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Valencene synthase from the heartwood of Nootka cypress (*Callitropsis nootkatensis*) for biotechnological production of valencene

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Summary

Nootkatone is one of the major terpenes in the heartwood of the Nootka cypress *Callitropsis nootkatensis*. It is an oxidized sesquiterpene, which has been postulated to be derived from valencene. Both valencene and nootkatone are used for flavouring citrus beverages and are considered among the most valuable terpenes used at commercial scale. Functional evaluation of putative terpene synthase genes sourced by large-scale EST sequencing from Nootka cypress wood revealed a valencene synthase gene (*CnVS*). *CnVS* expression in different tissues from the tree correlates well with nootkatone content, suggesting that *CnVS* represents the first dedicated gene in the nootkatone biosynthetic pathway in *C. nootkatensis* The gene belongs to the gymnosperm-specific TPS-d subfamily of terpenes synthases and its protein sequence has low similarity to known citrus valencene synthases. *In vitro*, CnVS displays high robustness under different pH and temperature regimes, potentially beneficial properties for application in different host and physiological conditions. Biotechnological production of sesquiterpenes has been shown to be feasible, but productivity of microbial strains expressing valencene synthase from Citrus is low, indicating that optimization of valencene synthase activity is needed. Indeed, expression of *CnVS* in *Saccharomyces cerevisiae* indicated potential for higher yields. In an optimized *Rhodobacter sphaeroides* strain, expression of CnVS increased valencene yields 14-fold to 352 mg/L, bringing production to levels with industrial potential.

Introduction

The Nootka cypress tree *Callitropsis nootkatensis* (also known as the Alaska yellow cedar) is known to produce several terpenes, including the monoterpene carvacrol and significant amounts of nootkatone (Figure 1a) (Erdtman and Hirose, 1962). Nootka cypress is native to the west coast of North America and is used in temperate regions of Europe as an ornamental conifer in gardens. The tree is known for its highly decay-resistant wood, which makes it suitable as timber, for example boat decks and Japanese temples (Kelsey *et al.*, 2005). The heartwood oil and several of its components have high fungicidal activity on notorious forest pests, such as *Phytophthora ramorum* and *P. lateralis* (Manter *et al.*, 2006). Nootkatone has been reported to be active against insects: it has significant repellent and toxic effects to Formosan subterranean termites (Zhu *et al.*, 2010) and also has repellent activities on ticks (Dietrich *et al.*, 2006).

Nootkatone is an oxidized sesquiterpene, which is obtained by the oxidation of (+) valencene (Figure 1a; Fraatz *et al.*, 2009). Valencene is present in various citrus species, for example in the essential oil of Valencia orange (*Citrus x sinensis*) (Hunter and Brogden, 1965a,b). Valencene imparts a woody citrus characteristic and is economically important because it gives a juicy impression in citrus flavourings and fragrances. Currently, the market volume for valencene is about 10 000 kg per year. Another 5000 kg of valencene per year is used for the production of nootkatone. Nootkatone is used to provide a grapefruit-like flavour to soft drinks.

Apart from Nootka cypress, nootkatone is found in low amounts in grapefruit oil (Hunter and Brogden, 1965a,b). Commercial nootkatone is currently made from valencene by oxidation, either by chemical means (Hunter and Brogden, 1965b), or enzymatically (4). Valencene is isolated from citrus oils by steam distillation. However, the valencene concentration in citrus fruits is low (0.2%–0.6% by weight), and quality, availability and price depend on seasonal influences and the harvest. Therefore, researchers have set out to produce valencene via biotechnology, similar to what has been described for production of the sesquiterpene amorphadiene, a precursor for antimalarials (Tsuruta *et al.*, 2009). Essential for such biotechnological approaches is the availability of a gene encoding an effective valencene synthase that performs well under industrial conditions. Several valencene synthases have been described, for

