energy reserves activated under starvation or rapid growth conditions (Penno et al., 2013). In some plant species oil bodies have also been associated with stress responses, hormone signaling, plant growth and development, and sterol biosynthesis (Fujimoto and Parton, 2011; Chapman et al., 2012; Silvestro et al., 2013). In liverworts (of the plant division \textit{Marchantiophyta}), these types of organelles have been suggested to cooccur with terpenoids (He et al., 2013). In vascular plants, oil body structures have been detected in several parts of the plant including seeds, leaves, flowers, pollen, and fruits. Oil bodies are considered to be derived from the endoplasmic reticulum, resulting from the accumulation of triacylglycerides between the endoplasmic reticulum bilayer, followed by budding of the cytoplasm-oriented membrane to form the newly generated oil bodies (Beller et al., 2010; Chapman et al., 2012; Murphy, 2012). Our findings of oil bodies with a heterogeneous composition including diterpenoids and localization to the cork of the \textit{C. forskohlii} root suggest additional functions of oil bodies in the sequestration of specialized metabolites and possibly plant defense (as discussed above).

Evolution of the \textit{C. forskohlii} diTPS Family Toward Chemical Diversity

Screening of the \textit{C. forskohlii} root transcriptome resulted in the identification of a small CTPS gene family. Single-copy class I and class II diTPS candidates
likely required for general metabolism, as well as pairs of duplicated class I and class II diTPS candidates potentially involved in specialized metabolism, have been identified. Based on phylogenetic relations and transcript profiles, we hypothesized involvement of CfTPS1, CfTPS2, CfTPS3, and CfTPS4 in specialized metabolism and a role for CfTPS14 and CfTPS15 in general metabolism of gibberellin phytohormone biosynthesis. DiTPS genes of general metabolism can serve as a template for gene duplication, which, in turn, could facilitate the evolution of genes with novel functions in specialized metabolism (for review, see Chen et al., 2011). Here, we traced such events from the phylogenetic relationships of the six different CfTPSs and substantiated them with functional characterization.

Originating from a class I/II bifunctional diTPS progenitor, angiosperm and gymnosperm diTPSs are thought to have evolved in two distinct clades, the class II or CPS and CPS-derived and the class I or EKS and EKS-derived enzymes (Chen et al., 2011). Additional clades of bifunctional or monofunctional diTPSs exist outside of the angiosperms (Zerbe et al., 2013). Within the two clades of class I and class II diTPSs, several branches of angiosperm diTPSs involved in specialized metabolism indicate events of lineage-specific diversification through gene duplication and neofunctionalization. Events of neofunctionalization may be limited to recruitment of the enzyme to a novel pathway, yet retaining its original CPS or EKS enzymatic function, or may have led to novel enzymatic functions (with or without loss of the original function). Examples are class II diTPSs involved in the metabolism of specialized diterpenes, such as *I. eriocaulyx* IeCPS2 (Li et al., 2012a) derived from a recent duplication of IeCPS1 and retaining ent-CPS activity. By contrast, *S. miltiorrhiza* SmCPS, tobacco NtLPPS, and *S. sclarea* ScSCS represent CPS-like diTPS with novel enzymatic functions (Gao et al., 2009; Caniard et al., 2012; Sallaud et al., 2012). Results from our work in *C. forskohlii* support a model of evolution through gene duplication, neofunctionalization, and loss of ancestral ent-CPS activity leading to CfTPS1 and CfTPS2.

For the class I clade of diTPS, separation of functionalities in general and specialized metabolism may be more challenging to assign. Enzymes of this clade may acquire the capacity to accept novel substrates while also retaining activity toward copalyl diphosphate. CfTPS14 appears to be a bona fide EKS with a likely function in gibberellin biosynthesis. Our results with both CfTPS3 and CfTPS4, when coupled with functionally distinct class II diTPS CfTPS1 or CfTPS2, indicate substrate promiscuity and possible redundancy between these two EKS-like enzymes in specialized metabolism. However, the distinct transcript profiles of CfTPS3 and CfTPS4 also indicated that the two enzymes may be involved in different functional contexts or pathways in *C. forskohlii*. CfTPS4 is mainly expressed in

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**Figure 9.** Scheme of the biosynthetic routes from GGPP to specialized and general diterpenoids of the abietane, labdane, and ent-kaurene class. Dashed arrows indicate reactions without experimental evidence in *C. forskohlii*. Detection of (+)-ferruginol in *C. forskohlii* was reported earlier (Hurley, 1998); CYP76AH1 from the close relative *S. miltiorrhiza* was proposed to convert miltiradiene to ferruginol (Guo et al., 2013).
the aerial tissues of the plant, where forskolin does not accumulate. It cannot be excluded though that CfTPS4 is coupled in these tissues with specific type II diTPSs not identified in our study.

