Norditerpenoids biosynthesized by variediene synthase-associated P450 machinery along with modifications by the host cell Aspergillus oryzae

Lan Jiang a,1, Kangjie Lv a,1, Guoliang Zhu a,1, Zhi Lin b, Xue Zhang a, Cuiping Xing a, Huanting Yang a, Weiyan Zhang a, Zhixin Wang c, Chengwei Liu c, Xudong Qu b, Tom Hsiang d, Lixin Zhang a, Xueting Liu a,1

a State Key Laboratory of Bio reactor Engineering, East China University of Science and Technology, Shanghai, China
b State Key Laboratory of Microbial Metabolism and School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, 200240, China
c Key Laboratory of Saline-alkali Vegetation Ecology Restoration, Ministry of Education, College of Life Sciences, Northeast Forestry University, Harbin, China
d School of Environmental Sciences, University of Guelph, 50 Stone Road East, Guelph, Ontario, Canada

ARTICLE INFO

Keywords:
Bifunctional terpene synthases
Norditerpenoids
Cytochrome P450
Aspergillus oryzae
Heterologous expression

ABSTRACT

The chemical diversity of terpenoids is typically established by terpene synthase-catalyzed cyclization and diversified by post-tailoring modifications. Fungal bifunctional terpene synthase (BFTS) associated P450 enzymes have shown significant catalytic potentials through the development of various new terpenoids with different biological activities. This study discovered the BFTS and its related gene cluster from the plant endophytic fungus Didymopistia variable 17020. Heterologous expression of the BFTS in Saccharomyces cerevisiae resulted in the characterization of a major product diterpene variediene (1), along with two new minor products neovariiediene and neoflexibilene. Further heterologous expression of the BFTS and one cytochrome P450 enzyme VndE (CYP6138B1) in Aspergillus oryzae NSAR1 led to the identification of seven norditerpenoids (19 carbons) with a structurally unique 5/5 bicyclic ring system. Interestingly, in vivo experiments suggested that the catalyzed variediene (1) was modified by VndE along with the endogenous enzymes from the host cell A. oryzae through serial chemical conversions, followed by multi-site hydroxylation via A. oryzae endogenous enzymes. Our work revealed that the two-enzymes biosynthetic system and host cell machinery could produce structurally unique terpenoids.

1. Introduction

Containing more than 80,000 members, terpenoids are the most structurally diverse family of natural products (NPs) [1,2], and are comprised of highly diverse biological activities that can be applied to many broad pharmaceutical uses [3]. The chemical diversity of terpenoids is generated by the combination of cyclization catalyzed by terpene synthases and a variety of post-cyclization tailoring modifications catalyzed by enzymes such as cytochrome P450 monoxygenases (P450s), methyltransferases, glycosyltransferases, and acetylases [4]. Among these, P450 enzymes, which are heme-containing proteins, catalyze many diverse reactions, including hydroxylation, dealkylation, epoxidation, reduction, dehalogenation, and C-C bond formation or cleavage [5–7].

Fungi are a well-known rich source of terpenoids [4]. Among fungi-derived terpene synthases, bifunctional terpene synthases (BFTSs) have been discovered as unique types of chimeric enzymes containing a prenyltransferase (PT) domain and a terpene cyclase (TC) domain, and show the possibility of catalyzing the formation of structurally diversified di/ Lesterterpenes [1,8,9]. Since 2007, a series of BFTS genes or BFTS-containing biosynthetic gene clusters (BGCs) have been obtained from various fungal species by using a combination of genome mining and heterologous expression in either Aspergillus oryzae (AO) or Saccharomyces cerevisiae (SC) [9–23]. Based on bioinformatics analysis and tandem heterologous expression, an anti-inflammatory 5/15 bicyclic sesterterpenoid was characterized from Bipolaris sorokiniana, and 5/6/7/3/5 pentacyclic sesterterpene synthases (FoFS and AtAS) from Fusarium oxysporum and A. terreus [18,19]. The FoFS catalyzed the
formation of fusoxypenes, which are enantiomeric with the precursor of the products of ATAS [18,19].

