Biochemical Conservation and Evolution of Germacrene A Oxidase in Asteraceae

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Sesquiterpene lactones (STLs) are characteristic natural products in Asteraceae which constitutes approximately 8% of all plant species. Despite their physiological and pharmaceutical importance, the biochemistry and evolution of STLs remain unexplored. Here we show that germacrene A oxidase (GAO), evolutionarily conserved in all major subfamilies of Asteraceae, catalyzes three consecutive oxidations of germacrene A to yield germacrene A acid. Furthermore, it is also capable of oxidizing non-natural substrate amorphadiene. Co-expression of lettuce GAO with germacrene synthase (GAS) in engineered yeast synthesized aberrant products, costic acids and ilicic acid, in an acidic condition. However, cultivation in a neutral condition allowed the de novo synthesis of a single novel compound, which was identified as germacrene A acid by GC-MS, LC-MS, and NMR analyses. In order to trace the evolutionary lineage of GAO in Asteraceae, homologous genes were further isolated from the representative species of three major subfamilies of Asteraceae (sunflower, chicory, and costus from Asteroideae, Cichorioideae, and Carduoideae, respectively) and also from the phylogenetically basal species, Barnadesia spinosa, from Barnadesioideae. The recombinant GAOs from these genes clearly showed germacrene A oxidase activities, suggesting that GAO activity is widely conserved in Asteraceae including the basal lineage. All GAOs could catalyze the three-step oxidation of non-natural substrate amorphadiene to artemisinic acid, whereas amorphadiene oxidase (AMO) diverged from GAO displayed negligible activity for germacrene A oxidation. The observed amorphadiene oxidase activity in GAOs suggests that the catalytic plasticity is embedded in ancestral GAO enzymes that may contribute to the chemical and catalytic diversity in nature.

Terpenoids, derived from isopentenyl diphosphate (IPP), are structurally the most diverse class of natural products with many known biological functions and commercial applications (1). One subclass of terpenoids is sesquiterpene lactone (STL) characterized by its α-methylene γ-lactone moiety on the 15-carbon core backbone (2). Although STLs are found in several plant families including Cupressaceae (3), liverwort (4,5), and even fungus (6,7), their occurrence in nature is by far the most frequent among Asteraceae (or Compositae) plants, the second largest plant family, after Orchidaceae, comprised of 23,000 plant species (8,9). Despite the structural complexities of STLs, the basic backbones of all STLs are constrained to several core skeletons, such as germacranolide, eudesmanolide, guaianolide, and helenanolide (2). This may suggest that STL biosynthesis is governed by a limited number of biochemical rules. Apparently, the chemical diversification of STLs is interlocked with plant speciation in Asteraceae, and hence the structural features of STLs have been routinely used as
chemosystematic markers in Asteraceae (10-12). Reported allelochemical, deterrent, dermal allergic, and insecticidal properties of STLs suggest important eco-physiological roles of STLs in inter-organism interactions (3). Thus, STLs are likely to be in part attributable to the evolutionary success of Asteraceae plants. In addition, several STLs have benefited human health and wellness as anti-inflammatory (e.g., parthenolide), sedative and analgesic (e.g., thapsigargin), and anti-malarial (e.g., artemisinin) medicines (13-18).

Although structural and bio-activity studies of STLs have been extensively performed, the biosynthetic route of STLs remains poorly understood at the molecular level. The proposed biosynthetic route of the simplest STL, costunolide, is depicted in Figure 1 (19,20). Farnesyl diphosphate (FPP) is cyclized to C15 hydrocarbon germacrene A by germacrene A synthase (GAS), and subsequently its C12 methyl group undergoes a three-step oxidation reaction to yield the germacrene A acid [germacra-1(10), 4, 11(13)-trien-12-oic acid]. An additional hydroxylation at C6 position of germacrene A acid facilitates non-enzymatic lactonization of C6 hydroxyl and C12 carboxylic group, yielding costunolide. The costunolide in turn serves as a framework of guaianolides, eudesmanolides, germacranolides, and other STLs by unknown mechanisms. Lastly, elaborate chemical decorations of STL scaffolds are carried out by P450s and several other modifying enzymes in order to produce more complex and biologically active STL end-products.

A number of GAS genes have been isolated and characterized in Asteraceae (21-24); however, the biochemical mechanism of germacrene A C12 oxidation and other downstream modifications have not been fully understood. Based on cell free assays in chicory, it has been proposed that three distinct enzymes, germacrene A C12 hydroxylase (P450), germacrene alcohol dehydrogenase, and germacrene aldehyde dehydrogenase, are involved in the biosynthesis of germacrene A acid in chicory (19). The P450-mediated biosynthesis of costunolide from germacrene A acid has also been demonstrated in chicory extract (20). Nonetheless, the corresponding genes responsible for these reactions have not been identified to date. Recently, the biochemical scenario of such three independent enzymes for germacrene A acid synthesis has been challenged by the discovery of multi-functional amorphadiene oxidase (AMO or CYP71AV1) which can catalyze three-step oxidations of amorphadiene to yield artemisinic acid in Asteraceae plant, Artemisia annua (Fig. 1) (25,26).

From the perspective of biochemical evolution, the artemisinic acid biosynthesis is a specific evolutionary event that only occurred in a single modern species A. annua. It can be, therefore, theorized that the biochemistry of artemisinic acid diverged from the more general germacrene A acid biosynthetic pathway. Comparative analysis of artemisinic acid and germacrene A acid biosynthesis would be an attractive model to understand the adaptive evolution of enzymes in terpenoid metabolism.