Taken together, results from in planta and in vitro assays show that a relatively small family of diTPS can generate an array of different diterpene core structures (Fig. 9) when individual enzymes are combined in higher level functional modules of pairs of class II and class I enzymes. The diterpene structures detected in our assays match those known to exist in C. forskohlii and represent the starting points for oxidative decocations in the plant.

Involvement of CfTPS2 and CfTPS3 in the Formation of (13R) Manoyl Oxide en Route to Forskolin in Root Cork Cells

We showed here that the two class I diTPS CfTPS3 and CfTPS4 can each be effectively coupled with either of the two class II enzymes, CfTPS1 and CfTPS2 (Fig. 9), highlighting the remarkable modularity of diterpene metabolism. CfTPS1 was also found highly expressed in the same tissue as CfTPS2 and CfTPS3, indicating that cork cells may have the capacity to produce a suite of both labdane- and abietane-type diterpenoids. Following recent gene duplication, resulting in the CfTPS3 and CfTPS4 pair, a change in the expression pattern could have resulted in novel functional modules of diTPSs in different tissues. This finding is supported by the gene expression studies together with the nontargeted metabolomic HPLC-MS and GC-MS analyses of the root cork metabolites, showing highly complex profiles with accurate masses characteristic of diterpenoids of labdane and abietane classes (Fig. 4; Table 1).

Detection of both forskolin and (13R) manoyl oxide along with the accumulation of CfTPS2 and CfTPS3 transcripts in the specialized root cork cell type, together with the in vitro and in vivo functional characterization of CfTPS2 and CfTPS3, suggest that these two enzymes are involved in the early steps of forskolin biosynthesis via (13R) manoyl oxide in the root of C. forskohlii (Fig. 9). Transcript profiles of CfTPS4 would make a similar conclusion for this diTPS less obvious. Racemic mixtures of (13R) and (13S) manoyl oxide have previously been reported as artifacts or side products of enzyme assays (Caniard et al., 2012; Zerbe et al., 2013), a result also described here in our in vitro assay with CfTPS2 and CfTPS4. However, the stereospecific cyclization of GGPP into the (13R) epimer of manoyl oxide by the functional module of CfTPS2 coupled with either CfTPS3 (or CfTPS4) in vivo in the N. benthamiana expression system is consistent with the stereochemical conformation of forskolin and the series of forskolin-related labdanes detected in C. forskohlii (Asada et al., 2012), as well as with the absence of the (13S) epimer of manoyl oxide in the plant tissues analyzed (Fig. 4).

While the significance of (13R) manoyl oxide is attributed to its role as a forskolin precursor, manoyl oxide itself exhibits a number of important properties, such as antibacterial or antiinflammatory activities or as a potent anticancer agent (de las Heras and Hoult, 1994; Demetzos et al., 1994; Dimas et al., 1999; Angelopoulou et al., 2001).

Functions for CfTPS1 and CfTPS3 or CfTPS4 in the Formation of Miltiradiene

Assays with modules of CfTPS1 coupled to either the CfTPS3 or CfTPS4 resulted both in vitro and in vivo in the formation of miltiradiene, an intermediate in the biosynthesis of tanshinones in the closely related S. miltiorrhiza (Gao et al., 2009). While miltiradiene readily rearranged to dehydroabietadiene in our experimental setup, the in planta route to the aromatic dehydroabietadiene-derived diterpenoids, which are the second dominant type in C. forskohlii (Alasbahi and Melzig, 2010a), remains unclear. Recently, enzymatic conversion of miltiradiene to ferruginol, a 10-hydroxy-dehydroabietadiene, by a member of the CYP76 family was reported from S. miltiorrhiza (Guo et al., 2013). The identification of cytochrome P450s involved in the oxidative decoration of (13R) manoyl oxide en route to forskolin and related labdane diterpenoids is now underway using existing C. forskohlii P450 candidate resources and through development of new transcriptome and proteome resources for the specialized cell type of the C. forskohlii root cork cells.