Two BFTSs, EvVS from *Emericella variecolor* [24] and AbVS from *A. brasilienensis* [25], which were both reported for the production of a tricyclic diterpene hydrocarbon varieidiene, have been characterized. The Dickschat group succeeded in expanding the enzymatic synthesis of heterologous expression in containing BGC from *Didymosphaeria variabile* 17020 (DV17020), through heterologous expression in *A. oryzae* NRAS1. Primary biosynthetic elucidation revealed that a single P450 enzyme (VndE) coupled with *A. oryzae* NRAS1 was responsible for producing these products from varieidiene (1). 2. Materials and methods

2.1. General experimental procedures

NMR spectra were acquired on an Agilent DD2 600 MHz spectrometer (600 MHz for $^1$H NMR and 150 MHz for $^{13}$C NMR, Santa Clara, CA, USA). NMR spectra were recorded in CDCl$_3$ (99.8 atom% enriched, Kanto), CD$_3$OD (99.5 atom% enriched, Kanto), and pyridine-d$_5$ (99.8 atom % enriched, Kanto). Chemical shifts were reported in δ value on residual signal of the solvent (CDCl$_3$, δ$_H$ 7.26 and δ$_C$ 77.1), (CD$_3$OD, δ$_H$ 7.15 and δ$_C$ 128.0) or (pyridine-d$_5$, δ$_H$ 8.74, 7.58, 7.22 and δ$_C$ 149.0, 135.3, 123.3) as references. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constants (Hz), and integration. HR-ESI-MS measurements were obtained on Waters GCT Premier mass spectrometer. A high-purity silica gel (Sigma 643) and 60 N silica gel (Kanto Chemicals) were used for column chromatography. LC-HR-MS(ESI) measurements were obtained on Thermo Q Exactive Orbitrap mass spectrometer coupling with a Shimadzu LC-20AOD UPLC equipped with a Waters ACQUITY BEH C18 column (2.1 × 100 mm, 1.7 μm particles). RP-HPLC was performed on an Agilent 1260 Series separation module with a diode array detector. Semipreparative HPLC was carried out using an ACE C18-PPF column (10 × 250 mm, 5 μm, 4 ml/min), an ACE Excel 5C18 column (10 × 250 mm, 5 μm, 4 ml/min), and a Cosmosil Cholesterol (10 × 250 mm, 5 μm, 4 ml/min) column. GC-MS analyses were conducted with QP2010SE GC-MS (Shimadzu, Kyoto, Japan). Chirascan circular dichroism spectrometer was used to record the CD spectra of isolated compounds using MeOH as solvent. Optical rotation was measured using Rudolph Research Analytical Autopol V Automatic Polarimeter or JASCO P-2200 digital polarimeter. Biological reagents, chemicals, and media were purchased from standard commercial sources unless stated otherwise.

2.2. Strains and media

*D. variabile* 17020 was kindly provided by Prof. Hsiang from the University of Guelph (Ontario, Canada). Strains were all cultivated on 2% potato dextrose agar (PDA, Fisher Scientific) at 25 °C for 5 days and used as a source for the BFTS and post-modified genes cloning. *S. cerevisiae* BJ5464-NpgA (MATa ura3-52 his3-D200 leu2-d1 trp1 pep4:: HIS3 prb1- Δ1.6R can1 GAL1) [27] was used for EvVS gene expression following standard recombinant DNA techniques. *A. oryzae* NRAS1 (niaD, sc, Δarg7::ade4) [28] was used as the host for gene cluster expression. The *A. oryzae* transformants strain was grown in DPF medium at 30 °C and 200 rpm for 5 days.

2.3. Construction of expression plasmids

The genomic DNA was extracted using Rapid Fungi Genomic DNA Isolation Kit (Sangon Biotech). The five genes orfA-C, vndD, and vnde were amplified from the genomic DNA of *D. variabile* 17020 with primers shown in Table S1. PCR reactions were performed with the Q5 High-Fidelity DNA Polymerase (New England Biolabs). ClonExpress®II One Step Cloning Kit (Vazyme biotech) was used to construct expression plasmids by inserting PCR product into the appropriate restriction site of pUA220 or pUSA2. The constructed plasmids are summarized in Table S2. The sequence of the five genes has been deposited in the National Center for Biotechnology Information (NCBI) with the accession number ON911568.

