Plant Biotechnology 35, 113–121 (2018)
DOI: 10.5511/plantbiotechnology.18.0324a

Original Paper

Identification and characterization of a novel sesquiterpene synthase, 4-amorphen-11-ol synthase, from *Artemisia maritima*

Paskorn Muangphrom[1], Hikaru Seki[1], Seiya Matsumoto[1], Mika Nishiwaki[1], Ery O. Fukushima[1,2], Toshiya Muranaka[1,*]

[1] Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871 Japan; [2] Center for Open Innovation Research and Education, Graduate School of Engineering, Osaka University, 2-1, Yamadaoka, Suita, Osaka 565-0871 Japan
* E-mail: muranaka@bio.eng.osaka-u.ac.jp Tel: +81-6-6879-7423 Fax: +81-6-6879-7426

Received January 19, 2018; accepted March 24, 2018 (Edited by M. Mizutani)

Abstract Artemisinin, a sesquiterpene lactone exhibiting effective antimalarial activity, is produced by only *Artemisia annua* plant. A key step in artemisinin biosynthesis is the cyclization of farnesyl pyrophosphate (FPP) to amorpha-4,11-diene catalyzed by amorpha-4,11-diene synthase (AaADS). Intriguingly, several non-artemisinin-producing *Artemisia* plants also express genes highly homologous to *AaADS*. Our previous functional analysis of these homologous enzymes revealed that they catalyzed the synthesis of rare natural sesquiterpenoids. In this study, we analyzed the function of another putative sesquiterpene synthase highly homologous to AaADS from *A. maritima*. Unlike AaADS, *in vivo* enzymatic assay showed that this enzyme cyclized FPP to 4-amorphen-11-ol, a precursor of several gastroprotective agents. The discovery of 4-amorphen-11-ol synthase (AmAOS) and the successful *de novo* production of 4-amorphen-11-ol in engineered yeast demonstrated herein provides insights into the methods used to enhance its production for future application.

Key words: 4-Amorphen-11-ol, 4-Amorphen-11-ol synthase, artemisinin, *Artemisia maritima*, sesquiterpene synthase.

Introduction

Sesquiterpenoids, a class of terpenoids with three five-carbon isoprene units, display a wide range of bioactivities, such as anticancer, anti-inflammatory, antioxidant, antifungal, anti-HIV-I, and antimicrobial properties (Brahmkshatriya and Brahmkshatriya 2013; Wang et al. 2005). A common precursor of all sesquiterpenoid natural compounds, farnesyl pyrophosphate (FPP), is biosynthesized via two major pathways: the mevalonic acid (MVA) and 2*C*-methyl-D-erythritol-4-phosphate (MEP) pathways (Wu et al. 2006). FPP is then either dephosphorylated to acyclic sesquiterpene or cyclized to mono-, bi-, tri-, or tetracyclic sesquiterpenes by the unique functions of sesquiterpene synthases (Degenhardt et al. 2009). Subsequently, these sesquiterpene skeletons are further oxidized and glycosylated into numerous sesquiterpenoid products of more than 19,000 different structures discovered in plants, animals, and microbes (Chadwick et al. 2013; Chapman & Hall/CRC Chemical Database 2017).

Among the highly diverse sesquiterpenoids, artemisinin, a sesquiterpene lactone isolated from the plant *Artemisia annua* L., is the most renowned sesquiterpene-based drug because of its use as a standard treatment for malaria (World Health Organization 2015). In *A. annua*, artemisinin is produced mainly in glandular secretory trichomes (Olofsson et al. 2011). The biosynthetic pathway of artemisinin has been studied for several decades (Figure 1). The first committed step in artemisinin biosynthesis is the cyclization of FPP to amorpha-4,11-diene by amorpha-4,11-diene synthase (AaADS) (Bouwmeester et al. 1999; Chang et al. 2000; Mercke et al. 2000). Amorpha-4,11-diene is subsequently oxidized to artemisinic alcohol and artemisinic aldehyde by amorpha-4,11-diene 12-monooxygenase (CYP71AV1) and alcohol dehydrogenase 1 (ADH1) (Polichuk et al. 2010; Teoh et al. 2006). Next, artemisinic aldehyde Δ11(13) reductase (DBR2) reduces artemisinic

Abbreviations: Aa, *Artemisia annua*; Aab, *Artemisia absinthium*; ADH1, alcohol dehydrogenase 1; ADS, amorpha-4,11-diene synthase; Ak, *Artemisia kurramensis*; ALDH1, aldehyde dehydrogenase 1; Am, *Artemisia maritima*; AOS, 4-amorphen-11-ol synthase; BOS, α-bisabolol synthase; CYP71AV1, amorpha-4,11-diene 12-monooxygenase; DBR2, artemisinic aldehyde Δ11(13) reductase; FPP, farnesyl pyrophosphate; GAS, germacrene A synthase; GC-MS, gas chromatography-mass spectrometry; KOS, koidzumiol synthase; MEP, 2*C*-methyl-D-erythritol-4-phosphate; MVA, mevalonic acid.
This article can be found at http://www.jspcmb.jp/
Published online June 23, 2018

Copyright © 2018 The Japanese Society for Plant Cell and Molecular Biology

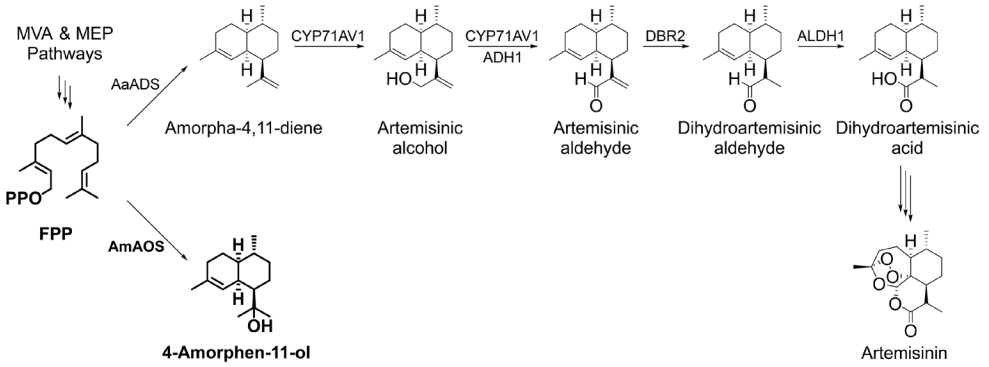

Figure 1. Biosynthetic pathways of artemisinin and 4-amorphen-11-ol. The enzymes responsible for each step are indicated along with the arrows. The full names of the enzymes are given in the text. Newly determined biosynthetic pathway in this study is highlighted in bold.

aldehyde to dihydroartemisinic aldehyde which is further oxidized to dihydroartemisinic acid by aldehyde dehydrogenase 1 (ALDH1) (Teoh et al. 2009; Zhang et al. 2008). Dihydroartemisinic acid is reportedly converted to artemisinin via spontaneous autoxidation and subsequent rearrangement (Brown and Sy 2004).

