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Substrate promiscuity of enzymes from the sesquiterpene biosynthetic pathways from *Artemisia annua* and *Tanacetum parthenium* allows for novel combinatorial sesquiterpene production

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ABSTRACT

The therapeutic properties of complex terpenes often depend on the stereochemistry of their functional groups. However, stereospecific chemical synthesis of terpenes is challenging. To overcome this challenge, metabolic engineering can be employed using enzymes with suitable stereospecific catalytic activity. Here we used a combinatorial metabolic engineering approach to explore the stereospecific modification activity of the *Artemisia annua* artemisinic aldehyde $\Delta 11(13)$ double bond reductase2 (AaDBR2) on products of the feverfew sesquiterpene biosynthesis pathway (GAS, GAO, COS and PTS). This allowed us to produce dihydrocostunolide and dihydroparthenolide. For dihydroparthenolide we demonstrate that the preferred order of biosynthesis of dihydroparthenolide is by reduction of the exocyclic methylene of parthenolide, rather than through C4-C5 epoxidation of dihydrocostunolide. Moreover, we demonstrate a promiscuous activity of feverfew CYP71CB1 on dihydrocostunolide and dihydroparthenolide for the production of 3 β -hydroxy-dihydrocostunolide and 3 β -hydroxy-dihydroparthenolide, respectively. Combined, these results offer new opportunities for engineering novel sesquiterpene lactones with potentially improved medicinal value.

1. Introduction

The terpenoids are a diverse and important group of secondary metabolites that are composed of C5 isoprene units. One important class of terpenoids, the sesquiterpene (C15) lactones (STLs), are mainly present in Asteraceae such as feverfew (*Tanacetum parthenium*), a well-known medicinal plant that has been used since ancient times for multiple afflictions from arthritis to insect bites, but mainly, as its name indicates, against fever. It is also used to treat migraine (Palevitch et al., 1997), and has been shown to have therapeutic effects on cancer (Guzman et al., 2005), by specifically inducing apoptotic cell death in cancer cells. Parthenolide is the main bioactive STL isolated from feverfew (Pareek et al., 2011). The chemical complexity of parthenolide (with its macrocyclic 10-membered ring, the C4-C5 epoxide and the attached α -methylene- γ -lactone) and of other STLs that have medicinal properties makes them difficult targets for chemical synthesis, especially if stereochemically pure compounds are needed. Recent studies

have demonstrated that several important bioactive compounds such as artemisinin (Khairul Ikram et al., 2017; Malhotra et al., 2016; Wang et al., 2016), costunolide (Liu et al., 2011) and parthenolide (Liu et al., 2014) can be produced, with the right stereochemistry, in microbial and plant production platforms through metabolic engineering, i.e. by expressing the pathway genes isolated from the original production host. Such pathway reconstruction in heterologous hosts also opens up the possibility of combinatorial biosynthesis, in which compatible biosynthesis genes from different organisms are combined with the potential to produce novel bioactive secondary metabolites (Julsing, 2006).

Several examples of combinatorial biosynthesis of secondary metabolites are based on the substrate promiscuity of cytochrome P450 monooxygenases that can act on various different substrates (Eudes et al., 2016; Mafu et al., 2016; O'Reilly et al., 2013). For example, CYP71AV8 from chicory (*Chicorium intybus*) can catalyse a triple oxidation of C12 of both amorpho-4,11-diene and germacrene A, resulting

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in artemisinic acid and germacrene A acid, respectively (Nguyen et al., 2010), while the same enzyme catalyses a double oxidation at C2 of (+)-valencene, such that nootkatone is produced (Cankar et al., 2011).

Feverfew contains several types of STLs (costunolide, parthenolide, artemisinic acid and santamarin) (Fischedick et al., 2012). The biosynthesis of parthenolide proceeds through epoxidation of the C4-C5 double bond of the precursor costunolide (Liu et al., 2014). Although chicory does not contain parthenolide, costunolide is also produced in chicory as an intermediate in the production of (6S,7S,11S)-11,13-dihydrocostunolide and leucodin (de Kraker et al., 2002). Some STLs with reduced α -methylene- γ -lactone such as 11,13- β -dihydroactinopicrin are more cytotoxic than their non-reduced counterpart (Ren et al., 2005). de Kraker et al. (2002) reported the presence of an enzymatic activity in chicory root extracts that reduces the exocyclic double bond of costunolide resulting in dihydrocostunolide. Interestingly, a reduced form of parthenolide, dihydroparthenolide, which shows bioactivity against multi-drug resistant *Mycobacterium tuberculosis* (causative agent of tuberculosis) and *Mycobacterium avium* (Rugutt et al., 2001), is present in feverfew extracts (Fischedick et al., 2012), suggesting that just as chicory, also feverfew possesses a double bond reductase (DBR) that can reduce the exocyclic double bond of these STLs. However, the genes encoding these putative DBRs in chicory and feverfew have not been identified.

Artemisia annua L. is also a member of the Asteraceae and known for production of the antimalarial drug artemisinin. The direct precursor of artemisinin is dihydroartemisinic acid (Fig. 1). An artemisinic aldehyde Δ 11(13) double bond reductase2 (AaDBR2) is responsible for reduction of the exocyclic double bond of artemisinic aldehyde to form dihydroartemisinic aldehyde, the precursor of dihydroartemisinic acid. In contrast to chicory and feverfew, the gene encoding DBR2 from *A. annua*, AaDBR2, has been identified (Zhang et al., 2008).