2.4. Transformation of *A. oryzae* and *S. cerevisiae*

The transformants AO-vndD (containing pUA220-vndD) and AO-orfABC/vnde (containing pUSA2-orfABC and pUA220-vnde) were transformed into protoplast polyethylene glycol method [29]. AO and AO-vndD are the abbreviations of *A. oryzae* and a transformant of *A. oryzae* harboring vndD gene, respectively. AO transformants listed in Table S2 were grown on MPY (Maltose Polypeptone Yeast extract medium; 3% maltose, 1% polypeptone, 0.5% yeast extract) medium containing 1% (NH$_4$)$_2$SO$_4$ and 0.01% adenine for 3–5 days at 30 °C.

Complementary DNA (cDNA) of DvVS was used as a template of the PCR reactions for direct sequencing and subcloning each gene into the pXW55 vector using SpeI and PmlI restriction sites to afford pXW55-DvVS. The methods of cDNA synthesis and transformants SC-DvVS construction were the same as those in our previous study [19]. Yeast competent cell preparation and transformation were performed with Frozen-EZ Yeast Transformation II kit (Zymo Research) according to the manufacturer’s protocols. Yeast plasmids were prepared by Zymoprep™ Yeast Plasmid Miniprep kit (Zymo Research) and transformed into Escherichia coli strain DH10b for propagation.

2.5. Isolation and purification of diterpenes (1–3) and norditerpenoids (4–10) and their physicochemical properties

Mycelia of BFTS transformant SC-DvVS was inoculated into 250 mL Erlenmeyer flasks containing 100 mL YPD medium (Yeast extract Peptone Dextrose medium; 1% yeast extract, 2% peptone, 2% glucose) to prepare the seed culture, respectively. Fermentation was then carried out in 10 Erlenmeyer flasks (3000 mL) containing 1000 mL YPD medium at 30 °C, 220 rpm for 3 days. Mycelia of the transformant AO-orfABC/vnde was inoculated into 250 mL Erlenmeyer flasks containing 100 mL of PMY medium containing 0.1% (NH$_4$)$_2$SO$_4$ and 0.01% adenine to prepare the seed culture. Fermentation was then carried out in 125 aseptic bags (each containing 80 g rice and 120 mL water), and cultivation methods were the same as those in our previous research [19] incubated stationary at 30 °C for 24 days. Culture broth (10 L) of SC-DvVS was centrifuged at 5000 rpm for 10 min to gain the cell pellet 310 g, which was further extracted with acetone (1 L) at room temperature overnight and then concentrated in vacuo. The residues were extracted with ethyl acetate (500 mL × 3). The combined organic layers were concentrated to yield a dark brown oil extract (230 mg), subjected to the silica gel column eluted by petroleum ether and afforded fractions G1–G9. G1 (3 mg) was further separated by semipreparative HPLC (100% ACN, 4 mL/min) over an ACE Excel 5C18 column to afford 3 (m$_r$ = 27 min, 1.0 mg). G2 (20 mg) was further separated by semipreparative HPLC (100% ACN, 4 mL/min) over an ACE Excel 5C18 column to afford 1 (m$_r$ = 21 min, 8.0 mg) and 2 (m$_r$ = 24.5 min, 3.0 mg).

The fermentation products of transformant AO-orfABC/vnde were extracted exhaustively with EtOAc and condensed in vacuo. The EtOAc layer (15 g after drying in vacuo) was fractionated by a Sephadex LH-20 column using MeOH as mobile phase to give nine sub-fractions.
The sub-fraction N2 (2.7 g) was further subjected to a Sephadex LH-20 column (MeOH 100%) to give 25 sub-fractions (N2N1–N2N25), fractions containing varinorterpenes (N2N2~N2N25), and varinorterpenes (control; ii and iv) transformed cells; (B) Structures of diterpenes 1–3 characterized from SC-vndD transformant cells, and a proposed pathway for their cyclization.