Previous studies have suggested that artemisinin is also produced in other *Artemisia* plant species (Arab et al. 2006; Dilshad et al. 2015; El Maggar 2012; Ivanescu et al. 2015; Mannan et al. 2010). However, among over 400 *Artemisia* species distributed throughout the world (The Plant List 2013), neither successful purification of artemisinin nor clear evidence of the accumulation of this compound in *Artemisia* plant species other than *A. annua* has been reported. The chemodiversity of sesquiterpenoids is regulated by the unique functions of sesquiterpene synthases. Therefore, to determine the key factor controlling the artemisinin-producing ability in *A. annua*, we previously analyzed the expression and enzymatic function of putative sesquiterpene synthases highly homologous to *AaADS*, a sesquiterpene synthase involved in artemisinin biosynthesis, in 13 non-artemisinin-producing *Artemisia* plant species. Among these, *A. maritima* L., a perennial plant widely distributed in the northern hemisphere and mountainous regions that exhibits several pharmaceutical properties, including anthelmintic, antimicrobial, anti-inflammatory, bronchodilatory, and spasmolytic activities (Irum et al. 2015; Møller et al. 2013; Shah et al. 2011), expresses putative sesquiterpene synthases highly homologous to *AaADS* in both its leaves and roots (Muangphrom et al. 2016). We previously identified a homologous enzyme expressed in *A. maritima* leaves as (+)-α-bisabolol synthase (AmBOS) (Muangphrom et al. 2016). Herein we analyzed the function of the other putative sesquiterpene synthase isolated from the roots of *A. maritima*. Our study led to the discovery of a novel sesquiterpene synthase uniquely catalyzing the synthesis of 4-amorphen-11-ol, a precursor of several gastroprotective agents. Thus, we named this enzyme 4-amorphen-11-ol synthase (AmAOS).

Materials and methods

Plant material and yeast strain

Artemisia maritima (plant species no. 20134-1881) was cultivated in and collected from the Medicinal Plant Garden of Hoshi University, Tokyo, Japan in June 2012. All collected tissues were frozen and stored at −80°C until use. The yeast *Saccharomyces cerevisiae* EPY300 strain of genotype *MATαα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* P*GAL1-tHMGR* P*GAL1-upc2-1 erg9*::*PMET3-ERG9* P*GAL1-tHMGR* P*GAL1-ERG20* was previously developed to increase FPP production (Ro et al. 2006, 2008).

General experimental procedures

NMR spectroscopic data were recorded in $CDCl_3$ using a Bruker AVANCE III HD 600 MHz spectrometer (Bruker, Osaka, Japan). Chemical shifts are reported as δ (ppm) relative to those of the internal standard tetramethylsilane (δ_H=0 ppm) or $CDCl_3$ (δ_C=77.0 ppm). Gas chromatography-mass spectrometry (GC-MS) analysis was performed using a gas chromatograph 7890B (Agilent Technologies, Santa Clara, CA, USA) connected to a 5977 A MSD mass spectrometer (Agilent Technologies). GC was equipped with a DB-1MS capillary column (30 m×0.25 mm i.d., film thickness 0.25 μm; J&W Scientific, Santa Clara, CA, USA) coupled with a fused silica capillary tube (1 m×0.25 mm i.d., GL-Sciences, Tokyo, Japan). The temperature program began at 100°C, was held for 2 min, and increased at a rate of 3°C min^{-1} to 106°C. The temperature was increased at a rate of 2°C min^{-1} to 110°C and held for 5 min before increasing at a rate of 1°C min^{-1} to 113°C. After reaching 113°C, the temperature was increased again to 178°C at a rate of 5°C min^{-1} before ramping to 200°C at a rate of 22°C min^{-1}. The injection port temperature and interface temperature were both set to 250°C. The ion source temperature was maintained at 230°C. The carrier gas was He, and the flow rate was set at 0.7 ml min^{-1}. Split injection (1 μl) was conducted at a ratio of 1 : 10. The scanning speed of the mass spectrometer was 2.4 scans s^{-1} from m/z 40 to 350 at an electron voltage of 70 eV.

Copyright © 2018 The Japanese Society for Plant Cell and Molecular Biology

Optical rotation was determined using the JASCO P-1020 polarimeter (JASCO, Tokyo, Japan) with a 50-mm microcell (1 ml). Column chromatography was performed using silica gel 60N (spherical, neutral), with particle sizes 63–210 μm (Kanto Chemical, Tokyo, Japan) or 10% $AgNO_3$ impregnated silica gel (Wako, Osaka, Japan). The amorpha-4,11-diene and koidzumiol standards were prepared as described previously (Muangphrom et al. 2014, 2016). α-Bisabolol was purchased from Tokyo Chemical Industry (Tokyo, Japan). Nerolidol and farnesol were identified by comparison of their mass spectra with those available in the NIST mass spectral library (The NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library version 2.2 2014).

RT-PCR and cDNA isolation

Total RNA was extracted from the leaves and roots of *A. maritima* using RNAiso Plus (Takara Bio Inc., Shiga, Japan) and treated with RNase-free DNase (Takara Bio Inc.). RNA was further purified using the RNeasy Plant Mini Kit (Qiagen KK, Tokyo, Japan). First-strand cDNA was synthesized using the PrimeScript RT-PCR Kit (Takara Bio Inc.) with 1 μg total RNA as a template. To obtain the full-length open reading frame (ORF) encoding AmAOS, RT-PCR was performed using primers 1 and 2 as described previously (Muangphrom et al. 2016). The amplified product was cloned into pENTR/D-TOPO and sequenced. To analyze a specific expression, RT-PCR was performed using primers 3 and 4 for *AmAOS*, 5 and 6 for *AmBOS*, and 7 and 8 for *actin* (internal control) (Olofsson et al. 2011). Thermal cycling conditions were as follows: 98°C for 2 min, followed by 35 cycles of 98°C for 10 s, 58°C for 15 s, and 72°C for 30 s, and one cycle of 72°C for 10 min. The sequences of all primers used are listed in Supplemental Table S1.