Germacrene A acid is certainly a necessary chemical for in-depth investigation of STL metabolism in Asteraceae. However, this compound is difficult to obtain because germacrene A acid is a low abundant, transient intermediate in the STL biosynthetic pathway (27), and the chemical synthesis of terpenoid is difficult to achieve. One report showed that a minute amount (2 mg) of germacrene A acid could be purified from 300 g of costus (Saussurea lappa) (27). Thus, microbial de novo production of germacrene A acid using cDNA clones can be a convenient alternative to acquire germacrene A acid.

In this report, germacrene A oxidase (GAO) isolated from lettuce (Lactuca sativa) was expressed in an engineered yeast to synthesize germacrene A acid de novo. Furthermore, GAO cDNAs were isolated from several other Asteraceae plants representing major subfamilies, including the phylogenetically basal species Barnadesia spinosa. Resulting biochemical data provided evidence that GAO activity is highly conserved in Asteraceae. In addition, the cross-reactivity of GAOs towards amorphadiene highlighted the evolutionary significance of the catalytic plasticity encoded in GAOs.

**EXPERIMENTAL PROCEDURES**

**Plasmid construct for gene expression**- Sequence information at the start and stop codons of LsGAO was obtained from the Compositae Genome
Project Database at the University of California Davis (compgenomics.ucdavis.edu). LsGAO was amplified from the cDNA pool prepared from lettuce leaf by a forward primer, 5'-CGAGGTCTAGATGGAGCTTTCAATAACC ACC-3', and a reverse primer, 5'-GCCCTCTAGAGCAAAACTCGGTAGTACA ACAAC-3'. The amplified product was digested by XbaI and ligated into the SpeI site of pESC-Ura plasmid. Artemisia annua CPR in a pESC-Ura plasmid (25) was digested by BamHI and NheI, and the digested fragment was ligated to the corresponding sites in LsGAO::pESC-Ura, resulting in a dual expression plasmid, LsGAO/CPR::pESC-Ura. This plasmid and previously generated GAS::pESC-Leu plasmid (24) were co-transformed to the EPY300 strain (25,28). For chemical purification purpose, plasmid stability was enhanced by coding three genes in a single plasmid. This was achieved by amplifying the expression cassette of GAS from GAS::pESC-Leu plasmid by a forward primer, 5'-GTCAATCCTAGCTGAGTGACTAGTACGA GCGCCGA-3', and a reverse primer, 5'-GTCAATCCTAGCTGAGTGACTAGTACGA GCGCCGA-3'. The amplified product was digested by DraIII and NaeI, and the digested fragment was ligated into the corresponding sites of an empty pESC-Leu2d. This DNA manipulation freed two multiple cloning sites for further cloning. Two expression cassettes for LsGAO and CPR were digested from the LsGAO/CPR::pESC-Ura by PacI and Scal, and the digested fragment was ligated to the corresponding sites of the newly generated GAS::pESC-Leu2d, resulting in the triple expression plasmid named GAS/LsGAO/CPR::pESC-Leu2d. Bioinformatic analyses identified start and stop codons of chicory, and sunflower, and their ORF sequences were used to design appropriate primers. The Barnadesia clone ordered from the Arizona Genomics Institute at the University of Arizona encoded a full-length cDNA (clone no. CCHS24399). For costus SIGAO clone isolation, a 1.4-Kb fragment of SIGAO was first obtained from costus cDNA using primers designed at the highly conserved domains of other GAOs. The primer pair used was a forward primer, 5'-ACCGTGGCTCAAAGCTCTCAGTC-3', and a reverse primer, 5'-GACTCCCCATAATCGGTACACATGC-3'. Both 5'- and 3'-RACE were conducted to determine start and stop codons of SIGAO, followed by the recovery of a full-length cDNA. All the isolated GAOs were first cloned to pESC-Leu vector to make translational fusions to the FLAG epitope. For subcloning purposes, HaGAO was amplified using a forward primer, 5'-GCACCTAGATTGGAAGCTCTCCCTACCACTT C-3', and a reverse primer, 5'-GCATCTAGTGCAAAACTTTGGTACAAGCA ACAAC-3'. SIGAO was amplified using a forward primer, 5'-TAATCTAGAATGGAGCTTCTCACCCTCTCACT TCCATTGC-3', and a reverse primer, 5'-TATTCTAGACGAAAAACTAGGTACCAGTACCAAATGAGTCC-3'. CiGAO was amplified using a forward primer, 5'-ACGTCCTAGAATGGAGCTCTCACCCTACCACT TCCA-3', and a reverse primer, 5'-ACGTCCTAGAATGGAGCTCTCACCCTACCACT TCCA-3'. Both amplifications were ligated into the XbaI or Spel and cloned into the SpeI site of pESC-Leu vector. The entire ORFs in fusion with the FLAG epitope were digested by NotI and PacI, and the LsGAO in the triple expression vector was removed and replaced with the NotI- and PacI-digested GAO ORFs.

Yeast culture and metabolite sample preparation—For standard yeast culture, the transgenic yeast strain of interest was inoculated in 3 mL Synthetic Complete (SC) medium omitting appropriate amino acids with 2% Glc. The inoculums were cultured overnight at 30 °C and 200 rpm. The start culture was diluted 100-fold in SC medium omitting appropriate amino acids with 1.8% Gal and 0.2% Glc. One hundred mL medium was cultured for metabolite profiling, whereas 1.0 - 2.5 L medium was cultured for chemical purification and identification. Rearranged sesquiterpenoids (costic acids) were extracted and analyzed by GC-MS according to the published methods (28). For buffered neutral culture, culture medium was adjusted to have 100 mM HEPES/NaOH (pH 7.5). After cultivating yeast for 48-72 h at 30 °C, the culture medium was adjusted to pH 6.0 with 2 M HCl, and the medium was extracted with ethyl
acetate twice. The ethyl acetate fractions were evaporated in N₂ gas to concentrate samples to 1 mL, and 1 µL was analyzed by GC-MS. For LC-MS analyses, the solvent was replaced with methanol.