CONCLUSION

Here, we reported the functional characterization of a panel of class I and II diTPSs, including a specific pair of diTPS involved in the biosynthesis of (13R) manoyl oxide in C. forskohlii. Manoyl oxide, itself an anticancer compound, is a structurally unusual diterpenoid carrying an oxygen-containing heterocycle and represents the molecular core for a large series of bioactive derivatives including the cAMP modulator, forskolin. We have shown that both manoyl oxide and forskolin accumulate together in a specific root cork cell type in C. forskohlii, indicating a role of these cells in protective plant-environment interactions in the rhizosphere. Biosynthesis and accumulation of specialized diterpenoids in these cells may be supported by the presence of oil bodies, providing the equivalent of a compartment specialized for storage of hydrophobic bioactive metabolites. Our discovery of C. forskohlii diTPSs participating in the early steps of forskolin biosynthesis, as well as the involvement of oil bodies in the storage of diterpenoids, may have implications for the discovery of other biosynthetic pathways of terpenoids in nonmodel plants and in biotechnological applications aiming at the production of high-value terpenoid-based pharmaceuticals, flavors, fragrances, and other terpenoid bioproducts.
MATERIALS AND METHODS

Plant Growth and Microscopy

*Coleus forskohlii* (Lamiaceae) plants were grown in the greenhouse at the University of Copenhagen, Denmark under ambient photoperiod and 24°C day/17°C night temperatures. Transverse sections of roots (diameter of approximately 1–5 mm) were prepared for histochemical analysis. Sections were performed by hand or by vibrating blade microtome (100 μm; Microm HM 650 V) and observed unstained with a Leica DM 5000B or a Nikon Eclipse 80i light and fluorescence microscope.

Additionally, root samples were fixed in a solution containing 2.5% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde, and 0.1 M sodium cacodylate buffer, pH 7.2, for 24 h, thereafter, surface sections and cross sections from the root cork were incubated in 0.1 μg ml⁻¹ Nile Red for identification of lipid components (Elgroth et al., 2005). Images from intact cells were recorded on a Leica SP5X confocal laser scanning microscope. A 20× water immersion objective was used for all images. Nile Red was excited with the 514-nm line from the argon laser, and the emitted light was collected at 525 to 567 nm and at 648 to 698 nm, respectively. Autofluorescence was tested without dye using the same microscope settings. Contrast adjustments were carried out to improve clarity of images but did not alter overall appearance. Final image processing, cropping, and mounting of the images were done with Adobe Photoshop CS2 and Illustrator CS2.

Diterpene Profiling and Forskolin Quantification in *C. forskohlii* Tissues

Tissue was extracted as described in De Vos et al. (2007). Cold methanol acidified with formic acid (0.125% [v/v]) was added to ground and frozen tissue samples in a ratio of 3:1 (tissue: solvent). Samples were sonicated in an ultrasonic bath at 23°C for 15 min at 40 kHz (Branson, 3510), filtered using 96-well filter plates, and analyzed by HPLC equipped with an ELSD. All tissue types were extracted in triplicate. The HPLC-ELSD system was comprised of a Shimadzu LC-20AT pump, SIL-20A HT autosampler, and ELSD-LTTI detector. Samples were separated on a Synergi 2.5 μm Fusion-RP C18 Column (50 × 2 mm; Phenomenex) at a flow rate of 0.2 mL min⁻¹ with column temperature held at 25°C. The mobile phase consisted of water with 0.1% formic acid (v/v; solvent A) and acetonitrile with 0.1% formic acid (v/v; solvent B). The gradient program was 20% to 100% B over 35 min and 100% B for 1 min, followed by a return to starting conditions over 0.25 min, which was then held for 15 min to allow the column to reequilibrate. The ELSD drift tube temperature was held at 50°C, and the nitrogen drying gas pressure was 3.8 bar. Forskolin was quantified by comparison to a standard series of forskolin (Sigma).