Phylogenetic analysis

The alignment was performed using CLUSTALW (Thompson et al. 1994) with a gap open cost of 10 and a gap extension cost of 0.2. The phylogenetic tree was constructed in MEGA7 software (Kumar et al. 2016) using the neighbor-joining method (Saitou and Nei 1984) with a bootstrap analysis of 1,000 replicates. *A. annua* germacrene A synthase (AaGAS) was used as the outgroup.

Heterologous expression and in vivo enzymatic assay

The full-length ORF encoding AmAOS was transferred via pENTR/D-TOPO into pYES-DEST52 (Invitrogen, Carlsbad, CA, USA) using Gateway LR Clonase II Enzyme Mix (Invitrogen). The expression clone was introduced into the yeast *S. cerevisiae* EPY300 strain. Yeast harboring the pYES2/CT empty vector (Invitrogen) was used as a negative control. Transgenic yeast cells were cultured as described previously (Muangphrom et al. 2016) with minor modifications. Yeast cells were cultured in synthetic complete medium (2 ml) containing 2% glucose in the absence of histidine, methionine, and uracil (SC-His-Met-Ura) at 30°C with shaking at 180 rpm overnight before scale-up to 50 ml in the same culture medium and further incubated under the same condition for 1 day. Cells were then added to SC-His-Met-Ura (500 ml) containing 1.8% galactose, 0.2% glucose, and 1 mM methionine and cultured at 30°C with shaking at 180 rpm for 4 days. After disrupting the cells of the resulting cultures by sonication at room temperature for 30 min, they were extracted three times with EtOAc. The combined organic layer was washed with brine and dried over anhydrous Na_2SO_4. The samples were then concentrated under reduced pressure and flow of nitrogen gas before further purification and GC-MS analysis.

Purification and structural determination of 4-amorphen-11-ol

Crude extract (176.49 mg) from yeast expressing *AmAOS* (1 l scale) was subjected to silica gel column chromatography and eluted with EtOAc–hexane (0–70% v/v) to yield fractions A1–A4 after combination and solvent removal. Fraction A3 (31.5 mg) was further purified by column chromatography (10% silver nitrate-impregnated silica gel, 5% v/v EtOAc–hexane as the eluent), yielding 4-amorphen-11-ol (7.81 mg) as a colorless oil.; $[\alpha]_D^{25.1}$ −4.9 (*c* 0.022, $CHCl_3$); ^1H and ^{13}C NMR, see Supplemental Table S3; EIMS *m*/*z* 204 (64), 189 (38), 175 (3), 161, (100), 149 (26), 133 (14), 119 (29), 105 (27), 93 (35), 81 (44); HRFABMS *m*/*z* 245.1879 (calcd. for $C_{15}H_{26}ONa$ 245.1881).

Results

Expression analysis of AmAOS isolated from A. maritima roots

The cDNA of *AmAOS* amplified from the cDNA library of *A. maritima* roots was cloned and sequenced. Sequencing analysis revealed that it contains 1,641 base pairs (bp) full-length ORF (GenBank accession number LC153278) (Supplemental Figure S1, upper sequence) showing 96% nucleotide sequence identity with *AmBOS* (GenBank accession number LC106015). Its expression pattern as compared to *AmBOS* in *A. maritima* was then analyzed by RT-PCR with primers specific for each gene

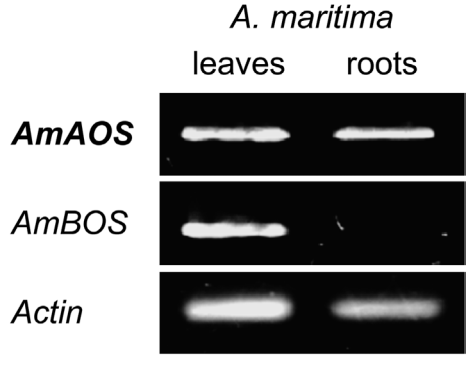

Figure 2. The expression patterns of *AmAOS* and *AmBOS*. Gene expression was analyzed using *AmAOS* and *AmBOS* specific primers. *Actin* was included as an internal control.