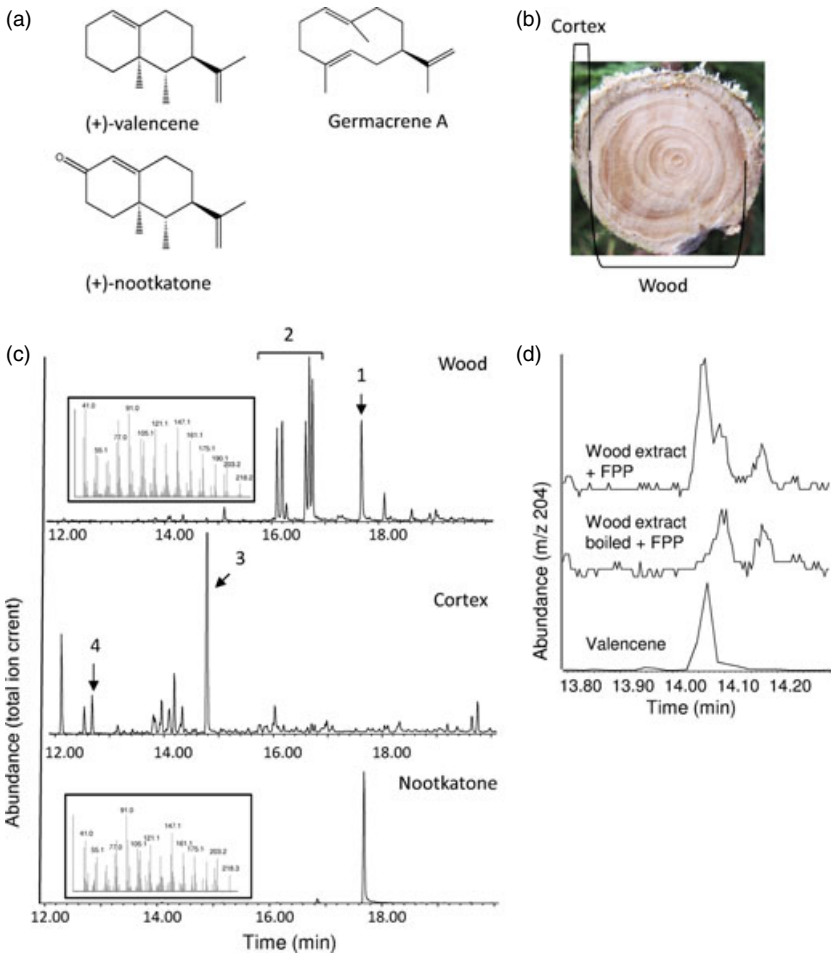

Figure 1 Chemical and enzymatic analysis of *Callitropsis nootkatensis*. (a) Chemical structures of valencene, germacrene A and nootkatone. (b) Cross section of *C. nootkatensis* trunk indicating wood and cortex. (c) Organic extracts of wood and cortex from *C. nootkatensis*, analysed on GC/MS. Peak identity: 1: nootkatone; 2: di-isopropyl naphtalenes; 3: nerolidol; 4: α-cubebene. (d) Terpene synthase activity of crude extracts of *C. nootkatensis* wood using active (top) or inactivated (middle) enzyme extract. Product formation was analysed by GC/MS. Shown is the chromatographic trace of the 204 m/z ion (R.U. = response units). The bottom trace shows chromatography of valencene. GC/MS chromatograms have identical intensity ranges.

example from *Citrus x sinensis*, *Citrus x paradisi* and *Vitis vinifera* (Lücker *et al.*, 2004; Schalk and Clark, 2008; Sharon-Asa *et al.*, 2003). These enzymes perform relatively inefficient in micro-organisms, as compared to other sesquiterpene synthases. In fermentation experiments with yeast optimized for substrate levels, CitrusVS was 3-5 times less efficient in producing sesquiterpene than other synthases such as those for patchoulol, premnaspirodiene and 5-epi-aristolochene and optimized fermentations reached maximal levels of 20 mg/L valencene (Asadollahi *et al.*, 2008; Takahashi *et al.*, 2007). In addition, valencene synthases display limited product specificity and synthesize significant amounts of side products such as germacrene A and (−)-7-epi-α-selinene (Chappell and Greenhagen, 2008; Lücker *et al.*, 2004).

In this work, the pathway towards nootkatone in Nootka cypress was studied. Valencene synthesis by Nootka cypress heart wood tissue was demonstrated. A valencene synthase gene encoding the first dedicated enzyme towards nootkatone was isolated by a large-scale sequence analysis strategy. The enzyme was characterized by its product specificity and its behaviour under different pH and temperature regimes. Its potential for biotechnological production of (+)-valencene was demonstrated by introducing it in the industrial bacterium *Rhodobacter sphaeroides*.

Results

Callitropsis nootkatensis wood contains nootkatone and valencene synthase activity

A stem section of *C. nootkatensis* was separated into a wood and a cortex part (Figure 1b). Both parts were extracted with dichloromethane, and extracts were analysed by GC/MS (Figure 1c). The cortex part showed many monoterpenes and sesquiterpenes, such as terpinolene, nerolidol (Figure 1c, peak 3) and α-cubebene (Figure 1c, peak 4). No valencene or nootkatone could be observed. In contrast, analysis of wood showed hardly any monoterpenes and very few sesquiterpenes. A clear peak (Figure 1c, peak 1) could be identified as nootkatone, by comparison of the mass spectrum and retention time to an authentic standard, while no valencene was detected. The other dominant peaks in the sesquiterpene area of the chromatogram were identified as di-isopropyl naphtalenes (Figure 1c, peak 2).

To investigate whether the identified nootkatone indeed originates from valencene synthesis in wood tissue, the presence

of sesquiterpene synthase activity was tested. An enzyme extract of wood was prepared and incubated with farnesyl diphosphate (FPP). After incubation, a low but clear peak could be detected at the retention time of valencene, while no other sesquiterpenes could be detected. The valencene peak was not detected when crude extracts had been cooked before incubation, or when FPP had been omitted from the reaction (Figure 1d). Also, no valencene synthesis could be observed using extracts from *C. nootkatensis* cortex. Thus, it was concluded that valencene synthase activity is present in *C. nootkatensis* wood, although activity was very low (0.4 nmol/min/g).

Isolation and functional characterization of *C. nootkatensis* valencene synthase

Following a large-scale cDNA-sequencing strategy combined with RACE-PCR, a candidate valencene synthase cDNA was identified using BLAST analysis based on its homology with known conifer terpene synthases. The open-reading frame of this cDNA was expressed in *E. coli,* and recombinant protein was tested for sesquiterpene synthase activity *in vitro*. By comparison with original standards, valencene was found to be the dominant product, while also germacrene A was detected (Figure 2). A clone expressing the valencene synthase from *Citrus x paradisi* (*CitrusVS*) was tested in parallel and showed a similar product spectrum (Figure 2). Thus, the cDNA from Nootka cypress was found to encode a valencene synthase, which is further referred to as *CnVS*. The gene was deposited in GenBank under accession number JX040471.