Here we assess whether AaDBR2 can act on STLs and pathway intermediates from feverfew to explore the options to produce novel STLs with potential medicinal value. DBRs have been shown to act on early (the aldehyde in *A. annua*, Fig. 1a (Zhang et al., 2008)), as well as later intermediates (costunolide in chicory) of STL biosynthesis (Fig. 1b (de Kraker et al., 2002)). We studied whether AaDBR2 can also reduce the exocyclic double bond of feverfew STLs and pathway intermediates, and if so whether the enzyme acts on early or late pathway intermediates, and what the products formed are. We discuss how specific reduction of the exocyclic methylene double bond of STLs offers new opportunities for engineering novel compounds with potentially improved medicinal value.

2. Materials and methods

2.1. Plasmid construction for gene expression in *N. benthamiana*

TpGAO (KC964544), CiCOS (JF816041), TpPTS (KC954155), Tp3 β -hydroxylase (KC954153) and AaDBR2 (JX898526) were previously cloned into pIV1A1.1. TpGAS (JF819848) was cloned into impact vector 1.5 (www.impactvector.com) between the CaMV35S promoter and the RBCS1 terminator. All individual constructs were later cloned into the plant binary vector, pBINplus. *Agrobacterium tumefaciens* strain AGL-0 was used for agroinfiltration. Agroinfiltration was performed according to the described method by (van Herpen et al., 2010). *Agrobacterium* constructs were grown at 28 °C for 48 h in a shaker incubator at 250 rpm in Luria broth medium supplied with 50 mg L⁻¹ kanamycin and 37.5 mg L⁻¹ rifampicin. Cells were then harvested at 3500 $\times$ g for 15 min at room temperature. 20 mL of 10 mM MES buffer (2-(N-morpholino) ethanesulfonic acid), 20 mL of 10 mM MgCl₂ and 1 mL of 100 mM acetosyringone (4'-Hydroxy-3',5'-dimethoxyacetophenone) (to a final volume of 1 L) was used as agroinfiltration buffer to resuspend cells. The OD₆₀₀ of these cells were adjusted to 0.5 by addition of agroinfiltration buffer. After mixing of the cultures for co-expression studies they were incubated on a roller mix for 120 min.

The mixtures were infiltrated with a 1 mL syringe to the abaxial side of *N. benthamiana* fully developed leaves. Plants were kept in a day-night temperature of 22–20 °C for 3 or 5 days for substrate feeding experiments or metabolites extraction, respectively.

2.2. Metabolite extraction from *N. benthamiana* leaves

Agroinfiltrated leaves were harvested and snap-frozen in liquid N₂ for metabolites extraction. For substrate feeding, 2 leaf disks were punched from each leaf and vacuum infiltrated with 20 μ M parthenolide or costunolide and kept for 2 h at room temperature. Then the leaves were ground into a fine powder using a tissuelyser and glass beads. 100 mg of fine ground material was extracted with 300 μ L of MeOH:Formic acid (1000:1). Samples were sonicated for 15 min and centrifuged for 15 min at 13500 $\times$ g (5 °C). Later the supernatant was filtered through a 0.45 μ m filter (Minisart® RC4, Sartorius, Germany) for metabolites analysis by LC-MS. For GC-MS analysis, samples were extracted 2 times with 2 mL of ethyl acetate (EtAc), sonicated for 15 min and centrifuged for 15 min at 3500 $\times$ g (10 °C). The upper phase (EtAc) was passed through a Pasteur pipette filled with anhydrous Na₂SO₄ to dehydrate the samples before analysis.

2.3. Plasmid construction for gene expression in yeast

TpPTS (KC954155), Tp3 β -hydroxylase (KC954153) and TpKLS (MF197558) were previously cloned into the pESC-Ura vector under the control of the galactose-inducible promoter by addition of *Bam*HI and *Not*I restriction sites (Liu et al., 2018, 2014). Microsomal fraction isolation was performed according to the method described by Liu et al. (2018).

2.4. Yeast microsome assay and metabolites extraction

In order to perform yeast in-vitro assays microsomal fractions (72 μ L), NADPH (100 μ L of 10 mM stock), potassium buffer (20 μ L of 1 mM, pH = 7.5) and 288 μ L of distilled water were mixed with substrate (10 μ L of 10 mM stock solution). The mixture was incubated for 150 min at 25 °C in a shaker incubator (250 rpm). The enzymatic mixtures were then centrifuged for 15 min at 13500 $\times$ g (5 °C). The supernatant was filtered through a 0.45 μ m filter (Minisart® RC4, Sartorius, Germany) for metabolites analysis by LC-MS.

2.5. Metabolites analysis by LC-Orbitrap-FTMS

In order to analyse the agro-infiltrated *N. benthamiana* leaf extracts or yeast microsome enzyme assays we used a LC-LTQ-Orbitrap-FTMS system (Thermo Scientific). This system a HPLC, an Accela photodiode array detector (PDA). This was connected to an LTQ/Orbitrap hybrid MS detector coupled with an ESI source. An analytical column of Luna 3 μ m C18/2 100 Å; 2.0 $\times$ 150 mm (Phenomenex, USA) was used for chromatographic separation. We used HPLC H₂O: formic acid (1000:1, v-v) as eluent A while eluent B was combination of acetonitrile: formic acid (1000:1, v-v). Flow rate was set to 0.19 mL min⁻¹ and the gradient was from 5% to 75% acetonitrile in a 45 min gradient. This was then followed by a washing step of 15 min and later equilibrated. Full scan mass analysis was done at a resolution equal to 60000. FTMS calibration was externally done in negative ionisation mode by CH₃NaO₂. Injection volume for each sample was set to be 5 μ L.