Copyright © 2018 The Japanese Society for Plant Cell and Molecular Biology

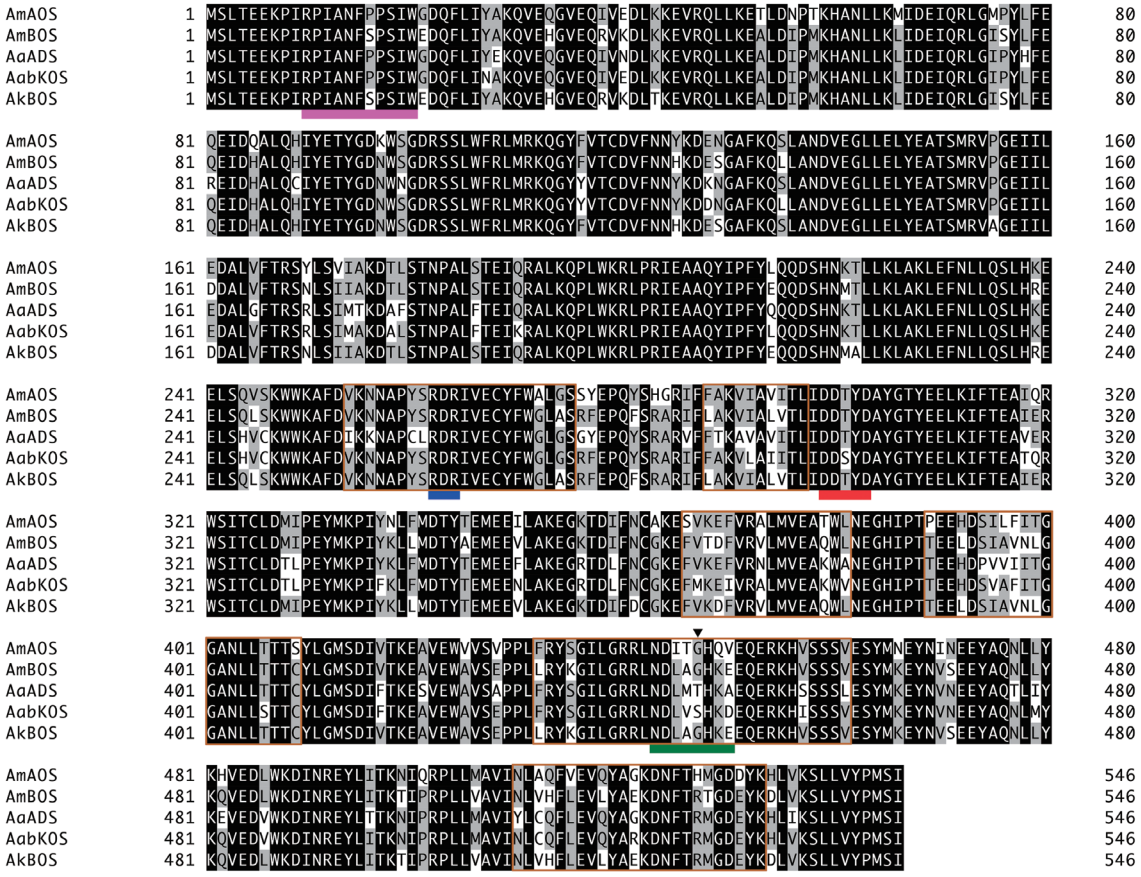

Figure 3. Amino acid sequence alignment of AmAOS and its homologous enzymes. Typical motifs of sesquiterpene synthases are underlined: R(R,P)$(X)_8$W, pink; RXR, blue; DDXXD, red; and NSE/DTE, green. Putative catalytic residues located within 12 Å from the active site as reported by Salmon et al. (2015) are indicated by brown boxes. Black shading indicates conserved amino acid residues. Gray shading indicates amino acid residues with >51% conservation. Black triangle indicates the magnesium ligands of the NSE/DTE motif (residue 447). GenBank accession numbers of the aligned enzymes; see SI.

(Supplemental Table S1). As shown in Figure 2, unlike *AmBOS*, *AmAOS* is expressed in both leaves and roots.

Highly conserved motifs of sesquiterpene synthases

The encoded protein of AmAOS contains all typical motifs of sesquiterpene synthase, i.e., R(R,P)$(X)_8$W, RXR, DDXXD, and NSE/DTE motifs, and shows 86% amino acid sequence identity to AmBOS (Christianson 2006; Ee et al. 2014; Tholl et al. 2005; Figure 3, Supplemental Table S2). The RXR and DDXXD motifs of this enzyme and those of AmBOS are identical. However, their R(R,P)$(X)_8$W and NSE/DTE motifs differ by one and four amino acids, respectively. The magnesium ligands of the NSE/DTE motifs (Christianson 2006) of both enzymes possesses the same substitution: serine/threonine to glycine at residue 447 (indicated by black triangles in Figures 3 and S2).

BLASTP analysis revealed that the amino acid sequence of AmAOS is highly homologous to AaADS, *A. annua* α-bisabolol synthase (AaBOS), *A. absinthium* koidzumiol synthase (AabKOS), *A. kurramensis* (+)-α-bisabolol synthase (AkBOS) and AmBOS with higher than 80% amino acid sequence identity (Supplemental Table S2). Although the putative catalytic residues of this enzyme are quite unique when compared to other sesquiterpene synthases (Salmon et al. 2015; Figure 3, Supplemental Figure S2), phylogenetic analysis of *Artemisia* spp. sesquiterpene synthases showed that it is located within the same branch as AaADS, AabKOS, and BOSs, all of which catalyze the initial cyclization of FPP via 1,6-ring closure (Supplemental Figure S3). These results suggested that this enzyme possesses a unique function but still catalyzes 1,6-cyclization of FPP as the first step as AaADS, AabKOS, and BOSs (Muangphrom et al. 2016).

In vivo enzymatic assay

The full-length ORF of *AmAOS* was cloned into a yeast expression vector and introduced into the yeast *S. cerevisiae* EPY300 strain, which has been metabolically engineered to synthesize an elevated level of FPP (Ro et al. 2006, 2008). After culture and extraction using EtOAc, crude extracts were analyzed by GC-MS. The retention