**Microsome preparation, immunoblot analysis, and in vitro enzyme assay**- Microsomes were prepared according to the published protocol (29) except that a micro-beadbeater (Biospec Products, Bartlesville, USA) was used for 90 s with glass beads (500 µm diameter). For immunoblot analysis, microsomal proteins were separated on a 10% SDS-PAGE and transferred onto a Polyvinylidene Fluoride (PVDF) membrane. The membrane was blocked by 5% non-fat milk in TBST buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) for at least 1 h, incubated with anti-FLAG M2 antibodies (Sigma-Aldrich) in 1:5000 dilution, washed three time with TBST, and incubated with goat anti-mouse secondary antibody (GE Healthcare) in 1:5000 dilution. After washing the membrane three times with TBST, the bound secondary antibodies were detected with ECL Plus detection reagents (GE Healthcare). For *in vitro* enzyme assay, the protease-deficient *S. cerevisiae* YPL 154C:Pep4 KO strain was transformed with LsG AO/CPR::pESC-Leu2d and CPR::pESC-Leu2d. After 15 h cultivation in Glc, the yeast culture was shifted to fresh medium with 2% Gal, and the yeasts were further cultivated for 24 h. The *in vitro* enzyme reactions were carried out in 3 mL of 50 mM HEPES/NaOH (pH 7.5) buffer containing 3 mg of microsomal protein, 200 µM germacrene A, 500 µM NADPH, and an NADPH regeneration system (10 mM G-6-phosphate and three units of G-6-phosphate dehydrogenase). The reaction occurred at 23 °C for 4 h with gentle agitation. The reaction product was acidified with 2 M HCl to pH 6.0 and extracted with ethyl acetate. The extract solvent was then replaced with methanol for LC-MS analysis.

**GC-MS analysis of sesquiterpenoids**- Products obtained by the expression of the GAS/LsG AO/CPR::pESC-Leu2d plasmid were analysed on an Agilent 6890N gas chromatography system coupled to an Agilent 5975B mass spectrometer. One µl samples were injected at a port temperature of 150°C and analyzed on a DB5-MS column (30 m x 250 µm i.d. x 0.25 µm film thickness). Helium was used as carrier gas with a constant flow rate of 1 ml min⁻¹.

The temperature program was set to 40°C for 1 min, followed by a linear gradient of 10°C min⁻¹ to 250°C. For separating α- and β-costic acids, 2 µl samples were analyzed on an Agilent chiral Cyclodex-B column (30 m x 250 µm i.d. x 0.25 µm film thickness). The temperature program used was 1 min hold at 40 °C, 10 °C min⁻¹ increments from 40 °C to 100 °C, and 3 °C min⁻¹ increments from 100 °C to 245 °C. Retention indices (RI) for methyl esters of α-, β-, γ- costic acid, and ilicic acid were: in DB-5 column, 1807, 1805, 1788, and 1966, respectively; in Cyclodex-B column, 1914, 1920, 1889, and 2208, respectively. RI values for native forms of α-, β-, γ- costic acid and ilicic acid were: 1873, 1870, 1852, and 2103, respectively, in DB5 column. Cope-rearranged product, elemenoic acid, showed a RI value of 1762, and EI-MS relative ion intensity as follows: M⁺ 234 (1), 81 (100), 67 (49), 91 (38), 105 (34), 53 (33), 121 (29), 133 (18), 147 (17), 177 (17), 189 (14), 219 (8), 161 (9), 207 (4).

**NMR analyses**- For costic acids and ilicic acid, NMR spectra were recorded in 3 mm standard NMR tubes on a Varian Unity Inova 500 MHz spectrometer equipped with a 3 mm ID-PFG probe. The ¹H and ¹³C NMR chemical shifts were referenced to solvent signals at δ_H 7.14/127.68 (CDCl₃) relative to TMS. ¹H and ¹³C homonuclear NMR spectra were measured with standard Varian pulse sequences, and the experiments performed included gCOSY, TOCSY, ROESY, gHSQCAD, and gHMBCAD. Adiabatic broadband and band-selective gHSQCAD and gHMBCAD spectra were recorded using CHEMPACK 4.0 pulse sequences (implemented in Varian Vnmrj 2.1B spectrometer software). For germacrene A acid, ¹H and ¹³C NMR spectra were acquired in 5 mm standard NMR tubes at 400.13 and 100.6 MHz on a Bruker AVANCE 400 Spectrometer equipped with 5mm inverse probe with triple axis gradients. Chemical shifts (δ) were referenced to internal TMS for both ¹³C and ¹H. Spectra were recorded with standard Bruker pulse sequences under Xwinmr. Experiments performed included 1D proton, 1D ¹³C with proton decoupling, ¹³C attached proton test with proton decoupling, COSY with double quantum filter, TOCSY with 60 ms mixing time, HSQC, and HMBC.