2.6. GC-MS analysis

GC-MS analysis using a 7809A gas chromatograph, equipped with a 30 m $\times$ 0.25 mm $\times$ 0.25 μ m film thickness column (DB-5), coupled to a 5975 C Triple-Axis detector (Agilent) was used to analyse the EtAc extracts. The flow rate was adjusted to 1 mL min⁻¹ and helium was used as the carrier gas. No splitting was used for injection and inlet

Fig. 1. Biosynthesis pathway of artemisinin in *Artemisia annua* (a) and of costunolide and parthenolide in *Tanacetum parthenium* (feverfew) as well as proposed combinatorial biosynthesis of a number of dihydro-derivatives of sesquiterpene lactones (b). ADS: amorphadiene synthase; CYP71AV1: amorphadiene oxidase; ALDH1: alcohol dehydrogenase; DBR2: double bond reductase; GAS: germacrene A synthase; GAO: germacrene A oxidase; COS: costunolide synthase; PTS: parthenolide synthase. Broken arrows indicate possible enzymatic steps that were not experimentally verified in this study.

temperature set to 250 °C. The initial oven temperature was 45 °C for 1 min, and increased to 300 °C after 1 min at a rate of 10 °C min⁻¹ which was held for 5 min.

2.7. Synthesis of (6S,7S,11S)-11,13-dihydrocostunolide

Costunolide (13.6 mg, 0.058 mmol, 1.0 eq.) was dissolved in dry MeOH (450 µl) and a catalytic amount of Pd/C (< 1 mg) was added. The resulting mixture was stirred in an H₂ atmosphere (1 bar) until the starting material was consumed (90 min). The catalyst was filtered off. Column chromatography on reversed phase silica gel (MeOH: H₂O 1:1 > 3:1) resulted in the title compound (11 mg, 0.047 mmol, 81%). NMR data are given in Table S1.

2.8. NMR analysis

NMR spectra were recorded on a Bruker (Billerica, MA, USA)

Avance I (500 MHz), Avance III HD Prodigy (500 MHz) or an Avance III HD Cryo (700 MHz) NMR spectrometer. Spectra were referenced against the solvent signal (¹H-NMR, residual proton signal of C₆D₆ δ = 7.16; ¹³C-NMR: C₆D₆ δ = 128.06).

2.9. Accession numbers

Gene bank accession numbers of the genes used in this study were KC964544 (TpGAO); JF816041 (CiCOS); KC954155 (TpPTS); KC954153 (Tp3β-hydroxylase); JX898526 (AaDBR2) and MF197558 (TpKLS). The cytochrome P450s were previously deposited to David Nelson's cytochrome P450 database (<http://drnelson.uthsc.edu/cytochromeP450.html>) with the assigned names of CYP71CA1 (TpPTS); CYP71CB1 (Tp-3βhydroxylase); CYP71BL3 and CYP71BL3 (CiCOS) (Nelson, 2009).

Fig. 2. AaDBR2 functions as a costunolide reductase in *N. benthamiana*. (a). Q-ExactivePlus Orbitrap LC-MS/MS chromatograms of *N. benthamiana* leaves agroinfiltrated with the costunolide pathway (PW) (*AtHMGR*, *TpGAS*, *CiGAO* and *CiCOS*) with or without AaDBR2 (bottom chromatograms), dihydrocostunolide and co-injection of the dihydrocostunolide standard and the product of the costunolide PW + AaDBR2 (top chromatograms). EV represents the empty pBIN⁺ vector that was used instead of AaDBR2 as control. (b) MS spectrum of the peak at 42.39 min, the product of the costunolide PW + AaDBR2, the dihydrocostunolide standard and co-injection of these two. (c) MS/MS fragmentation of peak eluting at 42.39 min for dihydrocostunolide standard (top chromatogram) and the product of the costunolide PW + AaDBR2 (bottom). HMGR: 3-hydroxy-3-methylglutaryl coenzyme A synthase, GAS: germacrene A synthase, GAO: germacrene A oxidase, COS: costunolide synthase, AaDBR2: *Artemisia annua* double bond reductase 2.

3. Results

3.1. Functional characterization of AaDBR2 in the costunolide pathway

Incubation of costunolide with a chicory root extract results in the formation of dihydrocostunolide in an NADPH dependent manner (de Kraker et al., 2002). We supposed that dihydrocostunolide, a germacranolide type STL, is formed through the action of a reductase. Dihydrocostunolide and its C4-C5 epoxide (dihydroparthenolide) are detected in chicory and feverfew extracts, respectively, but a reductase that is responsible for the exocyclic double bond reduction in these compounds has not been identified in these two species. Hence we tested DBR2 from *A. annua*, which is active in a closely related artemisinin biosynthesis pathway. AaDBR2 reduces the exocyclic double bond of artemisinic aldehyde, resulting in the formation of dihydroartemisinic aldehyde en route to artemisinin (Ting et al., 2013;

Zhang et al., 2008). Here we tested whether AaDBR2 can also reduce the exocyclic double bond of costunolide. Hereto, the costunolide biosynthesis pathway genes (*GAS*, *GAO* and *COS*) were co-expressed with AaDBR2 using transient expression in *Nicotiana benthamiana* by agroinfiltration. To boost substrate availability, *Arabidopsis thaliana* 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*AtHMGR*) was also co-expressed. *AtHMGR* can boost mevalonate production which leads to higher FPP precursor input for STL production in *N. benthamiana* (Liu et al., 2014). Leaves were harvested 5 days post agro-infiltration (5 dpi) and metabolites were extracted with MeOH. A non-targeted metabolite profiling by LC/Q Exactive MS was used for product detection. Comparison of the chromatograms of samples that expressed the costunolide pathway genes with and without AaDBR2 revealed a new peak eluting at 42.32 min ($[M + H]^+ = 235.169$) (Fig. 2). This mass differs 2.01 Da from that of costunolide ($[M + H]^+ = 233.153$), which fits with a double bond reduction in costunolide. In *N. benthamiana* heterologous

Scheme 1. Synthesis of dihydrocostunolide by hydrogenation of costunolide.