Copyright © 2018 The Japanese Society for Plant Cell and Molecular Biology

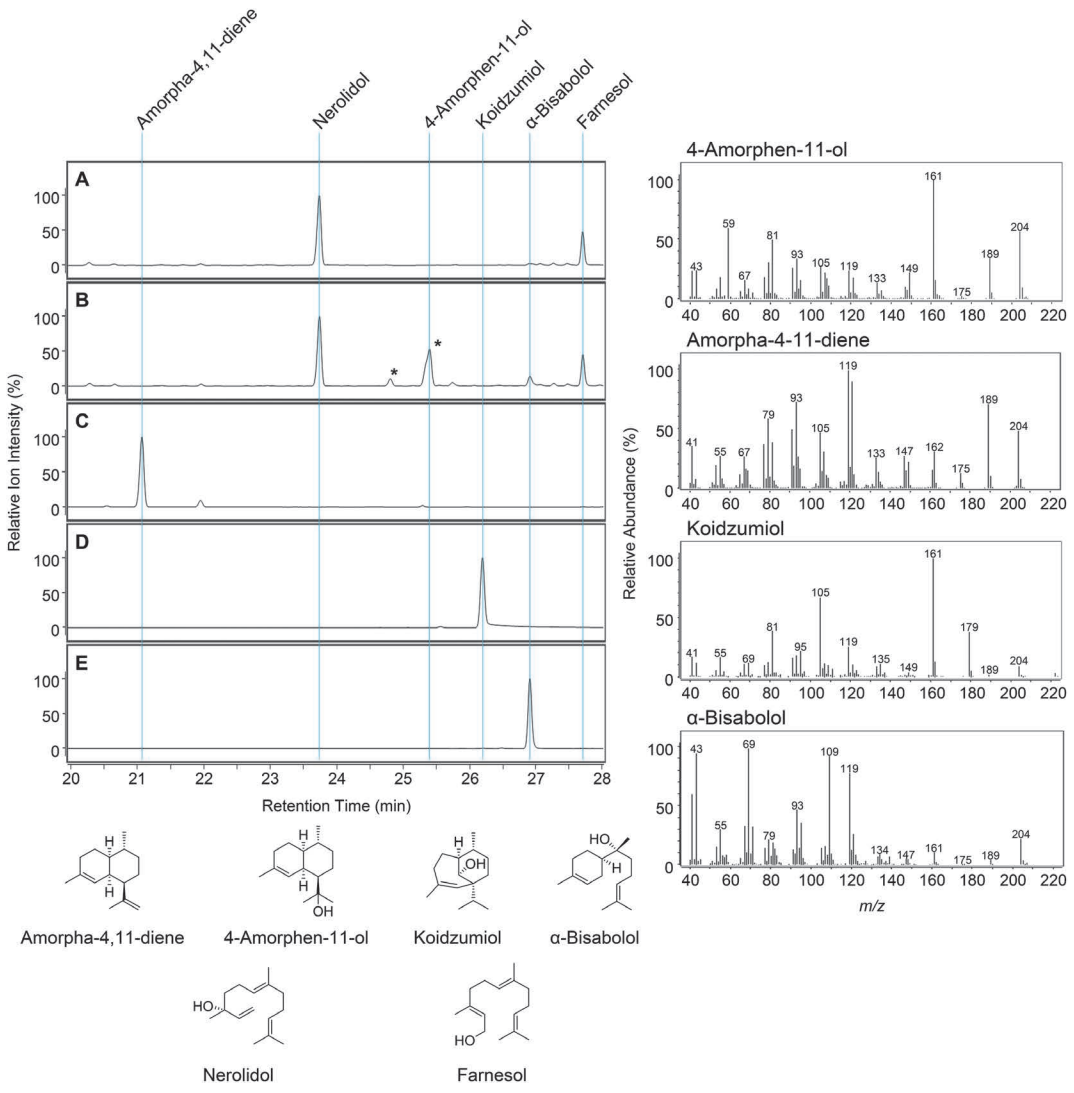

Figure 4. *In vivo* enzymatic assay of AmAOS analyzed by GC-MS. Total ion chromatograms display the extracts of yeast transformed with (A) empty vector (negative control) or (B) *AmAOS*, and the standards (C) amorpha-4,11-diene, (D) koidzumiol, and (E) α-bisabolol. A major product, 4-amorphen-11-ol, and the unknown sesquiterpenoid newly detected in the extract of yeast expressing *AmAOS* (B) are indicated by asterisks. Mass spectra of the major enzymatic product and the standards are shown. Nerolidol and farnesol are endogenous sesquiterpenoids of yeast detected even in the extracts of the empty vector control line (A). The structures of all compounds are shown under the GC chromatograms.

times and mass spectra of the enzymatic products (Figure 4B) were compared to those of the standards amorpha-4,11-diene (Figure 4C), koidzumiol (Figure 4D), and α-bisabolol (Figure 4E). As shown in Figure 4B, this recombinant expression led to the production of two unknown major products (indicated by asterisks) that differed in both retention time and mass fragmentation pattern from products of other sesquiterpene synthases highly homologous to AaADS (Muangphrom et al. 2016). α-Bisabolol was also detected as a minor product in the extracts (Figure 4B). After increased the culture volume to 1 l enabling isolation of the major product (ca. 8 mg; a peak at retention time 25.4 min) in quantities allowing NMR analysis, its structure was then elucidated to be 4-amorphen-11-ol (Supplemental Figures S4–S6, Supplemental Table S3). Despite increasing the culture volume, the amount of the other newly detected sesquiterpenoid (a peak at retention time 24.8 min) remained inadequate for NMR analysis, and its structure thus remains unknown.

Discussion

The unique functions of sesquiterpene synthases have led to the synthesis of highly diverse structure of sesquiterpenoids. In artemisinin biosynthesis, AaADS is a sesquiterpene synthase controlling the first step cyclization of FPP to amorpha-4,11-diene via a two-step cyclization (1,6- and subsequent 1,10-ring closure) following by a regioselective deprotonation (Kim et al. 2006; Picaud et al. 2006; Figure 5). We previously identified several sesquiterpene synthases which