**Isolation of ilicic acid (4)**- The EtOAc extract was concentrated to 100 µl in a vacuum concentrator...
Isolation of costic acids (1, 3). The same TLC separation was conducted as described above. In addition to the ilicic acid band, another band on the TLC plate showing strong absorbance at UV 254 nm with a Rf value of 0.62 was identified to contain 4 (peak 4) in Fig. 2 by GC–MS analysis. This band was excised, eluted with EtOAc and further separated by HPLC as described above. Compound 1 (γ-costic acid) was eluted at 9.6 min with a shoulder peak. Separate collection of the shoulder peak and subsequent NMR analysis afforded β-costic acid (3).

Isolation of germacrene A acid- For purification of germacrene A acid, the culture medium was extracted with EtOAc, concentrated to 100 µL and fractionated by reverse-phase column, Sep-Pak Plus C18 cartridge 55-105 µm column (Waters, Massachusetts, USA). Step gradients of elution solvents (water and acetonitrile), starting from 100% water with 10% increments of acetonitrile, were used to isolate germacrene A acid. A total of 56 fractions (0.5 mL each) were collected, and the presence of germacrene A acid was visualized as a blue spot on TLC plate using the method described by de Kraker et al (2001b). Fractions showing blue spots on TLC were further monitored by HPLC fractionation [P580 Dionex Liquid Chromatography system, Grom-Sil 120 ODS-5 ST column, 5 µm, 4.6 x 250 mm (Grom, Herrenberg, Germany)]. The mobile phase used was 40% acetonitrile acidified to pH 2.5 with TFA, and compounds were detected with Dionex DAD UVD340S. Compound 4 eluted at 9.9 min.

LC-MS analyses of sesquiterpenoids- Metabolite mass profiles were generated by Agilent 1200 Rapid Resolution LC (RRLC) system coupled with an Agilent 6410 MS using 10 µL injections of samples onto a reverse phase C18 column (2.1 x 50 mm, 1.8 µm, Eclipse plus C18 Zorbax) with a solvent gradient of 80:20 (A:B) to 20:80 (A:B) over 12 min at 0.4 mL min⁻¹ at 40 °C column temperature (A: H₂O with 1% acetic acid; B: 100% acetonitrile). The initial 30 s of LC was operated at an isocratic mode with solvent composition 80:20 (A:B). Total ion scans were used in both negative and positive mode and specific ion masses were selected for further mass analysis. For germacrene A acid detection in B. spinosa, one hundred mg of ground fresh tissue (a mixture of flower and leaf that were used for the cDNA library preparation in the Compositae genomics project) was provided from the Center for Genomics and Bioinformatics, Indiana University. The sample was extracted with 5 mL ethyl acetate, filtered, dried, and dissolved in methanol. The same RRLC program as above was employed using the Zorbax SB-C18 column (2.1 x 30 mm, 3.5 µm).

Phylogenetic analysis- GAO and AMO amino acid sequences were aligned by ClustalW algorithm. Phylogenetic analysis was performed using the Phylogenetic Analysis Using Parsimony (PAUP) 4.0 software (Sinauer Associates Inc., Sunderland, MA). The first 21 amino acids corresponding to the membrane domain were excluded. Characters were reweighted according to the rescaled consistency index. Parsimony analysis was performed using the tree-bisection-reconnection (TBR) algorithm. 1000 replicates of the bootstrap analysis were performed to evaluate the statistical significance of each node.

Sequence deposition- The DNA sequences for the GAO genes have been deposited in the GenBank data library under the following accession numbers: GU198171 (Lactuca sativa), GU256644 (Cichorium intybus), GU256645 (Helianthus annuus), GU256646 (Sassurea lappa), and GU256647 (Barnadesia spinosa).

RESULTS

Isolation of GAO and pathway reconstitution in engineered yeast. Lettuce (Lactuca sativa) accumulates STLs inside its laticiferous cells in
stems and leaves (30), and hence we first aimed at functionally identifying a gene encoding the enzyme for germacrene A oxidation from lettuce. A bioinformatics screening of the lettuce express sequence tag (EST) database using *Artemisia annua AMO* (or *CYP71AV1*) identified a gene highly homologous to *AMO* (25,26). PCR-amplification using primers designed at the start and stop codons of the identified ESTs allowed us to isolate a full-length gene from lettuce leaf cDNA. The isolated cDNA encodes a polypeptide of 488 amino acids with a predicted molecular mass of 54.9 kDa. The deduced amino acid sequence from this gene showed 86.7% identity to that from *A. annua AMO*. This P450 gene was designated as germacrene A oxidase (*GAO*) based on its catalytic property (see below).

In order to assess the enzymatic activity of *GAO*, yeast strain EPY300 engineered to produce a markedly increased level of farnesyl diphosphate (FPP, an immediate precursor of germacrene A) served as a platform strain. The production of the hydrocarbon germacrene A in the EPY300 strain expressing previously characterized lettuce *GAS* has been demonstrated (21,24). For *in vivo* catalytic coupling of *GAS* and *GAO*, open reading frames of *GAO* with FLAG epitope tag and *A. annua* cytochrome P450 reductase (CPR) with cMyc epitope tag under Gal10 and Gal1 promoters, respectively, were placed in the *pESC-URA* dual expression vector. This plasmid and previously generated *GAS::pESC-Leu* were co-transformed in EPY300 resulting in simultaneous expression of *GAS*, *GAO*, and *CPR* in FPP abundant cells. Upon induction of transgenes, the presence of *GAO* and *CPR* recombinant proteins was confirmed by immunoblot analysis with FLAG and cMyc antibodies (data not shown).