CnVS encodes a protein of 589 amino acids. The protein does not contain a predicted N-terminal targeting sequence, and therefore, it is putatively localized in the cytosol, as expected for a sesquiterpene synthase. *CnVS* displays low-protein sequence similarity with other known sesquiterpene synthases. The closest homologue of *CnVS* in the GenBank nonredundant protein database is the longifolene synthase from the Norway spruce *Picea abies* (Martin *et al.*, 2004), with 44% sequence identity. The CitrusVS is even more distantly related to the CnVS, with only 32% identity on the amino acid level (Figure 3a). An alignment of protein sequences from both *CnVS* and *CitrusVS* to representatives of the terpene synthase family showed that *CnVS* belongs to the gymnosperm-specific subfamily TPS-d (Figure 3b), while *CitrusVS* belongs to dicot sesquiterpene synthase subfamily TPS-a1 (Bohlmann *et al.*, 1998; Chen *et al.*, 2011). Both *Citrus VS* and *CnVS* have a βα didomain structural organization with the N-terminus folding back to form part of the catalytic C-terminal α domain (Gao *et al.*, 2012).

Gene expression analysis of CnVS in *C. nootkatensis*

The expression of *CnVS* in different tissues of *C. nootkatensis* was assessed (Table 1). Samples were collected from a 6-year-old tree (Figure S1), and quantitative RT-PCR was performed on total RNA. In parallel, GC/MS analysis was performed on ethylacetate extracts from the same tissue samples. In tissues where nootkatone was detected, gene expression of *CnVS* was highest, indicating a relation between expression of *CnVS* and nootkatone formation in *C. nootkatensis.*

CnVS is a robust enzyme

Kinetic constants for both CnVS and CitrusVS were evaluated. Km values at pH = 7.0 and 30 °C for both enzymes were comparable (Table 2), and also Kcat and Kcat/Km values differed less than twofold.

The tolerance of CnVS to different conditions was evaluated *in vitro*. CnVS and CitrusVS were compared for the effect of buffer pH on the synthesis of valencene and germacrene A (Figure 4a). For CitrusVS, synthesis of valencene had a rather sharp maximum at pH 7. At more alkaline pH conditions, the valencene production quickly diminished, and the production of germacrene A increased with a maximum production of this side product between pH 8 and pH 9. This is in agreement with observations reported before (Chappell and Greenhagen, 2008). In contrast to the CitrusVS, the CnVS enzyme showed a stable and high valencene production over a broad pH range. For both enzymes, the optimal ratio between valencene and germacrene A production was at pH 7, where germacrene A accounted for approximately 6% of the total sesquiterpenes produced. With increasing pH, the amount of side-product germacrene A also increased for CnVS, but the valencene to germacrene A ratio remains favourable compared with the CitrusVS.

CnVS was characterized for its temperature optimum (Figure 4b). The optimal activity was observed at 40 °C, while at 4 °C and at 52 °C, significant valencene production could still be

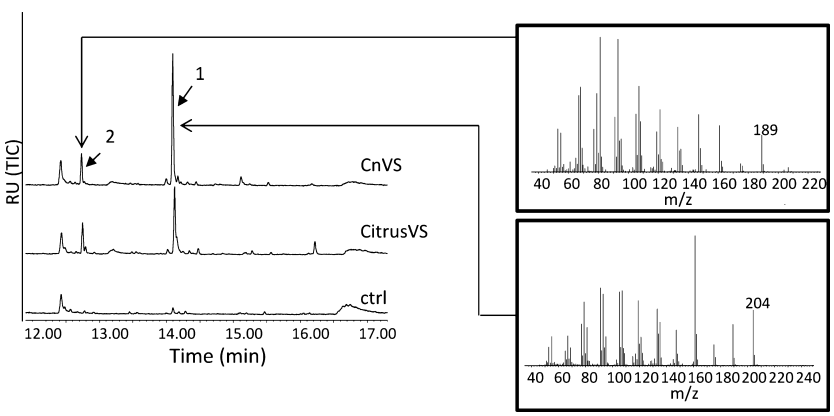

Figure 2 Activity of recombinant valencene synthases *in vitro*. Protein extracts from *E. coli* cultures expressing *CnVS* (top), *CitrusVS* (middle) or control (bottom) were incubated with FPP as substrate. Shown are analysis of products in GC/MS chromatograms (total ions). GC/MS chromatograms have identical intensity ranges. Boxes show mass spectra of β-elemene, the cope rearrangement product of germacrene A (peak 2: top spectrum) and valencene (peak 1: bottom spectrum) peaks.