Fig. 3. AaDBR2 is also a parthenolide reductase. (a) LC-Orbitrap-FTMS chromatograms of the products of the parthenolide pathway (PW) (*AtHMGR*, *TpGAS*, *CiGAO*, *CiCOS* and *TpPTS*) without AaDBR2 (bottom), the parthenolide PW with AaDBR2 (middle) and a dihydroparthenolide standard (top). A similar peak to the dihydroparthenolide standard eluted at 29.47 min in the parthenolide PW + AaDBR2 which was absent in the control. (b) in source spectrum of dihydroparthenolide (top) and the product of the parthenolide PW + AaDBR2 eluting at 29.47 min. The grey highlighted box is indicating the parent ion $[M+H]^+ = 251.16472$. HMGR: 3-hydroxy-3-methylglutaryl coenzyme A synthase, GAS: germacrene A synthase, GAO: germacrene A oxidase, COS: costunolide synthase, PTS: parthenolide synthase, AaDBR2: *Artemisia annua* double bond reductase 2.

costunolide is detoxified by conjugation to glutathione (GSH) resulting in costunolide^{GSH}, which over time may break down to the costunolide-cysteine (costunolide^C) conjugate. This GSH conjugation supposedly takes place at the methylene group adjacent to the carbonyl group of the lactone ring of costunolide (Liu et al., 2011). Expression of the costunolide pathway genes with AaDBR2 in *N. benthamiana* resulted in a 70% reduction in costunolide^{GSH} and costunolide^C levels, providing additional support for AaDBR2 activity on costunolide.

Since a commercial standard of dihydrocostunolide is not available, we used chemical synthesis to produce dihydrocostunolide to confirm the identity and stereochemistry of the novel product in *N. benthamiana*. Reduction of the 11,13-double bond in costunolide was done as described in the methods section. This reaction including the workup with a NH_4Cl solution resulted in the formation of a complex product mixture from which the cadinenes, dihydro- α -cyclocostunolide (1) and dihydro- β -cyclocostunolide (2) could be isolated in low yields (Scheme 1) and their structure confirmed using NMR (see Suppl Information). The reduction of costunolide using NaBH_4 is known to produce both stereoisomers, (11S)- and (11R)-dihydrocostunolide (G. Appendino, personal communication). The cadinenes likely formed from these two stereo-isomers during column chromatography on the slightly acidic silica gel, which is known to cause acid-induced cyclisation in

germacrenes (de Kraker et al., 2001). Catalytic hydrogenation of costunolide using palladium on charcoal and purification on reversed phase silica gel resulted in the efficient formation of the target compound (6S,7S,11S)-11,13-dihydrocostunolide (dihydrocostunolide) as a single stereoisomer with 81% yield. The synthetic compound had the same mass spectrum and eluted at the same retention time as the metabolite observed in *N. benthamiana* (Fig. 2). These results show that AaDBR2 can indeed stereospecifically reduce the exocyclic double bond of costunolide to produce (6S,7S,11S)-11,13-dihydrocostunolide (dihydrocostunolide).

3.2. Reduction of parthenolide by AaDBR2

In order to test whether AaDBR2 can also act on parthenolide, all the parthenolide biosynthesis pathway genes (*GAS*, *GAO*, *COS* and *PTS*) (Liu et al., 2014) were co-expressed with AaDBR2. Again, boosting of FPP production was achieved by co-expression of *AtHMGR1*. Agro-infiltrated leaves were harvested at 5 dpi and methanolic extracts were injected into LC-Orbitrap-FTMS to analyse product formation. Comparison of the chromatograms of extracts from samples expressing the parthenolide pathway with and without AaDBR2 showed a new peak at $\text{RT} = 29.47$ min ($[M+H]^+ = 251.16$). The mass of the new compound

Fig. 4. AaDBR2 produces dihydrocostunolide from costunolide in *N. benthamiana*. (a) LC-Orbitrap-FTMS chromatograms of *N. benthamiana* leaf extracts. *N. benthamiana* leaves were agroinfiltrated with AaDBR2 or pBIN + (EV as the control). After 3 days (3dpi), discs of these agroinfiltrated leaves were vacuum infiltrated with a costunolide solution and incubated for 2 h. Chromatograms represent costunolide infiltration into discs expressing EV (bottom) or AaDBR2 (middle) and a dihydrocostunolide standard. Dihydrocostunolide, regardless of the presence of AaDBR2, was produced in *N. benthamiana* leaves. (b) Mass spectra of a dihydrocostunolide standard and the peak at 37.29 min produced upon costunolide infiltration into leaf discs expressing AaDBR2 and EV, demonstrating identical parent ion $[M+H]^+ = 235.16981$ and fragmentation pattern. AaDBR2: *Artemisia annua* double bond reductase 2.

fits that of dihydroparthenolide and the retention time shift between parthenolide and the putative dihydroparthenolide of about 0.4 min is similar to the retention time shift between costunolide and dihydrocostunolide (Fig. S5). The identity of the novel compound was confirmed by comparison with an authentic dihydroparthenolide standard, isolated from feverfew extracts (Fischedick et al., 2012) (Fig. 3).