Terpenoids were extracted, derivatized as described previously (25), and subjected to the GC-MS analysis. Unexpectedly, four novel compounds, which were not present in the yeast expressing either *GAS* or *GAO/CPR*, were identified in the yeast expressing *GAS*, *GAO*, and *CPR* (Fig. 2A). The four compounds (1, 2, 3 and 4) showed the same parental mass of *m/z* 248 (methyl ester form) but distinct fragmentation patterns in EI-MS (Fig. 2B). The EI-MS analysis of their native (non-methylated) forms revealed an identical parent mass of *m/z* 234 in each case. The observation of *m/z* 248 (methyl ester) and 234 (native form) suggested that the hydrocarbon germacrene A (*M*, 204) is sequentially oxidized three times by GAO as is the case for the biosynthesis of artemisinic acid by AMO (Fig. 1).

(-/+)LC ESI-MS analyses of 1, 2 and 3 displayed their [M-H]· ions at *m/z* 233 and their [M+H]· ions at *m/z* 235 confirming the above findings. However, the (-)ESI-MS of 4 revealed a quasi-molecular ion [M-H]· at *m/z* 251. In the (+)LC ESI-MS, compound 4 displayed an abundant ion at *m/z* 235 which appears to be derived from the loss of H₂O from the unobserved parent ion [M+H]+ at *m/z* 253. In summary, the expression of lettuce *GAO* with *GAS* and *CPR* in EPY300 led to the production of four new compounds, and their MS analyses showed that 1, 2, and 3 have parent masses of 234, but 4 of 252.

**Structure analyses of products from yeast culture under acidic conditions.** We pursued the chemical purification of the new compounds for structural analysis. Prior to purification, plasmid stabilities were enhanced by coding all three genes (*GAS*, *GAO*, and *CPR*) in a single plasmid in *pESC-Leu2d* vector. The EPY300 strain transgenic for this new construct was cultured in 2.5 L, and the newly synthesized compounds were purified through TLC and HPLC. Compound 1, 3, and 4 were successfully purified and subjected to extensive 1D and 2D NMR studies (see Experimental Procedures for details). The structures of compounds 1 and 4 were unambiguously determined to be γ-castic acid and ilicic acid, respectively (Supplemental Table 1, Fig. 2C). Due to limited amount of 3, the 1H-NMR and indirectly detected 13C-signal of peak 3 could only be partially assigned. Nevertheless, structure elucidation indicated this compound to be β-castic acid. Compounds 1, 3, and 4 represent cyclization products of germacrene A acid (Fig. 2C). Although purification of 2 was not achieved, one can assume that this compound could be α-castic acid, the third form of cyclized product from germacrene A acid, in accordance with the observed molecular mass of 234 amu. Compound 4 (ilicic acid) is believed to be derived from hydration of the double-bonds in costic acids.

**Altered metabolite profile from the pH adjusted yeast cultivation.** We speculated that the cyclization of germacrene A acid occurs in the course of *de novo* germacrene A acid synthesis in an acidic culture condition. When pH values of the
yeast culture were monitored, the yeast medium (initially pH 6) became very acidic after a 48 h cultivation (pH ~3). In order to overcome the medium acidity, the pH was stabilized by supplementing the medium with 100 mM HEPES buffer (pH 7.5). In the buffered medium, the pH of the medium decreased to 6 after a 48 h cultivation. In the GC-MS analysis of the metabolites, the four peaks identified in the acidic condition almost disappeared, and instead a very broad, new peak with m/z 234 (without methylation) appeared (Fig. 3A, top). The broad peak was also observed previously when the hydrocarbon germacrene A was subjected to GC-MS analysis (27). This broadening was attributed to the on-column heat induced Cope-rearrangement of germacrene A into faster migrating β-elemene.

Before chemical purification, we evaluated if this new compound (broad peak) was indeed the precursor of the costic acids detected previously. To assess this possibility, the compound from neutral cultivation was treated with trichloroacetic acid (TCA). When the acidified samples were reanalyzed by GC-MS, the three early eluting peaks identified as costic acids were clearly detected with complete disappearance of the broad peak (Fig. 3A, middle). In addition, when the compound from the neutral cultivation was injected at 330 °C GC inlet temperature (previously 150 °C), the minute peak preceding the broad peak increased by 19-fold (Fig. 3A, arrow). GC-MS analysis suggested that the fast migrating compound was β-elemenoic acid by the heat-induced Cope-rearrangement in agreement with the published data (27) (Fig. 2D, see Experimental Procedures for full MS spectrum). In LC-MS analysis, the samples prepared from non-buffered medium showed the presence of costic acids (m/z 233) and ilicic acid (m/z 251) with a negligible amount of putative germacrene A acid (m/z 233) (Fig. 3B, top). However, in the samples prepared from the buffered medium, the amount of costic acids and ilicic acid decreased to 15% and 34%, respectively, relative to their levels in non-buffered culture. Concomitantly, a new peak of m/z 233 at a slightly earlier retention time than costic acids increased by 44-fold (Fig. 3B, bottom). These GC- and LC-MS results showed that simply cultivating the transgenic yeast in buffered medium dramatically alters the sesquiterpenoid profile from aberrant rearranged products (costic acids and ilicic acid) to a new peak with m/z 233 in negative ion.