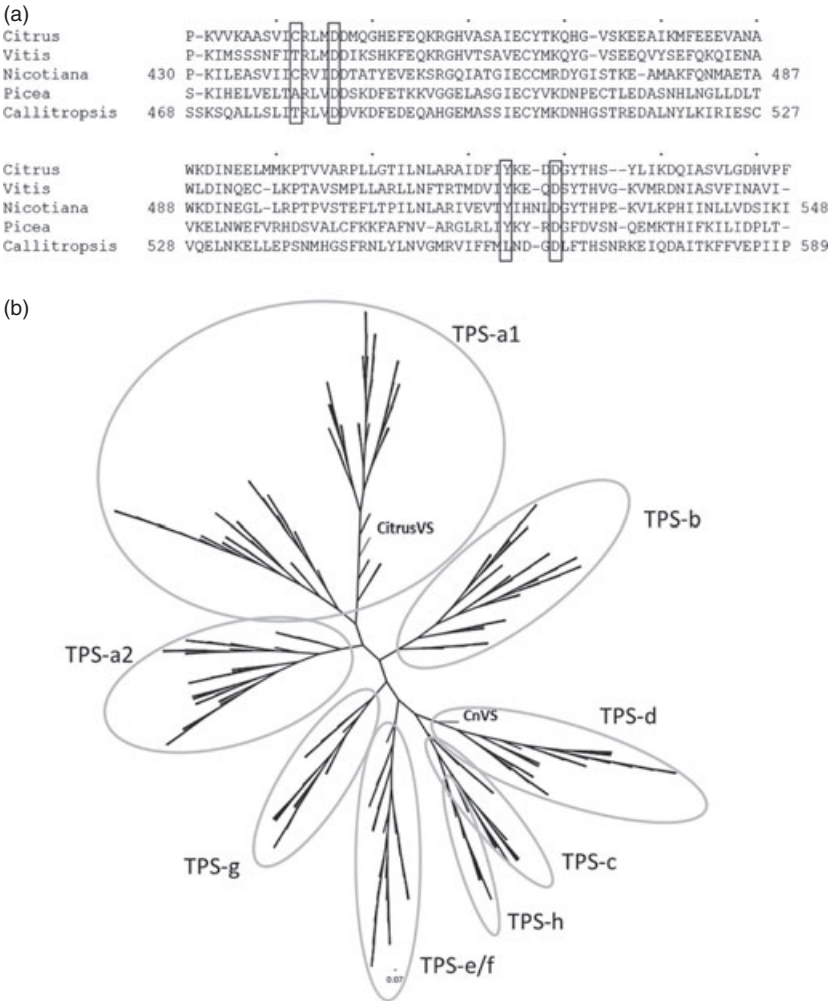

Figure 3 Analysis of the CnVS protein sequence. (a) Representative part of the alignment between CnVS and other sesquiterpene synthases. Shown is an alignment of protein sequences of valencene synthase from *Citrus x paradisi* (genbank accession CAG29905), valencene synthase from *Vitis vinifera* (ACO36239), epi-aristiolochene synthase from *Nicotiana tabacum* (Q40577), longifolene synthase from *Picea abies* (AAS47695) and CnVS. The residues discussed in the text as important for the second cyclization reaction are marked with boxes. (b) Unrooted phylogenetic tree of terpene synthase proteins. Sequences included are from Chen *et al.* (2011) with the addition of CitrusVS and CnVS.

Table 1 Expression of *CnVS* in *Callitropsis nootkatensis* tissue measured by quantitative RT-PCR

Tissue	*CnVS* expression*	Nootkatone (μg/g tissue)
Wood bottom stem	6.72 ± 0.97	1.1 ± 0.1
Wood top stem	4.29 ± 0.60	1.6 ± 0.2
Root	n.d.†	n.d.
Leaf	0.062 ± 0.006	n.d.
Epidermis bottom stem	1.34 ± 0.26	n.d.
Cortex bottom stem	n.d.	n.d.
Cortex top stem	0.065 ± 0.006	n.d.
Cone	0.41 ± 0.02	n.d.

*Expression is listed as expression relative to actin and is multiplied by 1000. Standard deviations apply to triplicate measurements. †n.d., not detected. Tissues are shown in supplemental material.

observed. In contrast, the activity of the citrus enzyme at the latter temperatures was hardly detectable. The germacrene A production by both CnVS and CitrusVS was highest at 45 °C. For CnVS, germacrene A did not exceed 25% of total terpene production, while CitrusVS enzyme produced 55% of total terpene as germacrene A at 45 °C.

Table 2 Kinetic constants of CnVS and Citrus VS. Kinetic constants were determined in triplicate; variation is shown as standard deviation

	Km (μM)	Kcat (/s)	Kcat/Km (M/s)
CnVS	1.04 ± 0.36	0.0032 ± 0.0012	$3.4 \times 10^3 \pm 1.0 \times 10^3$
Citrus VS	0.52 ± 0.10	0.0025 ± 0.0008	$5.0 \times 10^3 \pm 2.7 \times 10^3$

In vivo production of valencene in microbial hosts

To evaluate whether the versatility observed *in vitro* is reflected *in vivo*, the performance of the enzyme was tested in different prokaryotic and eukaryotic hosts. For the expression of CnVS and CitrusVS in *Saccharomyces cerevisiae*, the open-reading frames were recloned in yeast vector pYES3, under control of the GAL1 promoter. The constructs were used for expression in the yeast strain WAT11 (a strain not optimized for sesquiterpene production), in a shaker culture overlaid with n-dodecane. After 3 days of fermentation, the n-dodecane layers were analysed by gas chromatography. Valencene production by CnVS was at 1.36 ± 0.05 mg/L yeast culture (Table 3). The yeast harbouring CitrusVS produced only trace amounts of valencene and valencene was absent from a WAT11 culture harbouring an empty plasmid. Thus, CnVS was found to strongly improve production of valencene in a nonoptimized yeast strain.