Copyright © 2018 The Japanese Society for Plant Cell and Molecular Biology

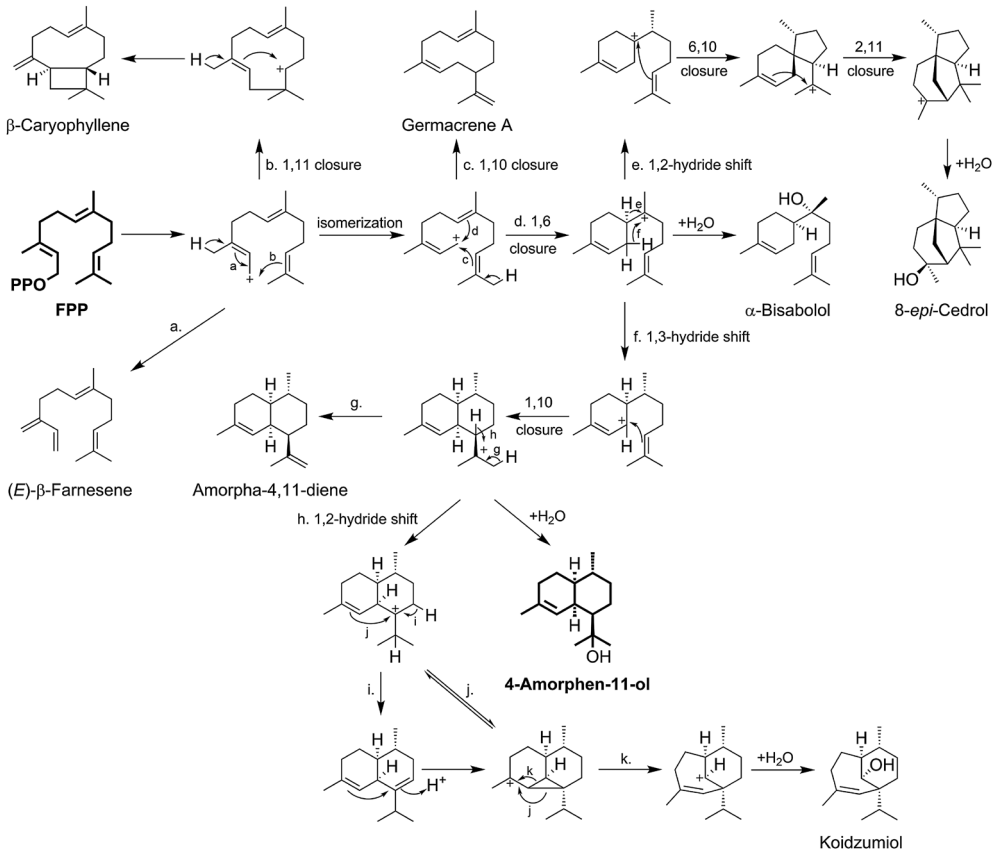

Figure 5. Biosynthetic mechanism of sesquiterpenoids catalyzed by all characterized sesquiterpene synthases from *Artemisia* spp. The reaction mechanism begins with the ionization of farnesyl pyrophosphate (FPP). Enzymes responsible for the biosyntheses of amorpha-4,11-diene, koidzumiol, α-bisabolol, (*E*)-β-farnesene, β-caryophyllene, germacrene A, and 8-*epi*-cedrol were reported previously (Bertea et al. 2006; Bouwmeester et al. 1999; Cai et al. 2002; Chang et al. 2000; Hua and Matsuda 1999; Li et al. 2013; Mercke et al. 2000; Muangphrom et al. 2016; Picaud et al. 2005). Only major sesquiterpenoid products synthesized from each responsible sesquiterpene synthase are shown. The starting material FPP and a key compound in this study 4-amorphen-11-ol are highlighted in bold.

are highly homologous and exhibit similar catalytic mechanism to AaADS (Muangphrom et al. 2016). In this study, we analyzed the function of a novel putative sesquiterpene synthase, highly homologous to AaADS, of *A. maritima*. We found that it also shares the same catalytic mechanism as AaADS but exhibits a different product specificity on the synthesis of 4-amorphen-11-ol. Thus we named it 4-amorphen-11-ol synthase (AmAOS).

Initial cyclization of FPP catalyzed by sesquiterpene synthases can be proceeded via several ring closure types (Degenhardt et al. 2009; Salmon et al. 2015; Figure 5). In previous report, 4-amorphen-11-ol was tentatively detected in the extracts of yeast expressing *AaADS*, and its biosynthetic mechanism was proposed with 1,6-ring closure as the initial cyclization (Mercke et al. 2000). This finding, together with the existence of a minor product detected in this study (α-bisabolol, Figure 4B), coincides with our speculation from the phylogenetic analysis, as AmAOS was located in the same branch as other sesquiterpene synthases that also catalyze the first cyclization via 1,6-ring closure (Supplemental Figure S3). The cyclization mechanisms exhibited by characterized sesquiterpene synthases from *Artemisia* plant species are summarized in Figure 5.

We previously reported that putative sesquiterpene synthases highly homologous to *AaADS* were expressed in both leaves and roots of *A. maritima*. The one amplified from leaves has been identified as *AmBOS* (Muangphrom et al. 2016), and the other one amplified from roots is identified as *AmAOS* in this study. Using *AmAOS* and *AmBOS* specific primers, we detected *AmAOS* expression in both leaves and roots, while *AmBOS* was expressed only in leaves (Figure 2). Therefore, we sought to analyze the accumulation of 4-amorphen-11-ol in both tissues of *A. maritima*. However, we could not detect any trace of this compound or its derivatives thereof in the crude extracts (data not shown). When recombinant *AmAOS* was expressed in FPP high producing *S. cerevisiae* EPY300 strain, only ca. 8 mg l^{-1} of 4-amorphen-11-ol was obtained. This indicates that AmAOS may have a low catalytic capacity, and the quantity of 4-amorphen-11-ol produced by this enzyme in the plant may be very low. Other endogenous enzymes of *A. maritima* might also be able to derivatize

Copyright © 2018 The Japanese Society for Plant Cell and Molecular Biology

4-amorphen-11-ol thus further lowering the yield of this compound. Still the production of these derivatives *in planta* are limited by the available yield of their substrate. Owing to these, therefore, the quantities of both 4-amorphen-11-ol and its derivatives in the plant are probably inadequate for our analysis.

Although 4-amorphen-11-ol has not been reported as a major sesquiterpenoid in *Artemisia* spp., it is one of the major natural compounds accumulated in *Fabiana imbricata* (Brown 1994a), a flowering plant in the family Solanaceae native to Southern Chile (Brown and Shill 1993). Several derivatives of this compound, either isolated from *F. imbricata* or chemically synthesized using 4-amorphen-11-ol as the starting material, have been reported (Brown 1994b; Reyes et al. 2005; Schmeda-Hirschmann and Papastergiou 1994). Among these, 11-hydroxy-4-amorphen-15-oic acid and its derivatives are highly protective against gastric lesions and toxic to the human gastric adenocarcinoma cell line (AGS) (ATCC CRL-1739) (Reyes et al. 2005). In addition, 4-amorphen-11-ol can be converted to peroxofabianane via either chemical synthesis or autoxidation/rearrangement under the presence of singlet oxygen (Jung and Youn 1996; Ngo and Brown 1999a). Although the bioactivity of peroxofabianane has not been studied, this compound shares structural similarities to artemisinin, in terms of the overall stereochemistry and the endoperoxide bridge required for antimalarial activity (de Ridder et al. 2008; Klonis et al. 2013; van Agtmael et al. 1999; Wang et al. 2015; Willcox 2009). The chemical structures of notable 4-amorphen-11-ol derivatives and peroxofabianane are shown in Supplemental Figure S7.

Total synthesis of 4-amorphen-11-ol has been reported (Ngo and Brown 1999b). However, the separation of the products of each step from the by-products was rather difficult and the overall production yield was less than 20%. Here, we identified a 4-amorphen-11-ol synthase from *A. maritima* (AmAOS). Although 4-amorphen-11-ol accumulation in transgenic yeast was low (ca. 8 mgl^{-1}), successful *de novo* production was nonetheless confirmed. Our findings will aid in large-scale production of 4-amorphen-11-ol, which can be further modified to yield several bioactive derivatives.

