Two residues determine the product profile of the class II diterpene synthases TPS14 and TPS21 of *Tripterygium wilfordii*

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ABSTRACT

The medicinal plant *Tripterygium wilfordii* (Celastraceae) contains a pair of class II diterpene synthases (diTPS) of specialized labdane-type metabolism that, despite remarkably close homology, form strikingly different products. *TwTPS21* catalyzes bicyclization of the linear C20 precursor geranylgeranyl diphosphate to *ent*-copal-8-ol diphosphate, while *TwTPS14* forms kolavenyl diphosphate. To determine the amino acid signature controlling the functional divergence of the homologues, we modeled their structures based on an existing crystal structure of the *Arabidopsis* *ent*-copalyl diphosphate synthase, archetypical of diTPSs in general metabolism of gibberellin phytohormones. Of the residues differing between *TwTPS21* and *TwTPS14* two located to the predicted active site, and we hypothesized that these control the functional differentiation of the enzymes. Using site-directed mutagenesis, we generated a panel of six variants, where one, or both positions were exchanged between the enzymes. In coupled heterologous assays with a corresponding class I diTPS, *TwTPS2*, complete product interchange was observed in variants with both reciprocal mutations, while substitutions of either residue gave mixed product profiles. Two mutants, *TwTPS14*:Y265H and *TwTPS21*:A325V, also produced *ent*-copalyl diphosphate, highlighting the evolutionary potential of enzymes of this family to drive rapid diversification of plant diterpene biosynthesis through neo-functionalization. Our study contributes to the understanding of structure-function relation in plant class II diTPSs and complements previous mutational studies of *Arabidopsis* *ent*-copalyl diphosphate synthase with additional examples from the specialized metabolism of *T. wilfordii*.

Key words: *Tripterygium wilfordii*, Celastraceae, diterpene synthase, homology model, structure-function relation.

1. Introduction

With over 50,000 terpenoid structures known, the plant kingdom constitutes the largest reservoir of the 7,000 labdane-type diterpenoids sharing a characteristic bicyclic decalin core (Pateraki et al., 2015; Zi et al., 2014). The chemical diversity of labdane-type diterpenoids is reflected in a broad range of bioactivities covering both general and specialized metabolism, including regulation of growth in all higher plants (gibberellins), allelopathy in rice (momilactones) and defense in trees (diterpene resin acids). Class II diterpene synthases (diTPSs) catalyze the biosynthetic entry point to labdane-type diterpenoids through bi-cyclisation of geranylgeranyl diphosphate (GGDP, **1**) and shaping of the decalin core (Peters, 2010). The enzymes define in particular the stereochemistry at carbons C-9 and C-10, which is preserved in the final diterpene scaffold, and may also introduce hydroxyl functional groups (Caniard et al., 2012; Peters, 2010). The diphosphate intermediates are subsequently converted in reactions catalyzed by class I diTPSs to diterpene olefins, alcohols or epoxy-labdane scaffolds (Fig. 1). While angiosperm class II and class I diTPS-functionalities reside in distinct monofunctional enzymes, bifunctional class II/I diTPSs are well known in gymnosperms and evolutionary older plant lineages (e.g. Hayashi et al., 2006; Peters et al., 2000; Vogel et al., 1996). The mechanistic understanding of class II diTPS has rapidly progressed with reported crystal-structures and detailed mutational studies of the model diTPS *ent*-copalyl diphosphate synthase AtCPS involved in general metabolism of *Arabidopsis thaliana* (Köksal et al., 2011; Köksal et al., 2014; Potter et al., 2014; Potter et al., 2016b). Illustrative examples of mutational studies on class II diTPSs of specialized metabolism are limited to the bifunctional class II/I abietadiene synthase from grand fir (*Abies grandis*) and orthologs of related gymnosperm species (Keeling et al., 2008; Wilderman and Peters, 2007; Zhou et al., 2012), the rice (*Oryza sativa*) *syn*-copalyl diphosphate synthase OsCPS4 (Potter et al., 2016a) and the peregrinol diphosphate synthase MvCPS1 of *Marrubium vulgare* (Mafu et al., 2016). In the active site of plant class II diTPSs that locates to the $\lambda\beta$ -domain intersection of a conserved $\gamma\beta\alpha$ -domain fold and contains a catalytic aspartate rich DxDD motif, three general steps take place:

(1) acid-initiated protonation at the terminal C=C bond of GGDP (Köksal et al., 2014; Prsic et al., 2007), (2) carbocation migrations with concerted 1,2-hydride and possibly methyl shifts, concomitant with cycloisomerization (e.g. Potter et al., 2016a; Potter et al., 2016b) and (3) base-catalyzed deprotonation or quenching of the carbocation by addition of water (Criswell et al., 2012). While the catalytic acid is the middle aspartic acid of the DxDD motif, the catalytic base varies with enzymatic product outcome (Zhou et al., 2012).

We recently reported that two class II diTPSs from the medicinal plant *Tripterygium wilfordii*, *TwTPS21* and *TwTPS14*, form the structurally distinct products *ent*-copal-8-ol diphosphate (**2**) and kolavenyl diphosphate (**3**), respectively (Fig. 1; Andersen-Ranberg et al., 2016; Hansen et al., 2016). To investigate in detail the structure-functional features controlling their distinct product patterns, we modeled the structures of both diTPSs based on homology to *AtCPS* and subsequently identified amino acid differences specific to the active site.

By superimposing the modeled structures we identified two residues at positions 265 and 325 that both differ between the enzymes and reside to the active site cavity. We exchanged the residues between the enzymes using site directed mutagenesis and showed that they together are responsible for the differential product profiles of *TwTPS21* and *TwTPS14*. We further show that variants of the diTPSs with single residue exchanges yielded mixed product profiles, in addition to formation of the gibberellin precursor *ent*-copalyl diphosphate (**4**) in two variants.

2. Results and discussion

TwTPS14 and *TwTPS21* share with only 13 amino acids difference over their total length of 815 amino acids a remarkable homology. Intrigued by their chemically distinct and dominating products, a hydroxylated conventional labdane-type diphosphate (**2**) for *TwTPS21* and a re-arranged (4→5, 10→9) clerodane-type diterpenoid diphosphate (**3**) for *TwTPS14* (Hansen et al., 2016), we set out to investigate the amino acid

signature directing the product specificity of the enzymes. We prioritized residues differing between *TwTPS21* and *TwTPS14* by predicted proximity to the active site, hypothesizing, that those are likely to affect the specificity of the enzymes. The protein folds were modeled by homology (Nielsen et al., 2010) with the crystal structure of *AtCPS* (PDB accession: 3PYB.B; 55% sequence identity) selected as template fold, consistent with products in stereochemical *ent*-configuration formed by *AtCPS* and *TwTPS21*. From a subsequent structure-based alignment of the models it became evident that two of the residues differentiating *TwTPS21* and *TwTPS14* lined the active site cavity at the $\lambda\beta$ -domain intersection (Fig. 2). The residues were identified as H265 and A325 in *TwTPS21* to contrast Y265 and V325 in *TwTPS14*. These residues confer a side-chain change of a positively charged 5-membered aromatic ring (His) in position 265 of *TwTPS21* to the sterically larger and more hydrophobic 6-membered phenyl-group (Tyr) in this position of *TwTPS14* in addition to an increased size of the hydrophobic residue in position 325 (Ala to Val; *TwTPS21* to *TwTPS14*). Notably, these positions partially overlap with the recently identified catalytic base coordinating dyad in *AtCPS*, H263 and N322, that binds and activates a water molecule (Potter et al., 2014). Mechanistic features of the mutant variants *AtCPS*:H263Y/F that form (-)-kolavenyl diphosphate (Potter et al., 2016b) may be shared by *TwTPS14*, while mechanisms behind formation of 8-hydroxylated-*ent*-CDP by two other mutants, *AtCPS*:H263A and *AtCPS*:N322A (Potter et al., 2014), may be shared by *TwTPS21*.