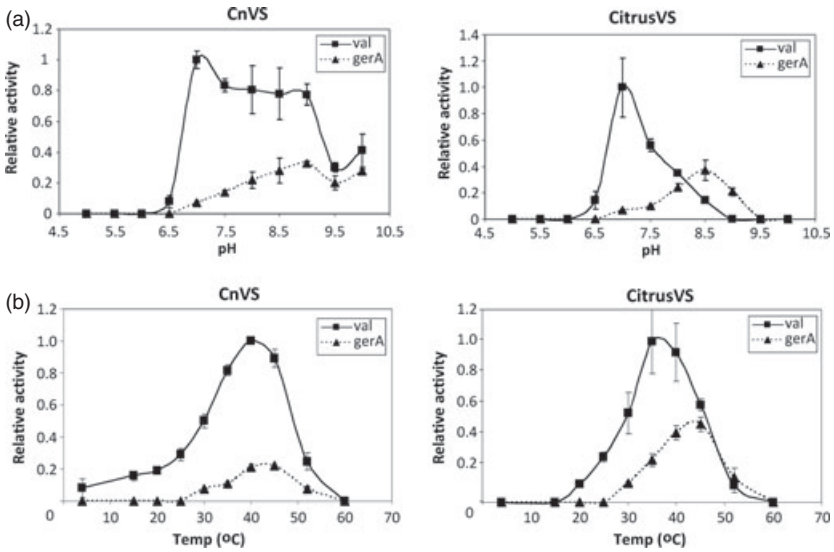

Figure 4 The dependence of activity of CnVS (left) and CitrusVS (right) enzymes on pH (a) and temperature (b). Formation of valencene (solid lines) and germacrene A (dashed lines) was tested *in vitro*. Error bars indicate the standard deviation among three independent measurements.

Table 3 Valencene production in mg/L culture in shaker flask cultures of different constructs in yeast (*Saccharomyces cerevisiae*) and bacterial (*Rhodobacter sphaeroides*) cultures

Construct + host	CnVS (mg/L culture)	CitrusVS (mg/L culture)
pYES + *S. cerevisiae*	1.36 ± 0.05	0.0021 ± 0.0002
pBBR + *R. sphaeroides*	57.5 ± 3.5	2.5 ± 0.1
pBBR-MEV + *R. sphaeroides*	352 ± 37	24.9 ± 1.3

To test the performance of *CnVS* in a prokaryote, the bacterium *R. sphaeroides* was used to produce valencene using the *CnVS* and *CitrusVS genes*. Both *CnVS* and *CitrusVS* were codon optimized for expression in *R. sphaeroides* (Figure S2) and placed in frame with an N-terminal MBP coding sequence, to minimize differences in translation initiation. Constructs were introduced in *R. sphaeroides*, and valencene production was monitored in 25 mL cultures in shaker flasks, with an overlay of n-dodecane. After 3 days of fermentation, the valencene present in the dodecane layer was quantified by GC/MS. The *CnVS* gene afforded >20-times higher productivity compared with the *CitrusVS* (Table 3). Most relevantly, when concentrations of CnVS and CitrusVS proteins were tested by Western blot, we observed far more soluble and insoluble synthase in CnVS cells than in CitrusVS cells (Figure S3). To further improve yields, plasmids were constructed in which the mevalonate operon from *Paracoccus zeaxanthinifaciens* is co-expressed with the valencene synthases to increase the availability of FPP substrate (Hümbelin *et al.*, 2002). Expression of CnVS in this engineered *R. sphaeroides* strain in shaker flasks resulted again in a strongly (six-fold) improved valencene production (Table 3). The final titre reached using *CnVS* in the engineered strain, even in a low-cell density culturing system, was 352 mg valencene per litre culture broth.

Discussion

Valencene distilled from citrus peel is an economically important sesquiterpene giving a juicy impression in food flavourings and fragrances. In this paper, we describe a novel valence synthase from the Nootka cypress with high biotechnological potential. The enzyme appears more robust than the valencene synthase from citrus itself. Our results show that CnVS performs well in both an eukaryotic (*Saccharomyces*) and a prokaryotic (*Rhodobacter*) micro-organism. *Rhodobacter* is a purple Gram-negative bacterium, which is used for the extraction of carotenoids for applications in food (Gu *et al.*, 2008). The valencene yield of CitrusVS in a nonoptimized yeast is hardly detectable, whereas in the same set-up, CnVS reaches titres of 1.36 mg/L (Table 3). CnVS is also more (14-fold compared with CitrusVS) productive in *R. sphaeroides,* leading to 352 mg/L under nonoptimized fermentation conditions, showing its potential for biotechnological production of valencene for food applications.

Others have observed a low yield of valencene from yeast cultures expressing Citrus VS, in comparison with other sesquiterpenes (Asadollahi *et al.*, 2008; Takahashi *et al.*, 2007). Also, we observed this for Saccharomyces and now also for Rhodobacter. In contrast, the CnVS performs much better in both systems. This can hardly be attributed to differences in the kinetic properties of CitrusVS and CnVS under optimal conditions, given that these values do not differ more than a factor 2 (Table 2). When tested at suboptimal pH and temperature *in vitro*, CnVS displays a higher robustness and produces much more valencene (Figure 4). Notably, the concentration of synthase protein in *Rhodobacter* cultures for valencene production is considerably higher for CnVS than for CitrusVS (Figure S3), in spite of the fact that both genes were codon optimized and expressed as a translational fusion to MBP. Thus, higher productivity of CnVS in microbial systems, in comparison with Citrus VS, may result from higher enzyme accumulation and/or robustness.

The monocyclic Germacrene A is known as an intermediate in the biosynthesis of many bicyclic sesquiterpenes, including valencene (Chappell, 2004). Its presence as a side product indicates that the reaction mechanisms deployed by CnVS and CitrusVS are similar. Yet CnVS differs from CitrusVS in a number of conserved residues in the active site (Figure 3a). Residues implicated in the formation of bicyclic sesquiterpenes, such as valencene, have been postulated based on the crystal structure of the *Nicotiana* aristolochene synthase (TEAS) and comprise Tyrosine 520, Aspartic acid 525, Cysteine 440 and Aspartic acid 444

(TEAS-Nicotiana numbering; Starks *et al.*, 1997). Several of these residues have not been conserved in CnVS (Figure 3a). An extensive mutational analysis would be required to address the role of these and other residues lining the active site in the observed robustness of CnVS.