3.3. AaDBR2 reduces costunolide

Formation of dihydrocostunolide by co-expression of AaDBR2 and the costunolide pathway genes in the experiment described above may occur through reduction of costunolide as suggested before (de Kraker et al., 2002), but could also occur through reduction of costunolide pathway intermediates produced by GAS and/or GAO (Fig. 1b-1), particularly considering that the native function of AaDBR2 is to reduce artemisinic aldehyde. If AaDBR2 forms double-bond reduced costunolide pathway intermediates, such products may subsequently be converted by GAO and COS to dihydrocostunolide (Fig. 1b-2), provided these enzymes are promiscuous for dihydro-substrates. To test whether AaDBR2 acts on costunolide we supplied costunolide to *N. benthamiana* leaf discs transiently expressing only AaDBR2. At three dpi, leaf discs were taken from agro-infiltrated leaves and vacuum infiltrated with water containing costunolide. The infiltrated leaf disks were harvested after 2 h and extracted with methanol for product analysis by LC-Orbitrap-FTMS. Product analysis showed that dihydrocostunolide was formed, indicating that AaDBR2 acts on costunolide (Fig. 4). Control experiments with leaf discs infiltrated with EV showed a very low level of the conversion of costunolide to dihydrocostunolide (~60% less active than with expression of AaDBR2), suggesting the presence of some endogenous reductase activity with low activity on costunolide in *N. benthamiana*.

3.4. AaDBR2 also reduces parthenolide

Formation of dihydroparthenolide by co-expression of AaDBR2 with the parthenolide pathway genes in *N. benthamiana* may be the result of promiscuous activity of TpPTS oxidising both costunolide and dihydrocostunolide (Fig. 5e, route 1) or by promiscuous activity of AaDBR2,

reducing also the double bond of parthenolide (Fig. 5e, route 2). To test the promiscuity of TpPTS we used a yeast microsome feeding assay. Yeast WAT11 was transformed with TpPTS and used for isolation of the microsomal fraction containing TpPTS. The microsomal fractions were subsequently incubated with either dihydrocostunolide or costunolide for 2.5 h. As control, microsome fractions from yeast expressing EV were used. Product analysis of these *in vitro* enzyme assays by LC-Orbitrap-FTMS showed that PTS can use both costunolide and dihydrocostunolide as substrate, resulting in parthenolide and dihydroparthenolide, respectively (Fig. 5a and b). No parthenolide or dihydroparthenolide was detected in microsomes from yeast expressing EV, incubated with costunolide or dihydrocostunolide, respectively. The conversion of dihydrocostunolide to dihydroparthenolide by PTS is not very efficient as only ~2.5% of the infiltrated dihydrocostunolide was converted to dihydroparthenolide.

To test whether AaDBR2 can use parthenolide as substrate (Fig. 5e, route 2) *N. benthamiana* leaves were infiltrated with *A. tumefaciens* harbouring the AaDBR2 expression construct. Three dpi, discs were taken from agro-infiltrated leaves and infiltrated with water containing parthenolide. Vacuum infiltration of parthenolide in discs from leaves transformed with EV was used as negative control. Leaf discs were incubated for 2 h after which they were extracted with MeOH and analysed by LC-Orbitrap-FTMS. The results showed that AaDBR2 catalyses exocyclic double bond reduction in parthenolide (Fig. 5c and d). A tiny peak, eluting at the same time as the authentic dihydroparthenolide standard, was detected in leaves expressing EV (Fig. 5c). However, the mass spectrum of this product contains fragment masses $m/z = 231.16$, $m/z = 249.16$ and $m/z = 273.15$ of which only one ($m/z = 273.15$) fits with fragment masses of authentic dihydroparthenolide, suggesting that this is likely not dihydroparthenolide (Fig. 5d). More than 90% of infiltrated parthenolide was converted by AaDBR2 compared to the activity in leaves expressing EV (Fig. 5c). This shows that parthenolide is a good substrate for AaDBR2.

3.5. Characterization of substrate promiscuity of Tp β -hydroxylase

Feverfew also contains a β -hydroxylase, which catalyses the stereospecific β -hydroxylation of costunolide and parthenolide to form β -hydroxycostunolide and β -hydroxyparthenolide, respectively (Liu

(caption on next page)

Fig. 5. Bidirectional characterization of dihydroparthenolide biosynthesis in *N. benthamiana* and *Saccharomyces cerevisiae* (yeast) microsomes. (a) LC-Orbitrap-FTMS (positive ionisation mode) chromatograms of *S. cerevisiae* microsome enzymatic mixture extracts to investigate dihydroparthenolide *in-vitro* biosynthesis through enzymatic epoxidation of dihydrocostunolide by the activity of TpPTS (parthenolide synthase) (Fig. 5e, route 1). From bottom; feeding dihydrocostunolide to yeast microsomes expressing EV or expressing parthenolide synthase (TpPTS), a dihydroparthenolide standard and co-injection of the product of TpPTS + dihydrocostunolide and the dihydroparthenolide standard. A peak eluting at RT = 29.47 was present in mixtures of feeding assay of dihydrocostunolide to TpPTS which was similar to the dihydroparthenolide standard. (b) Spectrum of produced compound from feeding assay of dihydrocostunolide to TpPTS (bottom), dihydroparthenolide standard (middle) and co-injection of the product of TpPTS + dihydrocostunolide and the dihydroparthenolide standard (top). (c) LC-Orbitrap-FTMS (positive ionisation mode) chromatograms of *in-vivo* biosynthesis of dihydroparthenolide in *N. benthamiana* through enzymatic reduction of parthenolide by the activity of AaDBR2. A highly concentrated solution of parthenolide was vacuum infiltrated to the leaf disks of *N. benthamiana* expressing AaDBR2 or EV (control) 3 days after agroinfiltration. A peak eluting at RT = 29.47 was present in samples with and without AaDBR2 but at ~95% lower concentration in the latter. (d). The mass spectrum of the compound eluting at 29.47 min produced upon feeding of parthenolide to leaf discs expressing AaDBR2 (middle) was identical to that of the dihydroparthenolide standard (top). The fragmentation pattern of the product of parthenolide feeding to an EV expressing leaf disc was only partially similar, but with the representative mass of the dihydroparthenolide standard present ($[M+H]^+ = 251.16472$, arrow head). (e) proposed bidirectional biosynthesis of dihydroparthenolide in feverfew. Route 1 represents biosynthesis of dihydroparthenolide, through reduction of costunolide by AaDBR2 and then epoxidation of the C4-C5 double bond by TpPTS whereas route 2 represents first epoxidation of the C4-C5 double bond of costunolide and then reduction of parthenolide through the activity of AaDBR2.