(N1−N9). The sub-fraction N2 (2.7 g) was further subjected to a Sephadex LH-20 column (MeOH 100%) to give 25 sub-fractions (N2N1–N2N25), fractions containing varinorterpenes (N2N4~N2N13) were combined guided by LCMS analysis. After drying in vacuo, N2N4-13 (870 mg) was separated by a RP-C18 silica gel column to give seven sub-fractions (N2N4-13RP1~N2N4-13RP7). N2N4-13RP3 (70 mg) was purified by preparative RP-HPLC using an ACE C18-PFP (10 × 250 mm) column eluting at a flow rate of 4.0 mL/min using a gradient elution: 0 min, 40% MeOH-H2O; 30 min, 55% MeOH-H2O to yield 4 (3.0 mg, tR = 7.0 min), 6 (2.2 mg, tR = 25.5 min), 8 (3.0 mg, tR = 19.0 min), 9 (1.8 mg, tR = 24.0 min), 10 (1.3 mg, tR = 9.6 min). N2N4-13RP4 (36 mg) was purified by preparative RP-HPLC using a Cosmosil Cholster (10 × 250 mm) column eluting at a flow rate of 40.0 mL/min using an isocratic elution (57% MeOH-H2O) to yield 5 (1.2 mg, tR = 21.0 min) and 7 (21.7 mg, tR = 22.0 min).

2.6. Chemical computation details

The calculations were performed by using the density functional theory (DFT) as carried out in the Gaussian 09 [30]. The preliminary conformational distributions search was performed by Sybyl-X 2.0 software. All ground-state geometries were optimized at the B3LYP/6-31G(d) level. The stable conformations were then used in molecular orbital calculations according to the slope and intercept of the linear-squares functions, the magnetic shielding values were converted into chemical shifts. And through subtracting the calculated 13C NMR from the measured shifts, the relative errors of chemical shifts were determined. Solvent effects of methanol solution were evaluated at the same DFT level by using the SCRF/PCM method. The time-dependent density functional theory (TD-DFT) at B3LYP/6-31 + G(d) was employed to calculate the electronic excitation energies and rotational strengths in methanol.

2.7. Biotransformation experiments

AO or AO-vndE transformants were grown on the MPY agar medium containing appropriate nutrients for 3 days at 30 °C. Prepare PBS buffer (pH 5.5) and filter for sterilization. 1 mL PBS buffer containing substrate (300 μg each, ethyl acetate solution) was layered on top of the cells or the blank CD agar used as a control sample. The cells and control sample was incubated at 30 °C for 2 days and extracted with ethyl acetate. The organic phase was dried and dissolved in methanol and directly analyzed by an LC-HR-MS equipped with a Waters ACQUITY BEH C18 column at the following conditions: flow rate: 0.35 mL/min, solvent system: acetonitrile in H2O containing 0.1% of formic acid, 0–8 min: a linear gradient 5%–33%; 8–12 min: a linear gradient 33%–34%, 12–20 min: a linear gradient 34%–99%; 20–22 min: 99%.

3. Results and discussion

A BFTS gene from DV17020, predicted to encode a protein of 699 amino acids, was identified. Similar to the other characterized BFTS enzymes, DV17020-derived BFTS also contained both PT domain and TC domain. In addition, an N-terminal DDXXE motif and a C-terminal DDXD motif were found in its protein sequence, which were necessary for the binding of Mg2+ (Fig. S1) [9]. This BFTS gene had been associated with different post-modification enzymes, including an epoxide hydrolase, a dehydrogenase, a P450, and other three unknown enzymes. Initially, the catalytic function(s) of the candidate DV17020 BFTS were examined with experiments based on heterologous expression in S. cerevisiae BJ5464-NpgA. GC-MS analysis of the ethyl acetate extracts of yeast cells showed three peaks, all of which displayed an M+ molecular ion peak at m/z 272 and were uniquely present in the cells harboring the expression construct of the candidate DV17020 BFTS. Using chromatographic methods, the major product 1 (Fig. 1A) was obtained and characterized as variediene (Fig. S3), a known diterpene generated by EvVS catalyzed reaction [24] and AbVS [25]. This candidate was named as DvVS (D. variabile variediene synthase). DvVS shared a 31.0% and 33.0% identity with EvVS and AbVS, respectively. Interestingly, DvVS did not catalyze the cyclization of GPP to form any products derived from PaPS [31], a homolog sharing the highest identity (46.5%) with DvVS (Fig. S2).