Acknowledgements

We thank Dr. Seiji Nagumo and Dr. Hiroshi Sudo (Hoshi University) for providing *A. maritima*. This work was supported by Grant-in-Aid for Scientific Research on Innovative Areas, No. JP17H05442 to H.S.; Frontier Research Base for Global Young Researchers, Osaka University from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) to E.O.F.; and the Monbukagakusho Scholarship to P.M.

References

Arab HA, Rahbari S, Rassouli A, Moslemi MH, Khosravirad F (2006) Determination of artemisinin in *Artemisia sieberi* and anticoccidial effects of the plant extract in broiler chickens. *Trop Anim Health Prod* 38: 497–503

Bertea CM, Voster A, Verstappen FWA, Maffei M, Beekwilder J, Bouwmeester HJ (2006) Isoprenoid biosynthesis in *Artemisia annua*: Cloning and heterologous expression of a germacrene A synthase from a glandular trichome cDNA library. *Arch Biochem Biophys* 448: 3–12

Bouwmeester HJ, Wallaart TE, Janssen MHA, van Loo B, Jansen BJM, Posthumus MA, Schmidt CO, De Kraker JW, König WA, Franssen MCR (1999) Amorpha-4,11-diene synthase catalyses the first probabl step in artemisinin biosynthesis. *Phytochemistry* 52: 843–854

Brahmkshatriya PP, Brahmkshatriya PS (2013) Chapter 86 terpenes: Chemistry, biological role, and therapeutic applications. In: Ramawat KG, Mérillon JM (eds) *Natural Products Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*. Springer Verlag, Heidelberg, pp 2665-2691

Brown GD, Shill J (1993) Isolation of 3,11-amorphadiene from *Fabiana imbricata. Planta Med* 60: 495–496

Brown GD (1994a) The sesquiterpenes of *Fabiana imbricata. Phytochemistry* 35: 425–433

Brown GD (1994b) Fabianane, an unusual secoamorphane from *Fabiana imbricata. J Nat Prod* 57: 328–330

Brown GD, Sy LK (2004) *In vivo* transformations of dihydroartemisinic acid in *Artemisia annua* plants. *Tetrahedron* 60: 1139–1159

Cai Y, Jia JW, Crock J, Lin ZX, Chen XY, Croteau R (2002) A cDNA clone for β-caryophyllene synthase from *Artemisia annua. Phytochemistry* 61: 523–529

Chadwick M, Trewin H, Gawthrop F, Wagstaff C (2013) Sesquiterpenoids lactones: Benefits to plants and people. *Int J Mol Sci* 14: 12780–12805

Chang YJ, Song SH, Park SH, Kim SU (2000) Amorpha-4,11-diene synthase of *Artemisia annua*: cDNA isolation and bacterial expression of a terpene synthase involved in artemisinin biosynthesis. *Arch Biochem Biophys* 383: 178–184

Chapman & Hall/CRC Chemical Database (2017) Dictionary of Natural Products 26.1 Available from: http://dnp.chemnetbase.com/ [accessed October 4, 2017]

Christianson DW (2006) Structural biology and chemistry of the terpenoid cyclases. *Chem Rev* 106: 3412–3442

de Ridder S, van der Kooy F, Verpoorte R (2008) *Artemisia annua* as a self-reliant treatment for malaria in developing countries. *J Ethnopharmacol* 120: 302–314

Degenhardt J, Köllner TG, Gershenzon J (2009) Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry* 70: 1621–1637

Dilshad E, Cusido RM, Estrada KR, Bonfill M, Mirza B (2015) Gnetic transformation of *Artemisia carvifolia* Buch with *rol* genes enhances artemisinin accumulation. *PLoS ONE* 10: e0140266

Ee SF, Hussein ZAM, Othman R, Shaharuddin NA, Ismail I, Zainal Z (2014) Functional characterization of sesquiterpene synthase from *Polygonum minus. Sci World J* 2014: 840592

El Maggar EMB (2012) *Artemisia herba alba* & *Artemisia monosperma*: The discovery of the first potential Egyptian plant sources for the pharmaceutical commercial production of artemisinin and some of its related analogues. *J Appl Pharm Sci* 2: 77–91

Copyright © 2018 The Japanese Society for Plant Cell and Molecular Biology

Hua H, Matsuda SPT (1999) The molecular cloning of 8-epicedrol synthase from *Artemisia annua*. *Arch Biochem Biophys* 369: 208–212

Irum S, Ahmed H, Mukhtar M, Mushtaq M, Mirza B, Donskow-Łysoniewska K, Qayyum M, Simsek S (2015) Anthelmintic activity of*Artemisia vestita* Wall ex DC. and *Artemisia maritima* L. against *Haemonchus contortus* from sheep. *Vet Parasitol* 212: 451–455

Ivanescu B, Miron A, Corciova A (2015) Sesquiterpene lactones from *Artemisia* genus: Biological activities and methods of analysis. *J Anal Methods Chem* 2015: 247685

Jung M, Youn BH (1996) Conversion of artemisinic acid into (−)-fabianane. *Heterocycles* 43: 1587–1590

Kim SH, Heo K, Chang YJ, Park SH, Rhee SK, Kim SU (2006) Cyclization mechanism of amorpha-4,11-diene synthase, a key enzyme in artemisinin biosynthesis. *J Nat Prod* 69: 758–762

Klonis N, Creek DJ, Tilley L (2013) Iron and heme metabolism in *Plasmodium falciparum* and the mechanism of action of artemisinins. *Curr Opin Microbiol* 16: 722–727

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol* 33: 1870–1874

Li JX, Fang X, Zhao Q, Ruang JX, Yang CQ, Wang LJ, Miller DJ, Faraldos JA, Allemann RK, Chen XY, et al. (2013) Rational engineering of plasticity residues of sesquiterpene synthases from *Artemisia annua*: Product specificity and catalytic efficiency. *Biochem J* 451: 417–426

Mannan A, Ahmed I, Arshad W, Asim MF, Qureshi RA, Hussain I, Mirza B (2010) Survey of artemisinin production by diverse *Artemisia* species in northern Pakistan. *Malar J* 9: 310

Mercke P, Bengtsson M, Bouwmeester HJ, Posthumus MA, Brodelius PE (2000) Molecular cloning, expression and characterization of amorpha-4,11-diene synthase, a key enzyme of artemisinin biosynthesis in *Artemisia annua* L. *Arch Biochem Biophys* 381: 173–180