et al., 2014). We assessed whether the feverfew β -hydroxylase also accepts dihydrocostunolide as a substrate, just as TpPTS that can oxidise both costunolide and dihydrocostunolide as demonstrated above. Hereto, microsomal fractions isolated from yeast expressing the feverfew β -hydroxylase were incubated for 2 h with dihydrocostunolide. Products of the *in vitro* enzyme assay were extracted and analysed by LC-Orbitrap-FTMS. The resulting chromatograms show a new peak eluting at 23.80 min ($[M+H]^+ = 251.16472$) in samples from yeast expressing Tp β -hydroxylase incubated with dihydrocostunolide. This peak was not present in the control samples where dihydrocostunolide was fed to microsome fractions from yeast expressing EV (Fig. 6). The mass of this novel compounds fits with the expected mass of β -hydroxy-dihydrocostunolide. Also the retention time shift of this new peak compared with dihydrocostunolide is similar to the retention time shift between costunolide and β -hydroxycostunolide (~0.4 min, Fig. S5). A commercial standard of β -hydroxy-dihydrocostunolide is not available hampering unambiguous confirmation of the identity of this new compound as β -hydroxy-dihydrocostunolide.

Similar to dihydroparthenolide formation, a bidirectional biosynthesis pathway can also be postulated for β -hydroxy-dihydrocostunolide. In order to test whether β -hydroxycostunolide is the preferred substrate for AaDBR2 (Fig. 6d, Route 2), we infiltrated β -hydroxycostunolide to the *N. benthamiana* cells expressing AaDBR2 and analysed the EtAC extracts by GC-MS. A new peak eluting at 17.30 min was present in the samples expressing AaDBR2 and not in the EV samples. In order to detect possible traces of the proposed β -hydroxy-dihydrocostunolide in the EV samples, both samples were concentrated ($\times 20$) and re-injected into the GC-MS, where no new peak eluting at 17.30 min was identified in the EV samples (Fig. 6c).

Tp β -hydroxylase also acts on parthenolide to form β -hydroxyparthenolide (Liu et al., 2014) and we tested whether dihydroparthenolide is also a substrate for this enzyme. Since the amount of dihydroparthenolide we had available was low, we produced dihydroparthenolide *in planta* by feeding parthenolide to AaDBR2 expressing *N. benthamiana* leaves. The Tp β -hydroxylase was co-expressed with AaDBR2 or EV. At three dpi, parthenolide was vacuum infiltrated into the leaf disks and discs were extracted after 2 h incubation for product analysis by LC-Orbitrap-FTMS. Results showed that both in absence and presence of AaDBR2 a product was formed which elutes at 18.70 min ($[M+H]^+ = 265.14399$), which was identified as β -hydroxyparthenolide, confirming the enzyme activity of Tp β -hydroxylase on parthenolide. If double-bond reduced β -hydroxy-dihydroparthenolide is formed from β -hydroxyparthenolide through the action of AaDBR2, it should have mass $[M+H]^+ = 267.15964$ and an about 0.4 min later retention time. A peak with these characteristics was indeed detected in samples expressing AaDBR2 (RT shift 0.65 min). A similar but smaller peak was identified in the samples only expressing EV (10-fold lower peak intensity), indicating that an endogenous *N. benthamiana* enzyme may also catalyse this double bond reduction (data

not shown). Because we lack the standard for β -hydroxy-dihydroparthenolide we cannot unambiguously confirm product identity. However, the five main peaks in the mass spectrum of the novel product, were similar to the five main peaks in the mass spectrum of β -hydroxyparthenolide, but with a 2 D higher mass in the m/z for each of the five peaks for the new compound, suggesting that the novel compound is indeed β -hydroxy-dihydroparthenolide (Fig. S4).

Very much similar to the bidirectional biosynthesis pathways of costunolide to dihydroparthenolide and costunolide to β -hydroxy-dihydrocostunolide a bidirectional biosynthetic pathway can also be proposed for β -hydroxy-dihydroparthenolide formation from parthenolide (Fig. 7d). In one pathway the parthenolide exocyclic methylene double bond is first reduced after which β -hydroxylation occurs by Tp β hydroxylase (Fig. 7d, route 1), while in the other pathway Tp β hydroxylase first hydroxylates C3 of parthenolide to produce β -hydroxyparthenolide after which AaDBR reduces the exocyclic double bond to produce β -hydroxy-dihydroparthenolide. We had a small amount of authentic β -hydroxyparthenolide available and vacuum infiltrated that into *N. benthamiana* leaf discs expressing AaDBR2. Samples were extracted with EtAC and injected into the GC-MS. After concentration of samples ($\times 20$) two peaks were identified in samples expressing AaDBR2 at 21.20 and 21.70 min (Fig. 7a). The mass difference of the major peak in the spectrum of the peak eluting at 21.20 was 2 D, suggesting a double bond reduction of the peak eluting at 21.70 min (β -hydroxyparthenolide). After concentration of the samples, a tiny peak eluting at 21.19 min was also detected in the control samples (expressing EV) (Fig. 7a) indicating an intrinsic DBR activity of *N. benthamiana* which was observed also with other substrates infiltrated (see above) (Fig. S4).