Two minor products were also characterized, including an unprecedented 5/5/5/6 tetracyclic diterpene, named neovarienediene (2), and a new analog of a previously reported monocyclic diterpene flexibilene [32], named neoflexibilene (3), from the extracts based on the HRMS and NMR data (Fig. 1B and S4–S6; Tables S4 and S5). Two similar 15-membered-ring terpenoids were yielded from the cyclization catalyzed by the homologous EvVS [24] but was not by homolog AbVS [25]. Notably, the concentration of 2 in the crude extract increased during processing upon contact with CDCl3 and silica gel. Given that both CDCl3 and silica gel have acidic properties, it was speculated that 1 was not stable under acidic conditions and therefore would be converted to 2 via a non-enzymatic re-protonation (Fig. 1B). To support this speculation, an acid-catalyzed reaction along with HPLC and TLC analysis was conducted and finally revealed that 1 was converted to 2 in 2 min of exposure to hexane containing 10% trifluoroacetic acid (Fig. S7).

Production of variediene (1) synthesized by DvVS allowed for the speculation that three post-modification enzymes and one unknown enzyme encoded by genes adjacent to the DvVS gene (Fig. 3A; Table S6) may be involved in producing more complex products from 1. Among these four genes products, OrfB is a homolog of epoxide hydrolases, which were normally found in the transformation of the epoxides to the diols on some fungal secondary metabolites, such as asteletin and aurovertin E [33–36]. OrfB is a short-chain dehydrogenase/reductase, which shows the highest similarity to L-allo-threonine dehydrogenase homologous that were found in catalyzing the oxidation of L-allo–threonine to L-2-amino-3-keto-butyrate [37]. OrfC might act as a pH-response regulator protein to maintain an alkaline ambient pH in the...
organism, which was required for the proteolytic cleavage of the transcription factor pacC [38]. Overall, orfA could be found in a few characterized fungal secondary metabolic pathways while orfB and orfC were not. Considering that these three genes were adjacent to the DvVS gene, we co-expressed them with DvVS and vndE to further verify their functionalities. As many fungal biosynthetic pathways are revealed through heterologous expression of A. oryzae [17,39–42], these genes were cloned into the expression vectors pUSA2 and pUARA2 for heterologous expression in A. oryzae. Two expression plasmids, pUSA2-orfABC and pUARA2-vndDE, were prepared and transformed into A. oryzae to generate AO-orfABC/vndDE. This transformant was cultivated in rice medium, or MPY medium supplemented with adenine.

Subsequent large-scaled fermentation and comparison of LC-MS metabolite profiles revealed that seven new peaks possessing similar UV spectra (Fig. S8B) were found in the AO-orfABC/vndDE fermentation extract (Fig. 2A). Isolation of these selected components with various chromatographic methods led to the characterization of seven previously unreported 5/5 membered bicyclic norditerpenoids, named varienordienoids A–G (4–10, respectively) (Fig. 2B). Their planar structures and relative configuration were determined by extensive spectroscopic experiments and DP4 analysis (Fig. S9–S16; Table S7–S17; Supplementary text). The absolute configurations of 4 (3S,4R,5R,11S), 5 (3S,4S,5R,7R,11S), 6 (3S,4R,5R,6R,11S), 7 (3S,4S,5R,7S), 8 (3S,4R,5R,6R), 9 (3S,4R,5R,10S), and 10 (3S,4R,5R), were established by TD-DFT based ECD computation (Fig. S17). The comprehensive structural elucidation of these compounds can be found in the Supplementary text. The unprecedented 5/5 bicyclic ring systems of these seven compounds are highly distinguishable from those 5/6 and 6/6 bicyclic ring systems observed in diterpenoids that have been previously characterized [43].