The observed accumulation of terpenes in different tissues in *C. nootkatensis* is noteworthy. The cortex, or sapwood, from trees is known to produce resins (Figure 1b), and clearly, the majority of terpenes found in Nootka cypress occur in sapwood (Figure 1C). However, nootkatone was not found in the cortex, but instead, it is the dominant terpene present in heartwood. Nootkatone is the oxidation product of valencene, and indeed, *CnVS* mRNA (Table 1) is predominantly detected in heartwood and not in the other tissues. This indicates that conversion of FPP to valencene by CnVS represents the first dedicated step in the biosynthesis of nootkatone in *C. nootkatensis*. Heartwood, from which *CnVS* was sourced, is known for having an extremely low number of living cells, which often represent radial medullar meristems (Fink, 1982). Microscopic studies including localization of expression on the subtissue level should be performed to investigate the exact site of nootkatone biosynthesis in heartwood tissue.

The biosynthesis of nootkatone from valencene should further comprise an oxidative step. In a previous study, a cytochrome P450 enzyme from chicory was described which mediated the oxidation of valencene into nootkatol (Cankar *et al.*, 2011). As chicory is not known to contain any nootkatone or valencene, the activity of this P450 on valencene likely relates to its broad substrate specificity. Based on this finding, one may expect a similar cytochrome P450 enzyme to occur in Nootka cypress. No valencene was observed in heartwood, indicating that it is quantitatively oxidized to nootkatone.

The biosynthetic pathway to nootkatone could be of interest for the engineering of insect and/or fungal resistance in plants. Repelling effects on insects and inhibition of sporulation and growth of fungi have been reported for nootkatone (Jordan *et al.*, 2012; Manter *et al.*, 2006, 2007). Likely, these properties contribute to the high persistency of Nootka cypress heartwood itself (Kelsey *et al.*, 2005). Metabolic engineering of production of oxidized sesquiterpenes in plants leads to levels in the 1–100 μg/g range (van Herpen *et al.*, 2010; Wu *et al.*, 2006). The efficient CnVS may be useful as a tool to produce nootkatone and thereby engineer insect and fungal resistance in plants.

During the last decade, microbial platforms for industrial production of plant terpenoids have been developed. The biotechnological production of artemisinin precursors in yeast and *E. coli* represents as an important milestone (Tsuruta *et al.*, 2009; Westfall *et al.*, 2012). With these technologies, it seems feasible to harvest the biochemical diversity that is offered by the plant kingdom: even terpenes that only occur in low amounts in plants can be produced at commercial levels. In this paper, we demonstrate that the success of this approach can depend on the availability of robust terpene synthases that perform well in the heterologous host of choice. Tapping from the diversity in the plant kingdom can deliver the required sequence space of synthases from which such robust enzymes can be selected.

Experimental procedures

Analysis of plant material

Callitropsis nootkatensis pendula was purchased from Plantentuin Esveld in Boskoop (NL). For dichloromethane (DCM) extraction, 0.5 g of plant material was weighed in a precooled glass tube, and 4 mL of dichloromethane was added. The suspension was vortexed for 1 min, sonicated for 5 min in an ultrasonic bath and centrifuged for 5 min at 1500 ***g*** at room temperature. The supernatant was collected, filtered over a column of 1 g sodium sulphate and diluted four-fold in DCM before analysis on a GC/MS. About 2 μL was analysed by GC/MS using a gas chromatograph as described in detail by Cankar *et al.* (2011). Valencene and nootkatone were identified by the comparison of retention time and mass spectrum to authentic (+)-valencene and nootkatone standards (both from Fluka).

Enzyme extraction

One gram of frozen powder was homogenized in 10 mL ice-cold buffer A (25 mM MOPSO (pH 6.8), 20% (v/v) glycerol, 25 mM sodium ascorbate, 25 mM sodium meta-bisulphite, 10 mM $MgCl_2$, 5 mM DTT) and polymerized polyvinylpyrrolidone (PVPP; 0.1 g/g tissue) and Amberlite XAD-4 (0.5 g/g tissue) was added. The mix was stirred gently for 10 min at 4 °C. The homogenate was filtered through cheese cloth, and the filtrate centrifuged at 20 000 ***g*** for 20 min at 4 °C, and the supernatant again centrifuged at 100 000 ***g*** for 90 min at 4 °C. The supernatant was changed from buffer A to MOPSO buffer [15 mM MOPSO (pH 7.0), 10% (v/v) glycerol, 10 mM $MgCl_2$, 1 mM sodium ascorbate, 2 mM DTT, 5 mM sodium orthovanadate] over a PD-10 column (GE Healthcare; Bio-Sciences, Diegem, Belgium) and immediately used for an enzyme assay.

To assay valencene synthase activity, 20–50 μM farnesyl diphosphate (FPP) was added to 0.5–1 mL of the enzyme preparation in a 10-mL glass tube. The reaction mix was carefully overlayed with 1 mL of pentane and incubated at 30 °C for 60 min under gentle agitation. Control samples of the enzymatic preparation had been boiled for 5 min to inactivate the enzymes. After incubation, the reaction tube was vortexed well and centrifuged (5 min 1100 ***g***) to separate the phases. The pentane phase was collected, and the water phase was washed with another mL of pentane. The pentane phases were pooled and dehydrated over a sodium sulphate (anhydrous) column and analysed by GC/MS, as described previously, but the machine was programmed in the selective-ion mode (SIM), where only ions m/z 161 and 204 were detected. Valencene was detected at 14.070 min.