For the diterpene profiling of isolated oil bodies, extracts were lyophilized and then dissolved in methanol and analyzed by HPLC-electrospray ionization-high-resolution mass spectrometry. Separation was carried out on an Agilent 1100 Series HPLC unit with column and gradient as described above. The liquid chromatography (LC) unit was coupled to a Bruker microTOF mass spectrometer for accurate mass measurements.

Isolation of *C. forskohlii* Root Cork Oil Bodies

For isolation of oil bodies from root cork tissue, approximately 15 g of tissue was gently ground in 100 mL extraction buffer (20 mM Tricine, 250 mM Suc, 0.2 mM phenylmethylsulfonyl fluoride, pH 8.5); the homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 3,500 rpm for 10 min for separation of cellular debris. The supernatant was collected and transferred in centrifugation tubes. Buffer B (20% [v/v] Suc, 20 mM HEPES, 100 mM KCl, 2 mM MgCl₂, pH 10.5) was added with formic acid (0.125% [v/v]) was added to ground and frozen cork tissue samples in a ratio of 1:1 (tissue: solvent). Samples were sonicated in an ultrasonic bath at 23°C for 15 min at 40 kHz (Branson, 3510), filtered using 96-well filter plates, and analyzed by HPLC equipped with an ELSD. All tissue types were extracted in triplicate. The HPLC-ELSD system was comprised of a Shimadzu LC-20AT pump, SIL-20A HT autosampler, and ELSD-LTTI detector. Samples were separated on a Synergi 2.5 μm Fusion-RP C18 Column (50 × 2 mm; Phenomenex) at a flow rate of 0.2 mL min⁻¹ with column temperature held at 25°C. The mobile phase consisted of water with 0.1% formic acid (v/v; solvent A) and acetonitrile with 0.1% formic acid (v/v; solvent B). The gradient program was 20% to 100% B over 35 min and 100% B for 1 min, followed by a return to starting conditions over 0.25 min, which was then held for 15 min to allow the column to reequilibrate. The ELSD drift tube temperature was held at 50°C, and the nitrogen drying gas pressure was 3.8 bar. Forskolin was quantified by comparison to a standard series of forskolin (Sigma).

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Identification and Cloning of Full-Length diTPS Genes

Mining of the *C. forskohlii* database was performed as previously described (Zerbe et al., 2013) using tblASTs software and known angiosperm diTPSs as query (CPs and EKS) and guided full-length cloning of a number of putative class I and class II diTPS genes. Total RNA from *C. forskohlii* roots, extracted as previously described (Hamberger et al., 2011), was used for cDNA synthesis. First-strand cDNA was synthesized using the Takara PrimeScript First-Strand cDNA Synthesis Kit and oligo(dT) primer. Cloning of the putative diTPS genes was achieved after PCR amplification using gene-specific primers that were designed based on the in silico sequences of the identified CTPS genes (Supplemental Table S1). PCR products were cloned into the pJET1.2 vector and verified by sequencing.

Phylogenetic Analysis

The phylogenetic analyses are based on manually inspected amino acid alignments (DIALIGN-TX), which were analyzed by PhyML 3.1 (four rate substitution categories, Le Gascuel amino acid replacement matrix) and PAM substitution matrix (using BioNJ starting tree, 100 bootstrap repetitions) and visualization in treeview (rooted with the bifunctional copalyl diphosphate synthase/kaurane synthase from the moss *Physcomitrella patens* PpCPS/EKS [BAF61135]). The abbreviations and accession numbers of sequences used are given in Supplemental Table S2.