To characterize the functions of enzymes that produce 4–10, three A. oryzae transformants AO-vndDE, AO-orfABC/vndDE, and AO-orfAB/vndDE were constructed (Fig. 3B and S18). The metabolites from these three transformants were analyzed by LC-MS experiments. Subsequent large-scaled fermentation and comparison of LC-MS metabolite profiles revealed that seven new peaks possessing similar UV spectra (Fig. S8B) were found in the AO-orfABC/vndDE fermentation extract (Fig. 2A). Isolation of these selected components with various chromatographic methods led to the characterization of seven previously unreported 5/5 membered bicyclic norditerpenoids, named varienordienoids A–G (4–10, respectively) (Fig. 2B). Their planar structures and relative configuration were determined by extensive spectroscopic experiments and DP4 analysis (Fig. S9–S16; Table S7–S17; Supplementary text). The absolute configurations of 4 (3S,4R,5R,11S), 5 (3S,4S,5R,7R,11S), 6 (3S,4R,5R,6R,11S), 7 (3S,4S,5R,7S), 8 (3S,4R,5R,6R), 9 (3S,4R,5R,10S), and 10 (3S,4R,5R), were established by TD-DFT based ECD computation (Fig. S17). The comprehensive structural elucidation of these compounds can be found in the Supplementary text. The unprecedented 5/5 bicyclic ring systems of these seven compounds are highly distinguishable from those 5/6 and 6/6 bicyclic ring systems observed in diterpenoids that have been previously characterized [43].

To characterize the functions of enzymes that produce 4–10, three A. oryzae transformants AO-vndDE, AO-orfABC/vndDE, and AO-orfAB/vndDE were constructed (Fig. 3B and S18). The metabolites from these three A. oryzae transformants were analyzed by LC-MS experiments. Subsequently, LC-MS analysis showed that the AO-vndDE cells could produce 4–10 (Fig. 3B). 4–10 were also detected in the AO-orfA/vndDE and AO-orfAB/vndDE samples (Fig. S18). These findings clearly suggested that the putative P450 enzyme VndE (CYP6138B1) [44] might contribute to the formation of all seven of these norditerpenoids, while OrfA–C were not. To identify the function of the P450 enzyme (VndE),
the BFTS and VndE were then heterologously expressed in *S. cerevisiae* RC01, which was introduced an integrated copy of the *A. terreus* cytochrome P450 reductase (CPR) gene into the genome of *S. cerevisiae* BJ5464-NpgA [45]. However, no product was found in these *S. cerevisiae* transformant extracts (data not shown), possibly due to the incompatible CPR.

It is of significance that the AO-vndD transformant did not produce any of these norditerpenes (Fig. 3Bii), which indicated that the P450 enzyme VndE was indeed involved in the biosynthesis of these varie-nordienoids. Speculations include theories that the complex transformation process from 1 to 4–10 might result from integrated catalysis involving the P450 Vnde and endogenous *A. oryzae* enzyme(s), considering that unexpected oxidations on biosynthetic intermediates by *A. oryzae* have been reported recently, such as the heterologous production of prosolanapryrone [46] and 4-hydroxy-pyridines [47]. The unusual biotransformation process of these compounds in *A. oryzae* NSAR1 cells could require different reaction types and needs further extensive investigation to elucidate the corresponding mechanisms.

To verify that VndE is indispensable in the generation of 4–10, feeding experiments were conducted using the AO and AO-vndE strains (transformant harboring vndE gene) with 1 as the substrate. Using HR-ESI-MS analysis, only 4 was successfully observed in the AO-vndE transformant but not in AO (Fig. 4A and S19), showing that VndE was vital for transforming 1 into norditerpenoids. However, no other predicted compounds (i.e. 5–10) were detected, which might be attributed to the low conversion rate in this feeding experiment. Hence, 4 was directly used as the substrate in further feeding experiments, which resulted in the acquisition of compounds 5 and 6 in both AO and AO-vndE cells (Fig. 4B). Meanwhile, in the medium, 10 into 4 were found to spontaneous hydrate (Fig. 4Cii). In addition, both AO and AO-vndE cells could transform 10 into 4–9 (Fig. 4Ci and 4Cii). These results suggested that the multi-site hydroxylation of norditerpenoids occurred in the presence of AO endogenous enzymes. Interestingly, the non-enzymatic conversion of 10 in the MPY medium generated a small quantity of 4, which only accounted for 5% of the yield of this particular compound in AO cells supplemented with 10 (Fig. 4C and S20). In addition, 4 and its downstream products (5 and 6) displayed the 11S configuration, thus hinting that AO endogenous enzymes were involved in the stereo-selective hydration at C-11.