To investigate if the residue-differences, located at active-site positions 265 and 325, were responsible for product differences between *TwTPS21* and *TwTPS14*, we employed site-directed mutagenesis followed by functional testing via transient gene expression in *N. benthamiana*. Constructs were generated for all permutations resulting in six mutant variants. Transient expression in *N. benthamiana* of the mutant- and wild type enzymes was followed by hexane extraction of diterpene metabolites and profiling by GC-MS analysis (Fig. 3b). The class I diTPS *TwTPS2*, established to specifically accept the class II diTPS products copalyl diphosphate and copal-8-ol diphosphate in *ent*-configuration (Andersen-Ranberg et al., 2016), was co-expressed for

verification of these products (Fig. 1b). No plant class I diTPS is known to specifically accept kolavenyl diphosphate (**3**). Hence, this product was instead detected indirectly through kolavenol (**6**) that forms by hydrolysis of **3** by endogenous non-specific phosphatases in *N. benthamiana*, and through an additional compound (**6'**), presumably resulting from water loss from **6** (Hansen et al., 2016).

When both residues were interchanged between *TwTPS21* and *TwTPS14*, a complete product interchange resulted (Fig. 3b), i.e. *TwTPS21*:H265Y/A325V yielded kolavenol (**6**), while *TwTPS14*:Y265H/V325A in combination with *TwTPS2* specifically formed (13*R*)-*ent*-manoyl oxide (**5**). In all variants, the residue at position 325 was found to control the major product formation. Enzymes carrying V325 consistently yielded **6** and **6'** via kolavenyl diphosphate (**3**), unlike enzymes with A325 that predominantly afforded **5** via *ent*-copal-8-ol diphosphate (**2**, Fig. 1 and Fig. 3b). In the mutant enzymes with single amino acids exchanged and carrying the combination A325/Y265, traces of **6** and **6'** could be detected in addition to predominant accumulation of **5**. Assays of single mutants with V325/H265, a constellation identical with the canonical plant *ent*-copalyl diphosphate synthases such as *AtCPS* and *TwTPS3* (Fig. 3a), showed besides predominant detection of **6** and **6'** and lower levels of **5**, significant accumulation of the diterpenes *ent*-kaur-15-ene (**7**), *ent*-kaur-16-ene (**8**) and 16-hydroxy-*ent*-kaurane (**9**). Biosynthesis of these scaffolds is well established and involves the archetypical diphosphate intermediate *ent*-copalyl diphosphate (**4**) (Fig. 1 and Fig. 3b). These results suggest that A325 may permit water access to the carbocation *ent*-labda-13*E*-en-8-yl⁺ (**A**) resulting in water-quenching of **A** and formation *ent*-copal-8-ol diphosphate (**2**). In contrast, possibly due to steric hindrance of the larger V325, variants carrying this amino acid may restrict water access and thereby stabilize the carbocation in leading through **A** to **2**, or to undergo further 1,2 methyl and hydride-shifts before proton abstraction at C-3 of the resulting re-arranged clerodan-13*E*-en-4-yl⁺, leading to **3** (Fig. 1 and Fig. 3b).

Notably, compounds **6** and **6'** that imply formation of the diphosphate intermediate **3**, dominate the mixed product profile of the variants carrying H265/V325 (Fig. 3b). In previous work, exchange of the homologous

histidine of the catalytic base coordinating dyad of *AtCPS* with the larger aromatic amino acids tyrosine, or phenylalanine (*AtCPS*:H263Y/F), was required to engineer a functional kolavenyl diphosphate synthase (Potter et al., 2016b). In the present context, H265 is retained, while a threonine (T324) resides in the position of the conserved base coordinating asparagine of plant CPSs such as *AtCPS*, or *TwTPS3* (Fig. 3). Thus, guided by variation in class II diTPSs of *T. wilfordii*, we expand the residue-plasticity that allows for kolavenyl diphosphate formation at a key position to include histidine (H265).