RNA Extraction and Quantitative Real-Time PCR

Total RNA from *C. forskohlii* root cork was extracted according to Hamberger et al. (2011) and further purified using the Spectrum Plant Total RNA Kit (Sigma), while total RNA from leaves, flowers, stems, and root cortex and stele was extracted using the Spectrum Plant Total RNA Kit. RNA extraction was followed by on-column DNase I digestion. The integrity of RNA samples was evaluated using the RNA-nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). First-strand cDNAs were synthesized from 0.5 μg of total RNA, with oligo(dT) primer, using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The resulting cDNA was diluted 10-fold for the quantitative reverse transcription-PCR reactions. The absence of genomic DNA contamination was verified by primers designed in exon-intron spanning regions of the Translation Initiation Factor4a (TIF4a) and Elongation Factor1a (EF1a) reference genes. Quantitative real-time PCR reactions were performed with gene-specific primers (Supplemental Table S1) and Maxima SYBR Green/Fluoroscribe dPCR Master Mix (Fermentas) on a Roche-Gen Q cycler (Qiagen). The PCR reactions were performed using the following cycling parameters: 95°C for 7 min (enzyme activation), 35 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, followed by a melting curve cycle from 60°C to 90°C. TIF4a and EF1a were used as reference genes as they showed the lowest variation across different tissues. No statistically significant differences were observed between the results obtained from the two different reference genes. The results were normalized with TIF4a. Relative transcript abundance was calculated as the mean of three biological replications (three different plants), while the reactions were performed in three technical replicates. Amplification efficiency was calculated with the Real Time PCR Miner (http://www.miner.eindhoven.info/). Efficiency-corrected differential cycles over threshold for target to reference values were used to quantify relative differences in target gene transcript accumulation. Primer specificity was assessed by agarose gel analysis and sequencing of amplicons from representative reactions, as well as from melting curve analysis of every reaction.

Functional Characterization of CTPS: In Vitro Assays

For the expression of the CTPS1, CTPS2, CTPS3, CTPS4, and CTPS14 in *Escherichia coli*, pseudomutants varying in predicted plastidial target sequence were cloned into the pET28b+ vector. The software ChloroP was used for prediction of the plastidial target sequence (http://www.cbs.dtu.dk/services/ChloroP/; Emanuelsson et al., 1999). As the expression levels of the recombinant CTPS1 was very poor, a codon-optimized version was synthesized by GenScript USA and subsequently cloned into the same vector (sequence is given in Supplemental Table S3). pET28b+ constructs were transformed into E. coli BL21DE3-C41 cells and inoculated in a starter culture with lysogyogen broth media and 50 μg mL⁻¹ kanamycin. A starter culture was diluted 1:100 in a 50 mL terrycloth broth medium with 50 μg mL⁻¹ kanamycin and grown at 37°C and 180 rpm until the optical density at 600 nm reached 0.3 to 0.4. Cultures were cooled to 16°C, and expression was induced at optical density at 600 nm of approximately 0.6 with 0.2 mM isopropylthio-β-galactoside. Expression was done overnight, and cells were harvested by centrifugation. Binding buffer (20 mM HEPES, pH 7.5, 0.5 mM NaCl, 25 mM Imidazole, 5% [v/v] glycerol) per 1 kanamycin were added to the cell pellet, which was gently shaken for 30 min and subsequently lysed by sonication (Branson sonifier 250; duty cycle, 30%; output control, 2-3; 1/4” tip). The cell lysate was centrifuged for 25 min at 12,000g, and the supernatant was subsequently used for purification of the recombinant proteins. Proteins encoded by *C. forskohlii* diTPS and the characterized monoterpane cineole
supplemental from Greek sage (Salvia fruticosa [SCIN]); Kampranis et al., 2007) were purified on 1-mL His SpinTrap columns (GE Healthcare) using elution buffer (binding buffer with 325 mM imidazole and 5 mM dithiothreitol [DTT]) and desalted on PD MiniTrap G-25 columns (GE Healthcare) with a desalting buffer (20 mM HEPES, pH 7.2, 350 mM NaCl, 5 mM DTT, 1 mM MgCl2, 5% [v/v] glycerol). In vitro TPS assays were performed by adding 15 µg GGPP or GPP and 100 µg purified CTPSs (or SCIN) enzymes in 597 µL enzyme assay buffer (50 mM HEPES, pH 7.2, 7.5 mM MgCl2, 5% [v/v] glycerol, 5 mM DTT). Onto the reaction mix, 500 µL n-hexane (Fluka GC-MS grade) was gently added as an overlay. Assays were incubated for 60 min at 30°C and approximately 70 rpm, and the hexane overlay was subsequently removed for GC-MS analysis.