Møller AP, Flensted-Jensen E, Mardal W, Soler JJ (2013) Host-parasite relationship between colonial terns and bacteria is modified by a mutualism with a plant with antibacterial defenses. *Oecologia* 173: 169–178

Muangphrom P, Suzuki M, Seki H, Fukushima EO, Muranaka T (2014) Functional analysis of orthologous artemisinic aldehyde Δ11(13)-reductase reveals potential artemisinin producing activity in non-artemisinin-producing *Artemisia absinthium*. *Plant Biotechnol* 31: 483–491

Muangphrom P, Seki H, Suzuki M, Komori A, Nishiwaki M, Mikawa R, Fukushima EO, Muranaka T (2016) Functional analysis of amorpha-4,11-diene synthase (ADS) homologs from non-artemisinin-producing *Artemisia* species: The discovery of novel koidzumiol and (+)-*α*-bisabolol synthases. *Plant Cell Physiol* 57: 1678–1688

Ngo KS, Brown GD (1999a) Autoxidation of 4-amorphen-11-ol and the biogenesis of *nor*- and *seco*-morphane sesquiterpenes from *Fabiana imbricata*. *Tetrahedron* 55: 15109–15126

Ngo KS, Brown GD (1999b) Synthesis of amorphane and cadinane sesquiterpenes from *Fabiana imbricata*. *Tetrahedron* 55: 15099–15108

Olofsson L, Engström A, Lundgren A, Brodelius PE (2011) Relative expression of genes of terpene metabolism in different tissues of *Artemisia annua* L. *BMC Plant Biol* 11: 45

Picaud S, Brodelius M, Brodelius PE (2005) Expression, purification and characterization of recombinant (*E*)-*β*-farnesene synthase from *Artemisia annua*. *Phytochemistry* 66: 961–967

Picaud S, Mercke P, He X, Sterner O, Brodelius M, Cane DE, Brodelius PE (2006) Amorpha-4,11-diene synthase: Mechanism and stereochemistry of the enzymatic cyclization of farnesyl diphosphate. *Arch Biochem Biophys* 448: 150–155

Polichuk D, Teoh KH, Zhang Y, Ellens KW, Reed DW, Covello PS (2010) Nucleotide sequence encoding an alcohol dehydrogenase from *Artemisia annua* and uses thereof. Patent No. WO2010012074A1

Reyes M, Schmeda-Hirschmann G, Raxmilic I, Theoduloz C, Yáñez T, Rodríguez JA (2005) Gastroprotective activity of sesquiterpene derivatives from *Fabiana imbricata*. *Phytother Res* 19: 1038–1042

Ro DK, Paradis EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, et al. (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440: 940–943

Ro DK, Ouellet M, Paradise EM, Burd H, Eng D, Paddon CJ, Newman JD, Keasling JD (2008) Induction of multiple pleiotropic drug resistance genes in yeast engineered to produce an increased level of anti-malarial drug precursor, artemisinic acid. *BMC Biotechnol* 8: 83–96

Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406–425

Salmon M, Laurendon C, Vardakou M, Cheema J, Defernez M, Green S, Faraldos JA, O'Maille PE (2015) Emergence of terpene cyclization in *Artemisia annua*. *Nat Commun* 6: 6143

Schmeda-Hirschmann G, Papastergiou F (1994) Sesquiterpenes from *Fabiana imbricata*. *Phytochemistry* 36: 1439–1442

Shah AJ, Gilani AH, Abbas K, Rasheed M, Ahmed A, Ahmad VU (2011) Studies on the chemical composition and possible mechanisms underlying the antispasmodic and bronchodilatory activities of the essential oil of *Artemisia maritima* L. *Arch Pharm Res* 34: 1227–1238

Teoh KH, Polichuk DR, Reed DW, Nowak G, Covello PS (2006) *Artemisia annua* L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. *FEBS Lett* 580: 1411–1416

Teoh KH, Polichuk DR, Reed DW, Covello PS (2009) Molecular cloning of an aldehyde dehydrogenase implicated in artemisinin biosynthesis in *Artemisia annua*. *Botany* 87: 635–642

The NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library version 2.2 (2014) Software for use with the GC/MSD and LC/MS ChemStation and MassHunter, Agilent Technologies

The Plant List (2013) Version 1.1 Available from: http://www.theplantlist.org/ [accessed April 7, 2016]

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673–4680

Tholl D, Chen F, Petri J, Gershenzon J, Pichersky E (2005) Two sesquiterpene synthases are responsible for the complex mixture of sesquiterpenes emitted from *Arabidopsis* flowers. *Plant J* 45: 757–771

van Agtmael MA, Eggelte TA, van Boxtel CJ (1999) Artemisinin drugs in the treatment of malaria: From medicinal herb to registered medication. *Trends Pharmacol Sci* 20: 199–205

Wang G, Tang W, Bidigare RR (2005) Chapter 9 Terpenoids as Therapeutic Drugs and Pharmaceutical Agents. In: Zhang L, Demain AL (ed) *Natural Products: Drug Discovery and*

Copyright © 2018 The Japanese Society for Plant Cell and Molecular Biology

Therapeutic Medicine. Humana Press Inc., New Jersey, pp 197-227

Wang J, Zhang CJ, Chia WN, Loh CCY, Li Z, Lee YM, He Y, Yuan LX, Lim TK, Liu M, et al. (2015) Haem-activated promiscuous targeting of artemisinin in *Plasmodium falciparum*. *Nat Commun* 6: 10111

Willcox M (2009) *Artemisia* species: From traditional medicines to modern antimalarials–and back again. *J Altern Complement Med* 15: 101–109

World Health Organization (2015) World Malaria Report 2015. Available from: http://www.who.int/malaria/publications/world-malaria-report-2015/wmr2015-without-profiles.pdf [accessed April 8, 2016].

Wu S, Schalk M, Clark A, Miles RB, Coates R, Chappell J (2006) Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. *Nat Biotechnol* 24: 1441–1447

Zhang Y, Teoh KH, Reed DW, Maes L, Goossens A, Olson DJH, Ross ARS, Covello PS (2008) The molecular cloning of artemisinic aldehyde Δ11(13) reductase and its role in glandular trichome-dependent biosynthesis of artemisinin in *Artemisia annua*. *J Biol Chem* 283: 21501–21508

Copyright © 2018 The Japanese Society for Plant Cell and Molecular Biology