**Structure elucidation of germacrene A acid.**
Four mg of the new compound was purified from a 1-L neutralized medium by solid-phase extraction column (C18 reverse phase). HR FT-ICR-MS showed [M-H]⁻ ions at m/z 233.1547 corresponding to the molecular mass for germacrene A acid (calculated mass for C_{15}H_{21}O_{2}, 233.1547). Based on the complete signal assignment of the compound by standard 1D and 2D NMR experiments (see Experimental Procedures, Supplemental Table 1), the compound was determined to be germacrene A acid. The extensive peak broadening observed in the 'H NMR spectra was attributable to the presence of different conformers as were found in the NMR analysis of germacrene A (31). In this case, the broad signals are due to intermediate exchange on the NMR time scale by the various conformers.

The *in vitro* activity of the recombinant GAO was further confirmed using the microsomes isolated from the yeast expressing *GAO* and *CPR*. In a careful pH-controlled experiment (pH was > 6.0 in all experimental conditions except for 12-min HPLC run), (-)LC-MS analysis showed that 233 ions for germacrene A acid were clearly detectable, but the other 233 ions from costic acids could not be detected (Fig. 3C). The *in vivo, in vitro*, and chemical analysis data demonstrated that GAO encodes an enzyme for the conversion of germacrene A to germacrene A acid.

**GAO activity is highly conserved in Asteraceae.**
The *in vivo* system was used to trace the advent of *GAO* in various Asteraceae plant species. In order to study the evolutionary lineage of *GAO*, a combination of EST-mining, PCR on conserved regions, and RACE methods was used to isolate full-length clones of *AMO/GAO* homologs from selected Asteraceae plants. Sunflower (*Helianthus annuus*), chicory (*Cichorium intybus*), and costus (*Saussurea lappa*) were chosen as the representatives of Asteroidae, Cichorioideae, and Carduoideae, respectively; these are the core subfamilies which constitute 95% of all Asteraceae. *Barnadesia spinosa* was selected as the representative of the phylogenetic base lineage, subfamily Barnadesioideae (Fig. 4A)(9).

The enzymatic activities of the isolated GAO clones were examined in the yeast *in vivo* system.
by co-expressing GAS and CPR. In the (-)LC-MS analyses, the 233 ions from the germacrene A acid were detected in all samples tested, and quantitative analyses showed that comparable amounts of germacrene A acid were synthesized in yeast expressing either a lettuce, chicory, costus, or Barnadesia clone (Fig. 5A). Although the germacrene A acid from the yeast expressing sunflower clone was one order of magnitude lower (10-15 fold) than that of the others, semi-quantitative immunoblot analysis of isolated microsomes revealed that the sunflower recombinant protein was at least 10-fold lower than that from the other clones (Fig. 5B). Hence, the sunflower enzyme also resulted in a comparable level of catalytic activity.

Although the involvement of germacrene A acid is apparent in sesquiterpenoid metabolisms of sunflower, costus, chicory, and lettuce on the basis of chemical structures and/or in vitro enzyme assays (19,20,24,27), the biochemical relevance of germacrene A acid in B. spinosa has not been addressed. Thus, the presence of germacrene A acid was examined by (-)LC-SIM (selective ion mode) from the ethyl acetate extract of B. spinosa (leaf and flower). In the (-)233 ion monitoring, germacrene A acid together with costic acids was detected at the concentration of 0.013% (w/ fresh w) (Fig. 5C). This result showed that germacrene A acid is present in B. spinosa most likely as an intermediate.

Collectively, these results demonstrated that GAOs and their corresponding enzymatic activities (three consecutive oxidations of germacrene A C12) are highly conserved at the phylogenetic basal clade of Asteraceae (i.e., Barnadesioideae) and retained in three major subfamilies of Asteraceae.

**Phylogenetic analysis of GAO and AMO.** The deduced amino acids from these clones shared significant sequence identities ranging from 78.4% to 97.3% (Fig. 4C). Interestingly, AMO shared a higher degree of homology to the GAOs from lettuce, chicory, sunflower, and costus (84.2-86.8%) than BsGAO did with these GAOs (79.6-82.6%). To obtain a better insight into the AMO and GAO evolution, a phylogenetic tree was reconstructed from AMO and five GAOs using two cytochrome P450s for sesquiterpene oxidations as outgroups (32,33). The phylogenetic analysis showed that AMO forms a distinctive node from the major GAO clade within the lineage originated from the Barnadesia GAO (Fig. 4B). Although both sunflower and Artemisia belong to the same Asteroideae subfamily, sunflower GAO constitutes part of the major GAO clade (Fig. 4B, bracket) that can be distinguished from AMO by a strong statistical support. This data implied that AMO in Artemisia annua recently underwent a specific biochemical micro-evolution that was not mirrored by the overall speciation patterns of the subfamily Asteroideae.

**Cross-reactivities of AMO and GAO.** In the sequence alignment, after excluding the membrane-bound domain, only 21 amino acids in AMO were different from the conserved residues in GAO, and an additional 7 amino acids in AMO were unique residues that were not present among the variable residues of other GAOs (Fig. 4C). Such high sequence homology between AMO and GAOs intrigued the substrate specificities of these two types of enzymes and the molecular evolution of AMO in Asteraceae. To address these questions, cross-reactivities of GAOs towards amorphadiene and AMO towards germacrene A were investigated using in vivo system. Co-expression plasmids for the swapped gene pairs (GAS/AMO or ADS/GAOs) were constructed in the CPR::pESC-Leu2d plasmid and were transformed to the EPY300 strain. When co-expressed, the GAS and AMO gene pair displayed negligible activity (0.04%) for germacrene A acid synthesis, relative to the activity detected from the native enzyme pair, GAS and GAO (Table 1). However, when co-expressed with ADS, all five GAOs from various Asteraceae plants displayed \(-10^2\)-\(-10^3\) fold higher relative activities (5-40%) for artemisinic acid synthesis than that from the GAS and AMO pair. These results suggest that AMO appeared to have lost its ancestral capacity to oxidize germacrene A during its evolutionary path, but the GAOs (predecessors of AMO) from various Asteraceae species including B. spinosa possess the catalytic potential (or plasticity) to oxidize amorphadiene.