A putative biosynthetic pathway was thus proposed that could account for the production of 4–10 in *A. oryzae* cells (Scheme 1). The cyclized terpene 1 was transformed to 10 based on the modifications by the VndE and AO endogenous enzymes through a series of chemical transformations, such as oxidation, decarboxylation, and C-C bond cleavage. The putative mechanism of the VndE-catalyzed reaction is shown in Fig. S21. Subsequent hydroxylation of 10 at the C-6, C-7, and C-10 positions formed compounds 7–9, while hydration of 10 yielded 4, which could be further oxidized into hydroxylated products 5 and 6.

Norterpenoids have been discovered in other organisms [48,49], but most of their biosynthesis pathways are not yet fully understood.

In summary, a varienediene synthase DvVS was characterized from the plant endophytic fungus *D. variabile* 17020 and the major product diterpene varienediene (1) along with two new minor products (2 and 3) were identified. Unexpectedly, seven norditerpenoids (4–10) feature an unprecedented 5/5 bicyclic ring skeleton, generated by a simple two enzyme gene cluster comprised of one BFTS (DvVS) and a single P450 (Vnde) in *A. oryzae* cells. Vnde participated in the degradation processes (i.e. C-C bond cleavage, oxidation, and decarboxylation) of 1 but was not responsible for the multiple tailing hydroxylation. It is worth studying how the AO endogenous enzymes cooperate with VndE in the complicated modifications of 1, in addition to which endogenous enzymes are responsible for the hydroxylation at every specific site. Furthermore, the OrfA–C adjacent to BFTS DvVS, which were predicted to be epoxide hydrolase, dehydrogenase, and unknown enzymes, might be cryptic. Further investigation on the activation of these genes and characterization of their corresponding products is ongoing.

CRediT authorship contribution statement

**Lan Jiang**: Methodology, Investigation, Writing – original draft, Project administration. **Kangjie Lv**: Investigation, Methodology, Formal analysis. **Guoliang Zhu**: Investigation, Methodology, Formal analysis. **Zhi Lin**: Methodology. **Xue Zhang**: Investigation, Validation. **Cuiping Xing**: Investigation, Validation. **Huanting Yang**: Investigation, Validation. **Weiyan Zhang**: Investigation, Formal analysis. **Zhixin Wang**: Investigation, Validation. **Chengwei Liu**: Resources. **Xudong Qiu**: Resources. **Tom Hsiang**: Resources, Writing – review & editing. **Lixin Zhang**: Conceptualization, Resources, Writing – review & editing, Supervision. **Xueting Liu**: Conceptualization, Resources, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare no competing financial interests.

Acknowledgements

We sincerely acknowledge Prof. Jun-Ichi Maruyama at the University of Tokyo for the kind provision of *A. oryzae* NSAR1, Prof. Katsuya Gomi at Tohoku University provides vectors pUARA2 and pUS2A, and Prof. Yi Tang at the University of California provides *S. cerevisiae* strains and vector pXW55. We sincerely thank Prof. Hideaki Oikawa at the Hokkaido University and Prof. Pinghua Liu at the Boston University for their valuable suggestions. We gratefully acknowledge the financial support from the National Key Research and Development Program of China (2020YFA0907800 and 2019YFA0906200), the National Natural
Science Foundation of China (21907031, 21977029, 31720103901, 21877124), the Open Project Funding of the State Key Laboratory of Bioreactor Engineering, the 111 Project (B18022). Genome sequencing and assembly of strain DV17020 were supported by funding from the Natural Science and Engineering Research Council of Canada to Prof. T. Hsiang.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.08.002.

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