Functional Characterization of CTPSs: Transient Expression in Nicotiana benthamiana

Full-length CTPS cDNAs were cloned into the Agrobacterium tumefaciens binary vector for plant transformation pCAMBIA1300_35Ssu with gene-specific primers (Supplemental Table S1) by USER cloning described in Norsved et al. (2006). Transient expression of CTPS genes with the gene-silencing suppressor p19 protein (Voinnet et al., 2003) in N. benthamiana leaves and extraction of diterpenes were performed as recently described (Spanner et al., 2013). Hexane extracts of N. benthamiana expressing the gene-silencing suppressor p19 protein alone were used as controls. Compounds of interest were identified by comparison of GC-MS total ion chromatogram and extracted ion chromatograms (EICs) of mass-to-charge ratio (m/z) 275 and 272 from samples. The ion m/z 275 is characteristic of several labdane-type diterpenes, including manoyl oxide, whereas m/z 272 is characteristic of several other nonlabdane-type diterpenes such as abietane-like diterpenes. All extractions from N. benthamiana transiently expressing CTPSs were carried out in biological triplicates (different leaves per plants infiltrated with the same agrobacteria mixture).

Metabolite Analysis from in Vitro and in Planta Assays

For the GC-MS analysis of N. benthamiana leaves expressing the CTPSs and specific C. forskohlii tissues, 500 µL GC-MS-grade hexane were added to two leaf discs (diameter = 3 cm) in a 1.5-mL glass vial. Samples were incubated at room temperature for 1 h in a Roto-Shake Genie revolving at 40 cycles min−1. After extraction, the solvent was transferred into new 1.5-mL glass vials and stored at ~20°C until GC-MS analysis. One microliter of hexane extract was injected into a Shimadzu GC-MS-QP2010 Ultra. Separation was carried out using an Agilent DB-5MS column (30 m × 0.25 mm i.d., 0.25-µm film thickness) with purge flow of 1 mL min−1, using helium as carrier gas. The GC temperature program was 50°C for 2 min, ramp at rate 4°C min−1 to 110°C, ramp at rate 8°C min−1 to 250°C, ramp at rate 10°C min−1 to 310°C, and hold for 5 min. Injection temperature was set at 250°C in splitless mode. For the GC-MS analysis of hexane extracts from in vitro assay, the following GC-program was used: 100°C for 1 min, ramp at rate 10°C min−1 to 250°C, ramp at rate 20°C min−1 to 310°C, and hold for 2 min. Compound identification was done by comparison to authentic standards (dehydroabietadiene, abietadiene), reference spectra from literature, databases, and comparison of retention time (miltiadene, manoyl oxide, copalol, labd-13-en-8,15-diol and 13(16)-14-labdien-8-ol; John Wiley and Sons, 2006; Adams et al., 2007; Lane et al., 2010). The differentiation of the C-13 epimers (13R) and (13S) manoyl oxide was performed as previously described (Demetzos et al., 2002). For the GC-MS analysis of hexane extracts from in vitro assays using GPP as substrate, the parameters used were: 2 min at 50°C, ramp to 140°C with 20°C min−1, ramp to 320°C with 10°C min−1 to 320°C, and hold for 3 min. One microliter was injected in splitless mode at 250°C, and the system was set in constant velocity mode with a linear velocity of 59.8 cm sec−1 using He as carrier gas. Solvent cutoff was set to 3.5 min.

Sequence data from this article have been submitted to the GenBank/EBI Data Bank under the following accession numbers KF444505 (CTPS1), KF444507 (CTPS2), KF444508 (CTPS3), KF444509 (CTPS4), and KF471011 (CTPS15).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Bright field microscopy of C. forskohlii stem cross section.

Supplemental Figure S2. Alignment of the CTPSs protein sequences together with PpCPS1/EKS.

Supplemental Figure S3. GC-MS analysis of hexane extract from in vitro assays using GPP as substrate.

Supplemental Table S1. List of primers used in this study.

Supplemental Table S2. Annotation and GenBank accession numbers of the proteins used in the phylograms.

Supplemental Table S3. Alignment of the nucleotide sequences of the native CTPS3 (natCTPS3) and the synthetic variant (synCTPS3).

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LITERATURE CITED


Biosynthesis and Intracellular Storage of Bioactive Diterpenes


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