RNA isolation and sequence analysis

About 15 mL extraction buffer (2% hexadecyl-trimethylammonium bromide, 2% polyvinylpyrrolidinone K 30, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaC1, 0.5 g/L spermidine and 2% ß-mercaptoethanol) was warmed to 65 °C, after which 3 g ground tissue was added and mixed. The mixture was extracted two times with an equal volume of chloroform: isoamylalcohol (1 : 24), and one-fourth volume of 10 M LiC1 was added to the supernatant and mixed. The RNA was precipitated overnight at 4 °C and harvested by centrifugation at 10 000 ***g*** for 20 min. The pellet was dissolved in 500 μL of SSTE [1.0 M NaCl, 0.5% SDS, 10 mM Tris-HC1 (pH 8.0), 1 mM EDTA (pH 8.0)] and extracted once with an equal volume of chloroform: isoamylalcohol. Two volumes of ethanol were added to the supernatant, incubated for at least 2 h at −20 °C, centrifuged at 13 000 ***g*** and the supernatant removed. The pellet was air-dried and resuspended in water. Total RNA (60 μg) was shipped to Vertis Biotechnology AG (Freising, Germany). PolyA+ RNA was isolated, random primed

(RDP) cDNA synthesized using a randomized N6 adapter primer and M-MLV H-reverse transcriptase. The RDP cDNAs carry attached to their 5′ and 3′ ends the adaptor sequences A and B as specified by 454 Life Sciences/Roche and are ready for 454 sequencing. The material was subsequently analysed on a Roche GS FLX Sequencing device, using a long run. Overlapping sequences were assembled into 'contigs', using Newbler software (Roche). This resulted in 34 743 contigs. Contigs were between 120 and 1600 bp long.

Extending candidate gene fragments to full length

To extend the cDNA fragments to full-length cDNA sequences, the SMART RACE cDNA Amplification Kit from Clontech was used. Firstly, from 133 μg total RNA, 2.7 μg polyA+ RNA was isolated by using the mRNA Purification Kit (GE Healthcare; Bio-Sciences). This polyA+ RNA was used to generate 3′RACE and 5′RACE cDNA according to the Kit's descriptions. RACE amplifications were executed using the oligonucleotides CAGTGTAGTGCATTATCAGTGCGAGTG (for 3′RACE) and TCCAGTCCGACGAAGGGCGTCCTTCA (for 5′RACE), and using primers supplied with the kit. RACE products were cloned into vector pGEMT-easy (Promega, Leiden, the Netherlands). The sequence of two of the inserts per RACE fragment was analysed. Sequence information was each time assembled with all RACE sequences and all terpene synthase contigs, using the Seqman II program (DNAstar Inc., Madison, WI).

Phylogenetic analysis of terpene synthases

To determine the TPS family for CnVS a phylogenetic tree was constructed using sequences of CnVS, CitrusVS and TPS sequences analysed in a recent review on terpene synthases families in plants published by Chen *et al.* (2011). This included putative full-length TPS of five sequenced angiosperm genomes (*Vitis vinifera*, *Populus trichocarpa*, *Arabidopsis thaliana*, *Oryza sativa*, *Sorghum bicolor*), the lycophyte *Selaginella moellendorffii* and representative TPS from seven gymnosperm species (full list is given in Table S1 of Chen *et al.*, 2011). Multiple protein sequence alignments were performed using the ClustalW algorithm using ClustalX 2.1 software (Larkin *et al.*, 2007). Bootstrap N-J trees were generated by the ClustalX 2.1 software with a 1000 replicates of bootstrap analysis and exported as a Phyllip format tree. The phylogeny was visualized using the Figtree v1.3.1. software.

Recombinant enzyme expression

Oligonucleotides were designed to re-amplify a putative sesquiterpene synthase from the original cDNA library using Phusion polymerase, and oligonucleotides ATATAGGATCCGGC TGAAATGTTTAATGGAAATTCCAGC and ATATACTGCAGCTCTG GATCTATGGAATGATTGGTTCCAC. Restriction sites for BamHI and PstI were included in the oligonucleotides and were used for cloning of the fragment in pACYCDUET-1 (Novagen, Merck Chemicals B.V., Amsterdam, the Netherlands). Plasmids were tested by restriction analysis and the inserts of the vectors were checked by DETT sequencing with vector primers.

pACYC DUET1 constructs were transformed to *E. coli* BL21 AI (Invitrogen, Bleiswijk, the Netherlands). For expression, a 1 mL overnight culture of the recombinant *E. coli* strains was prepared, and 500 μL of that culture was transferred to 50 mL of LB medium with 30 mg/L chloramphenicol in a 250-mL Erlenmeyer flask and incubated at 37 °C, 250 rpm until the A600 was 0.4–0.6. Subsequently, 0.02% arabinose was added, and cultures were incubated overnight at 18 °C and 250 rpm. The next day, cells were harvested by centrifugation, medium was removed, and cells were resuspended in 1 mL Resuspension buffer (50 mM Tris-HCl pH = 8.0, 300 mM NaCl, 1.4 mM 2-mercaptoethanol; 4 °C). Cells were disrupted by sonication (on ice, five times 10 s with 10 s break, MSE Soniprep 150, amplitude 14 μm). Insoluble particles were subsequently removed by centrifugation (10 min 13 000 ***g***, 4 °C). Soluble protein was further purified using Qiagen Ni-NTA spin columns. For every construct, in total, 200 μL eluate in imidazole buffer was transferred to a Slide-A-Lyzer Mini Dialysis Unit (10 000 MWCO; Pierce, Rockford, IL) and dialysed for 3 h to 1 L Storage buffer (50 mM Tris-HCl pH = 7.5, 12.5% glycerol, 1.4 mM 2-mercaptoethanol) at 4 °C. After dialysis, enzyme was immediately used for enzyme assays.