In order to test the other route where parthenolide is first reduced by the AaDBR2 and then used by the Tp β hydroxylase, we expressed Tp β hydroxylase in *N. benthamiana* leaves and vacuum infiltrated dihydroparthenolide. Samples were extracted with EtAC, concentrated and injected into GC-MS for analysis. This showed that feeding dihydroparthenolide to the cells expressing Tp β hydroxylase results in formation of a new compound (proposed β -hydroxy-dihydroparthenolide) eluting at 21.21 min (Fig. 7c), similar to the one identified in Fig. 7a. Such a peak was not present in samples expressing EV.

3.6. Dihydro-costunolide is not the substrate for feverfew kauniolide synthase

Recently we also characterized a kauniolide synthase from feverfew (TpKLS), which converts costunolide into the basic guaianolide-type sesquiterpene lactone kauniolide (Liu et al., 2018). To assess whether TpKLS can use dihydrocostunolide as a substrate to produce dihydrokauniolide, we incubated dihydrocostunolide with microsomal fractions of yeast expressing KLS. However, there was no decrease in

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Fig. 6. Production of tentatively identified 3 β -hydroxy-dihydrocostunolide from dihydrocostunolide by the activity of feverfew 3 β -hydroxylase. (a) LC-Orbitrap-FTMS (positive ionisation mode) chromatograms of products of dihydrocostunolide feeding to Tp3 β -hydroxylase expressing yeast microsomes showing a peak eluting at 23.80 min ($[M+H]^+ = 251.16472$) 0.4 min later than 3 β -hydroxycostunolide, tentatively identified as 3 β -hydroxy-dihydrocostunolide. (b) fragmentation spectrum of the peak at 23.80 min. The grey highlighted box indicates the parent ion that could represent 3 β -hydroxy-dihydrocostunolide. (c) Production of tentative 3 β -hydroxy-dihydrocostunolide from 3 β -hydroxycostunolide by the activity of AaDBR2. GC-MS chromatograms represent extracts from *N. benthamiana* cells expressing AaDBR2 (top) and EV (bottom) that were infiltrated with 3 β -hydroxycostunolide. A new peak was detected at 17.30 min (mass spectrum in (d)) not present in the control. (e) proposed bidirectional biosynthesis of 3 β -hydroxy-dihydrocostunolide. Route 1 represents biosynthesis of 3 β -hydroxy-dihydrocostunolide through reduction of costunolide by AaDBR2 and then 3 β -hydroxylation by Tp3 β -hydroxylase while route 2 proposes first 3 β -hydroxylation by Tp3 β -hydroxylase followed by reduction of 3 β -hydroxycostunolide through the activity of AaDBR2.

the amount of dihydrocostunolide and no dihydrokaunolide was detected (data not shown) suggesting that the biosynthesis of dihydrokaunolide takes place by reduction of kaunolide itself and not its precursor, costunolide.

4. Discussion

Co-expression of terpene biosynthesis genes from different species in a single heterologous host can be used to explore the range of compatible substrate-enzyme combinations beyond the substrates that are present in the original host. Such combinatorial metabolic engineering also provides a powerful strategy to produce novel terpenes with new biological activity or can be used to produce desired compounds even when not all the genes encoding its biosynthesis pathway are available (but can be substituted by compatible enzymes from a different plant species). For instance, although sesquiterpene lactones with reduced exocyclic methylene double bond have been reported both in feverfew and chicory, full reconstruction of their biosynthesis pathway has not been achieved yet, as from neither species the double bond reductases have been characterized. In this paper we took advantage of two sets of available enzymes from *A. annua* and feverfew in a combinatorial metabolic engineering approach for biosynthesis of sesquiterpene lactones with reduced exocyclic double bond. This was possible due to substrate promiscuity for a number of the enzymes involved in sesquiterpene lactone biosynthesis in these two species.

The artemisinin biosynthesis pathway in *A. annua* L. is similar to the costunolide biosynthesis pathway in chicory and feverfew (Fig. 1). Indeed, it was previously demonstrated that germacrene A oxidase (GAO) from chicory can act on the non-native substrate amorphadiene from *A. annua*, although amorphadiene oxidase (CYP71AV1) from *A. annua* cannot oxidise germacrene A (Nguyen et al., 2010). We tested whether AaDBR2 is able to reduce the double bond in germacrene A alcohol, germacrene A aldehyde or germacrene A acid. However, neither these intermediates, nor their reduced forms could be detected in extracts (or the headspace) of the reaction mixtures (data not shown). Indeed, detection of these intermediates was also shown to be problematic when produced in a *N. benthamiana* heterologous expression system (Liu et al., 2011). However, we show that AaDBR2 can reduce the exocyclic methylene double bond of both costunolide and parthenolide, producing dihydrocostunolide and dihydroparthenolide, respectively (Figs. 4 and 5). Moreover, the reduced products are substrates for CYP71CB1 (Tp3 β -hydroxylase) of feverfew for production of 3 β -hydroxy-dihydrocostunolide and 3 β -hydroxy-dihydroparthenolide, respectively (Figs. 6 and 7). Production of 3 β -hydroxy-dihydrocostunolide and 3 β -hydroxy-dihydroparthenolide may proceed through two routes: first reduction and then C3-hydroxylation or first C3-hydroxylation and then reduction (Figs. 6d and 7e). Results from the bidirectional biosynthesis of dihydroparthenolide (through dihydrocostunolide by PTS or through parthenolide by DBR) suggests a preferred biosynthetic route (Fig. 5e, route 2). The efficiency of this route 2 (exocyclic double bond reduction of parthenolide) is ~90%, while the efficiency of route 1 (Fig. 5e) is calculated to be only ~2.5%.