**DISCUSSION**

In this work, GAO catalytic activity was unambiguously determined to be germacrene A oxidase using the pathway reconstitution in yeast system in vivo and a standard in vitro assay. Lack
of substrates and chemical standards often interfere with the progress of natural product biochemistry. Moreover, the instability of transient intermediates complicates biochemical studies. Both germacrene A and germacrene A acid are unstable, transient intermediates in the STL metabolic pathway in plants, and thus the acquisition of these compounds by purification from plant sources is difficult. In the absence of an authentic standard (germacrene A acid), the enzymatic production of germacrene A acid by \textit{in vitro} assays and subsequent purification to the level adequate for standard NMR analyses would be technically challenging. The results presented here demonstrated that pathway reconstruction in yeast is a convenient alternative for the characterization of gene function, mass production of natural products for chemical analysis, and substrate preparation for further biochemical studies.

The β- and γ-costic acids identified in this study are known natural products which can be derived from germacrene A acid \textit{in vitro} (27). It was therefore not surprising to detect these products in acidic yeast culture conditions, but the identification of ilicic acid was not expected from the yeast system. Ilicic acid and its hydroxyl derivatives have been reported as natural products in Asteraceae plants (34-36), and their anti-tumor and anti-inflammatory activities have been suggested (34,37). The formation of ilicic acid from costic acids could be mediated by non-specific yeast enzyme(s) due to the stereo-specific hydroxyl group at C4 position, but the nature of this chemical conversion was not further pursued in this study.

The origin and phylogeny of Asteraceae has been a subject of intensive systematic studies for the last 30 years. Based on fossil records and molecular systematic data, it is believed that Asteraceae originated about 30 million years ago in South America and radiated rapidly in all worlds except Antarctica (9,38,39). In 1987, it was discovered that a 22-Kb DNA inversion in the chloroplastic genome shared by all Asteraceae plants is lacking in the Barnadesioideae group, and thereafter this inversion data has served as key molecular evidence to support an ancient split between Barnadesioideae and the rest of the Asteraceae (39).
In directed evolution studies in vitro, enzyme evolvability towards non-natural substrates with decreased activity for the native substrate has been shown a number of times (45). Although rare in nature, a similar enzyme evolution is seen in the homologous enzymes - deoxyhypusine synthase (DHS) and homospermidine synthase (HSS) (47). The primordial DHS conserved in all eukaryotes can utilize the substrate of HSS which has evolved from DHS in some plants for pyrrolizidine alkaloid biosynthesis. However, the later evolved enzyme, HSS, cannot utilize DHS substrate, closely mirroring the results shown in this study. In most plants in Asteraceae, diversification and specification of primitive GAO may not be favoured due to the importance of the compounds derived from germacrene A acid, unless GAO is pressured to evolve by alteration of its substrate structure (e.g., amorphadiene in A. annua). Therefore, the naturally occurring gene pairs, GAS/GAO and ADS/AMO, evolved for the optimal catalytic coupling can be an excellent model system to infer the co-evolutionary process of the enzymes in a linear metabolic pathway.

Currently, the enzymatic conversion of amorphadiene to artesiminic acid is limited and incomplete in metabolically engineered E. coli and yeast (28,48). In this respect, the several GAOs identified here and other undiscovered Asteraceae GAOs could serve as ideal molecular templates to create catalytically more efficient amorphadiene oxidase through in vitro evolution and engineering methods.

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FOOTNOTES

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The abbreviations used are: STL, sesquiterpene lactone; P450, cytochrome P450 monooxygenase; GAO, germacrene A oxidase; AMO, amorphadiene oxidase; Glc, glucose; Gal, galactose; RACE, rapid amplification of cDNA end.

FIGURE LEGENDS

Figure 1. Sesquiterpene lactone biosynthetic pathways in Asteraceae. Left, the proposed biosynthetic pathway of general sesquiterpene lactones in Asteraceae; Right, the artemisinic acid biosynthetic pathway in Artemisia annua. FPP, farnesyl diphosphate; GAS, germacrene A synthase; ADS, amorphadiene synthase.

Figure 2. Analyses of the metabolites de novo synthesized from transgenic yeast. A. GC-MS chromatographs are shown for the sesquiterpenoids from yeast transformed with the indicated genes (GAS, germacrene A synthase; GAO, germacrene A oxidase, CPR, cytochrome P450 reductase). Line a and b are negative controls, and line c displays the metabolites unique to the yeast transformed with three genes (GAS, GAO, and CPR). Compound 2 and 3 were not separated by DB-5 MS column but were clearly separated by the chiral column (Cyclodex-B column) as shown in the inset. B. The mass fragmentation patterns of compound 1 to 4 are given. C. Proposed acid-induced rearrangements of germacrene A acid to α-, β-, and γ-costic acid and additional modification of costic acids to ilicic acid in yeast culture. Speculated structure of peak 2 as α-costic acid was also given with a question mark. D. Cope-rearrangement of germacrene A acid to elemenoic acid by heat.