Finally, the novel formation of *ent*-copalyl diphosphate (**4**) in two mutants with single residue changes (*TwTPS14*:Y265H and *TwTPS21*:A325V), not only highlights the evolutionary product plasticity of class II diTPSs, but may also provide further evidence for a low evolutionary distance between generalized metabolism of gibberellin phytohormones and specialized metabolism of labdane-type diterpenes.

3. Conclusions

Although studies have decoded important aspects of the structure-functional relationship of plant class II diTPSs, few enzymes have been probed by targeted mutagenesis. We compared homology models of two class II diTPSs of *T. wilfordii* (the *ent*-copal-8-ol diphosphate synthase, *TwTPS21*, and the kolavenyl diphosphate synthase, *TwTPS14*) to identify differences at the active sites. By mutational exchange and heterologous expression two residues were confirmed as determinants for the distinct product specificities of *TwTPS21* and *TwTPS14*. The residue-positions corresponded to the identical and directly adjacent positions of the conserved base coordinating dyad of *ent*-copalyl diphosphate synthases (CPSs) of gibberellic acid metabolism in higher plants, mechanistically well understood. We suggest accordingly, that the observed product differences between *TwTPS21* and *TwTPS14*, and the panel of variants engineered, are facilitated via distinctive reaction-

terminations. Specifically, water access to the carbocation intermediate appears to be controlled by subtle size differences of the aliphatic side-chain at position 325.

Kolavenyl diphosphate formation was recently demonstrated for mutant variants of AtCPS via specific exchanges of the histidine part of the base coordinating dyad for larger, hydrophobic aromatic residues (Phe and Tyr). Our results showed that histidine at this position allowed for kolavenyl diphosphate formation, in context of the two mutants, *TwTPS21:H265/A325V* and *TwTPS14:Y265H*. This knowledge may prove useful for future identification and engineering of plant diTPS forming re-arranged clerodane-type scaffolds.

4. Experimental

4.1. General experimental procedures.

GC-MS analyses were performed on a Shimadzu GCMS-QP2010 Ultra with a Agilent HP-5MS column (20m x 0.18mm i.d., 0.18µm film thickness). The injection volume was 1 µl at 250 °C (splitless mode) and the GC program was as follows: 60°C for 1min, ramp at rate 10°C min⁻¹ to 180°C, ramp at rate 5°C min⁻¹ to 220°C, ramp at rate 30°C min⁻¹ to 310°C, hold for 3min.

4.2 Protein homology modeling

TwTPS21 and TwTPS14 was homology modeled using the CPHmodels 3.2 Server Nielsen et al., 2010 with default settings (automatic template selection; Arabidopsis *ent*-copalyl diphosphate synthase, PDB accession 3PYBb). For graphical representation of the models, PyMOL was used (Schrodinger, 2015).

4.3 Site directed mutagenesis

Mutants of *TwTPS14* and *TwTPS21* were cloned via USER-cloning (Geu-Flores et al., 2007) into the binary vector, pCAMBIA130035Su, for heterologous expression, as previously described for native enzymes (Andersen-Ranberg et al., 2016). Desired nucleotide changes were introduced with primers designed using the online tool PHUSER (Olsen et al., 2011). Primers are listed in Supplementary Table 1 with introduced mutations underlined and deoxyuracil indicated as U.

4.4 Heterologous expression and diterpenoid extraction

WT and mutants of *TwTPS14* and *TwTPS21* were transiently expressed in *N. benthamiana* according to a previously established protocol (Bach et al., 2014). The following enzymes were co-expressed to increase expression level and elevate the GGDP pool: the p19 suppressor of gene silencing (Baulcombe and Molnar, 2004) and *C. forskohlii* 1-deoxy-d-xylulose 5-phosphate synthase (*CfDXS*) and GGDP synthase (*CfGGDPS*), as previously established (Andersen-Ranberg et al., 2016). 6-8 days post infiltration, metabolites of two leaf discs (3 cm diameter, 1 disc per leaf) were extracted in 0.8 ml n-hexane and analyzed. As controls, p19, *CfDXS* and *CfGGDPS* were co-expressed without *T. wilfordii* diTPS. Experiments were conducted in three biological replicates (three plants infiltrated and analyzed for each gene combination).