AaDBR2 produces only the 11S-isomers of dihydrocostunolide and dihydroparthenolide. Thus, the enzymatic reaction follows the same stereochemical course as the chemical hydrogenation, likely with attack of the olefinic double bond from the better accessible face and/or

producing the thermodynamically more stable stereoisomer. Stereoselective enzymatic reactions are interesting from a pharmaceutical point of view, as often only one of the stereoisomers shows medicinal properties (Julsing, 2006). Thalidomide (softenone) for example as a mixture of two enantiomers was used in medicine (as sedative); however one enantiomer is used as sedative while the other one is responsible for teratogenic side effects (Nguyen et al., 2006).

4.1. Product biosynthesis potential versus actual product accumulation

Our inventory of the promiscuity of sesquiterpene biosynthesis enzymes and the identification of sesquiterpene lactones in feverfew (Fischedick et al., 2012) provide insight in how competition between enzymes in feverfew may regulate accumulation of individual sesquiterpenes. For instance, dihydroparthenolide is detected in feverfew extracts (Fischedick et al., 2012), suggesting that feverfew has an enzyme similar to AaDBR2, which we name here TpDBR. In contrast, dihydrocostunolide is not detected in feverfew (Fischedick et al., 2012), while this is produced by AaDBR2 in our *N. benthamiana* assays (Fig. 2). This could be due to efficient capturing in feverfew of costunolide by TpPTS, preventing formation of dihydrocostunolide by the putative TpDBR and/or to a lower affinity of the putative TpDBR for costunolide. Indeed, the methylene group of parthenolide is efficiently reduced also by AaDBR2 in the *in planta* (*N. benthamiana*) assay and this pathway is more efficient than costunolide reduction to dihydrocostunolide and subsequent epoxidation by PTS to dihydroparthenolide (route 1, Fig. 5). It could be that the putative feverfew DBR also has a higher affinity for parthenolide as substrate than costunolide. Another explanation for the preferred production of dihydroparthenolide from parthenolide may be related to a spatial arrangement of COS, PTS and possibly also the likely cytosolic DBR, in/on the ER membrane, resulting in substrate channelling (metabolone).

We recently showed that Lipid Transfer Proteins (LTPs) may exhibit high selectivity for the transport/sequestration of specific sesquiterpene lactones to the apoplast, where they are no longer available for enzymatic conversion (Wang et al., 2016). Competition for feverfew pathway intermediates may thus not only occur at the enzyme level, but also through transport. We detected up to eight different LTPs in trichomes of feverfew where sesquiterpene biosynthesis takes place. For two of these (TpLTP1 and TpLTP2) we demonstrated involvement in extracellular accumulation of costunolide, while TpLTP3 was highly specific for parthenolide (Beyraghdar Kashkooli et al., submitted). Activity of these LTPs in feverfew would place costunolide and parthenolide out of reach of intracellular (cytosolic) enzymes for further conversion. Characterization of the putative TpDBR and other enzymes in combination with further characterization of the affinity of different feverfew trichome LTPs for pathway products may eventually provide a full understanding of product accumulation in feverfew.

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Fig. 7. Production of tentatively identified 3 β -hydroxy-dihydroparthenolide. (a) GC-MS chromatograms of extracts of leaf discs of *Nicotiana benthamiana* expressing AaDBR2 (top) or EV (bottom), vacuum infiltrated with 3 β -hydroxyparthenolide, showing two peaks eluting at 21.20 and 21.70 min. The tiny peak eluting at 21.20 min present in the EV samples, fed with 3 β -hydroxyparthenolide indicates the intrinsic DBR activity on the 3 β -hydroxyparthenolide exocyclic double bond. (b) mass spectra of the peaks at 21.20 min (top) representative of putative 3 β -hydroxy-dihydroparthenolide and 21.70 min (bottom) representing 3 β -hydroxyparthenolide. (c) Chromatogram of leaf discs expressing 3 β -hydroxylase or EV, vacuum infiltrated with dihydroparthenolide. A tiny peak was identified after concentration of samples in the EtAc extracts of leaves expressing Tp3 β hydroxylase at 21.21 min. This peak was not present in the samples expressing the EV. (d) mass spectra of the peaks at 21.20 min representative of putative 3 β -hydroxy-dihydroparthenolide. (e) proposed bidirectional biosynthetic pathway of 3 β -hydroxy-dihydroparthenolide. Route 1 represents formation of dihydroparthenolide through the activity of AaDBR2 and then C3 hydroxylation of dihydroparthenolide to produce 3 β -hydroxy-dihydroparthenolide while route 2 shows first formation of 3 β -hydroxyparthenolide by the activity of the Tp3 β hydroxylase and then reduction of the exocyclic double bond of 3 β -hydroxyparthenolide, resulting in formation of 3 β -hydroxy-dihydroparthenolide. AaDBR2: *Artemisia annua* double bond reductase2.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2019.01.007.

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