Figure 3. GC-MS and LC-MS analyses of sesquiterpenoids from yeast expressing GAS, GAO, and CPR in buffered neutral culture. A. GC-MS chromatographs of the sesquiterpenoid products. Inlet temperatures and chemical treatment are shown in front of the chromatograms. TCA, trichloroacetic acid; bracket, α-, β-, γ-costic acids; asterisk, germacrene A acid; arrow, heat-induced rearranged product of germacrene A acid. B. LC-MS ion traces from negative ions of m/z 171, 233, and 251. m/z 171 corresponds to the internal standard (IS), decanoic acid, with Rt 7.68 min; m/z 233 is to detect the germacrene A acid (*) with Rt 8.41 min and, α-, β-, γ-costic acids (in bracket) with Rt 9.02 min and 9.17 min (α-, β-costic acids co-migrate at 9.02 min); m/z 251 is to detect ilicic acid (triangle), with Rt 5.38 min. C. LC-MS chromatographs (negative m/z 233) of in vitro enzyme assay products.
Figure 4. Phylogenetic and sequence analyses of GAOs from various Asteraceae plants. The Asteraceae phylogeny simplified from the figure by Panero and Funk (2008) is shown. Asterisks indicate the four subfamilies where GAOs were isolated, and the parentheses indicate specific species names. B. Phylogenetic tree of AMO/GAO in Asteraceae. HPO and EAH used as outgroups are *Hyoscyamus muticus* prennaspirodiene oxygenase (HPO) and tobacco 5-epi-aristolochene dihydroxylase (EAH), respectively. Bootstrap values were shown in percentage value from 1,000 replicates. The bracket indicates the GAO clade that is clearly distinguished from BsGAO and AMO. C. Alignment of deduced amino acids from GAOs and AMO. Amino acid sequences were obtained from cDNAs deposited at NCBI. AMO, amorphadiene oxidase from *Artemisia annua* (DQ268763 or DQ315671); LsGAO, germacrene A oxidase from *Lactuca sativa* (GU198171), or from *Cichorium intybus* (Ci; GU256644), *Sassurea lappa* (Sl; GU256646), *Helianthus annuus* (Ha; GU256645), and *Barnadesia spinosa* (Bs; GU256647). Star marks indicate the residues conserved in GAO but different in AMO. Circled marks indicate the residues unique in AMO although not conserved in GAO. The alignment is shaded to a 50% consensus. Dark and light shading indicate identical and similar residues, respectively.

Figure 5. Biochemical analyses of GAOs from various Asteraceae plants. A. LC-MS chromatography at selective (-)233 ion for germacrene A acid and (-)171 ion for internal standard (IS), decanoic acid. The arrow is germacrene A acid, and the arrow head is IS. Yields of germacrene A acid from four independent transformants were given at the start of the chromatographs (mean ± SD). B. Immunoblot analysis of recombinant GAOs was shown. FLAG antibodies were used to detect the epitope tags at the C-terminals of GAOs. Loaded microsome amounts were indicated. In A and B, Ls: *Lactuca sativa*; Ci: *Cichorium intybus*; Sl: *Sassurea lappa*, Bs: *Barnadesia spinosa*; Ha: *Helianthus annuus*. C. The LC-MS chromatography of *B. spinosa* extract at (-)233 ion was shown in the top line. Standards for GAA (germacrene A acid) and costic acids were shown in the second and third line, respectively.
Table 1. Germacrene A acid or artemisinic acid production from the transgenic yeast in which terpene synthase (ADS or GAS) and cytochrome P450 (AMO or GAO) were co-expressed.

| Sesquiterene synthase | Cytochrome P450    | Germacrene A acid or artemisinic acid yield (μg mL⁻¹) | Relative activity (%) |
|-----------------------|-------------------|------------------------------------------------------|-----------------------|
| Lettuce GAS           | Lettuce GAO       | 22.0 ± 5.3\(^b\)                                      | 100                   |
| Lettuce GAS           | Artemisia AMO     | 0.003 ± 0.001\(^b\)                                   | 0.04 ± 0.02           |
| Artemisia ADS         | Artemisia AMO     | 27.0 ± 2.0\(^c\)                                      | 100                   |
| Artemisia ADS         | Lettuce GAO       | 9.3 ± 1.3\(^c\)                                       | 40 ± 16               |
| Artemisia ADS         | Chicory GAO       | 11.4 ± 1.7\(^c\)                                      | 36 ± 11               |
| Artemisia ADS         | Costus GAO        | 4.8 ± 0.8\(^c\)                                       | 25 ± 13               |
| Artemisia ADS         | Sunflower GAO     | 0.4 ± 0.1\(^c\)                                       | 7 ± 1                 |
| Artemisia ADS         | Barnadesia GAO    | 3.7 ± 0.2\(^c\)                                       | 5 ± 2                 |

\(^a\) Natural pairs of sesquiterpene synthase and P450 (e.g., lettuce GAS and lettuce GAO or Artemisia ADS and Artemisia AMO) were set as 100% activities. The production levels of germacrene A acid or artemisinic acid were normalized by relative P450 abundance estimated by immunoblot analysis. Values (mean ± SD) were obtained from at least three independent transformants.

\(^b\) Germacrene A acid measurement

\(^c\) Artemisinic acid measurement
Figure 2
Figure 3
Figure 4
Figure 5

A

B

C

Relative Ion Abundance
8-10 min
(-) LC-SIM at 233
B. spinosa
Germacrene A
Costic acids
Biochemical conservation and evolution of germacrene A oxidase in Asteraceae
Don Trinh Nguyen, Jens Christian Goepfert, Nobuhiro Ikezawa, Gillian MacNevin, Meena Kathiresan, Juergen Conrad, Otmar Spring and Dae-Kyun Ro

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