4.5 Product identification analysis by GC-MS

Hexane extracts were analyzed by GC-MS with instrument and settings described above (4.1.). Compound identification was based on matching retention time and mass spectrum to known products of *TwTPS14*, *TwTPS21* (Andersen-Ranberg et al., 2016) and the bifunctional *ent*-CPP/*ent*-kaurene synthase from *Physcomitrella patens*, *PpCPS/KS* (Hayashi et al., 2006; Zhan et al., 2015).

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Competing financial interests: Bj.H. is inventor on an international patent application (Patent Cooperation Treaty) No. PCT/DK2015/050021 covering 'Methods and materials for producing diterpenes' related to the manufacture of labdane and clerodane-type diterpenes and precursors thereof.

Figure legends:

Fig. 1. Reaction mechanisms of class II diTPSs (**a**), and labdane-type diterpenes resulting from coupled assays in *Nicotiana benthamiana* with the class I diTPS *TwTPS2* (**b**). **a**) The reaction mechanisms of *TwTPS21*, *TwTPS14* and of general metabolism diTPSs such as *TwTPS3* and *AtCPS*. **b**) Conversions of **2**, **3** and **4** in the heterologous (*N. benthamiana*) co-expression system. Class II diTPS products were indirectly confirmed in coupled assays with the class I diTPS *TwTPS2*. Activity of *TwTPS14* was detected as the product of dephosphorylation of **3** by endogenous phosphatase activity of *N. benthamiana*. The characteristic decalin core of labdane-type diterpenes is shown in bold in **5**.

ent-labda-13E-en-8-yl⁺ (**A**); labda-13E-en-8-yl⁺ (**B**), unknown stereoisform; geranylgeranyl diphosphate (**1**); *ent*-copal-8-ol diphosphate (**2**); kolavenyl diphosphate (**3**); *ent*-copalyl diphosphate (**4**); (13*R*)-*ent*-manoyl oxide (**5**); kolavenol (**6**); *ent*-kaur-15-ene (**7**); *ent*-kaur-16-ene (**8**); 16-hydroxy-*ent*-kaurane (**9**)

Fig. 2. Two residues at the active site cavity differ between *TwTPS21* and *TwTPS14*. Cartoon representation of homology models of *TwTPS21* and *TwTPS14* focused on the residues of the active site cavity that differ between the enzymes. Modeled with CPHmodels 3.2. Visualized in PyMOL: gamma-domain in forest green; beta-domain in hot pink; showing side chains of residues 265 and 325 colored according to atoms (carbon = green; nitrogen = blue; oxygen = red).

Fig. 3. Sequence alignment around positions 265 and 325 of *TwTPS14/21* (**a**) and GC-MS analysis of coupled assays with *TwTPS14/21* wild types and mutants (**b**). **a**) Aligned sequence windows (separated by dots) around residue 265 and 325 of *TwTPS14/21*. The starting residue of the first window and the last residue of the second window are numbered. Identical residues to the top sequence are represented by dashes. **b**) GC-MS Total Ion Chromatograms (TICs) of leaf diterpene extracts from heterologous co-expression assays in *Nicotiana benthamiana*. The residue identity at positions 265 and 325 are indicated as well as major peaks and an internal standard (IS).

13*R*-*ent*-manoyl oxide (**5**); kolavenol (**6**); putative water loss derivative of **6** (**6'**); *ent*-kaur-15-ene (**7**); *ent*-kaur-16-ene (**8**); 16-hydroxy-*ent*-kaurane (**9**); unknown product of putative oxidation of **5** in *N. benthamiana* (*)

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Figure

a

b

TwTPS21

TwTPS14

		265		325	
<i>TwTPS14</i>	263	LLYSL	...	VPTVYP	327
<i>TwTPS21</i>	263	--H--	...	---A--	327
<i>TwTPS3</i>	266	--H--	...	--N--	330
<i>AtCPS</i>	261	--H--	...	--N-F-	325

Two active site located residues functionally differentiates a pair of class II diterpene synthases of *Tripterygium wilfordii*
***Graphical Abstract**