Enzyme assay

In a glass tube, a mix was made of 800 μL of MOPSO buffer, 175 μL of purified enzyme solution and 5 μL of farnesyl diphosphate (10 mM, Sigma FPP, St Louis, MO). This mix was carefully overlayed with 500 μL of pentane and incubated at 30 °C with mild agitation for 2 h, unless another temperature was indicated. Subsequently, the pentane was collected. The remaining water phase was extracted with 1 mL ethylacetate. Ethylacetate and pentane phases were combined, centrifuged at 1200 ***g***, dried over a sodium sulphate column and analysed by GC/MS, as described previously. Buffers used for testing valencene synthase activity at various pH's were used at 100 mM, and included, sodium acetate (pH = 5.0 and 5.5), 2-(N-morpholino) ethanesulfonic acid (MES; pH = 6.0, 6.5 and 7.0), Tris-HCl (pH = 7.5, 8.0, 8.5 and 9.0) and ethanolamine (pH = 9.5 and 10.0). Protein concentrations of CnVS and Citrus valencene synthase in enzyme extracts were determined by SDS-PAGE analysis on a 12% gel, using SYPRO ruby staining and QuantityONE software (Promega) for quantification. Ratios of recombinant protein between CnVS and CitrusVS preparations were confirmed by Western blotting of serial dilutions of the enzyme extracts, using anti-His6-PO antibody (Roche Diagnostic GmbH, Basel, Switzerland). Km was determined by performing the assays at 8 different FPP concentrations ranging from 0.5 to 50 μM, using an enzyme concentration (0.5 μg enzyme per mL) and incubation time (40 min) under which <50% of the substrate was turned over, and Eadie Hofstee plotting.

Gene expression analysis of CnVS

One microgram of total RNA was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, the Netherlands). Real-time PCRs were carried out in triplicate in a total volume of 20 μL containing 10 μL of 2× iQ SYBR Green Supermix (Bio-Rad), 0.3 μM of forward and reverse primer and 10 ng of cDNA in a MyiQ real-Time PCR machine from Bio-Rad. The following PCR program was used: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primers used were CnVs-Fw (AAGTTCAAAGGTGAAGAAGGACA) and CnVs-Re (TTTTGCATCTTGATCGCTAGATT), and Actin Fw (TGACAATGGAACCGGAATG) and Actin Re (CACTGCCCTTGGAGCATC). Relative gene expression was calculated as: $2^{-\Delta C_t}$, where $\Delta C_t = C_t$ CnVS $- C_t$ actin.

Expression of ValC in *Saccharomyces cerevisiae*

The full-length open-reading frame encoding CnVS was amplified from the plasmid pACYC-DUET plasmid using Phusion polymer-

ase and primers TATATGGATCCATGGCTGAAATGTTTAATG GAAATTCCAGC and GATTATGCGGCCGTGTACAA. The obtained PCR fragment was introduced into the yeast expression vector pYES3/CT (Invitrogen) using restriction enzymes BamHI and NotI. The plasmid pYES-CnVS plasmid was transformed into yeast strain WAT11 (Urban *et al.*, 1997) using standard protocols (22). Yeast WAT11 cultures transformed with the empty pYES3/CT vector were used as controls. The recombinant yeast colonies were selected on solid Synthetic Dextrose minimal (SD) medium omitting L-tryptophan for auxotrophic selection. A single yeast colony containing pYES-CnVS was inoculated into 5 mL of liquid Synthetic Galactose minimal (SG) medium, which was identical to SD medium but glucose was replaced by 2% D-galactose, and the starter yeast culture was grown overnight at 30 °C and 200 rpm. After overnight incubation, the cultures were subsequently diluted to OD600 of 0.05 in 50 mL of SG medium and incubated at 200 rpm and 30 °C. The cultures were overlaid with 5 mL of n-dodecane when the OD600 was in the range from 0.8 to 1, and cultivation was continued for 3 days. After 3 days of fermentation, the n-dodecane layer was separated from the yeast cultures by a glass separation funnel, diluted 1 : 2 (v/v) in ethylacetate and used for GC/MS analysis, as described previously.

Expression of valencene synthases in *R. Sphaeroides*

For expression in *R. sphaeroides*, both *CitrusVS* and *CnVS* were codon optimized for expression in *R. sphaeroides*. By using NdeI and BamHI restriction sites, both fragments were cloned into shuttle vector pBBR-K-PcrtE (Berry *et al.*, 2002), which contains a 270 bp fragment, derived from a region upstream of the *Paracoccus* sp. R114 crtE gene, including a promoter region, ribosome binding site and start codon. Terpene synthases were expressed in frame and downstream of maltose binding protein. Plasmids were transformed to *E. coli* S17-1, and transferred to *R. sphaeroides* Rs-265-9c by conjugation (Parke, 1990). Recombinant bacteria were selected on medium containing 50 mg/L kanamycin at 30 °C for 2–3 nights.

The recombinant *R. sphaeroides* strains were grown in precultures of 25 mL RS102 medium (20 g/L yeast extract; 0.5 g/L NaCl; 0.5 g/L $MgSO_4.7H_2O$; 33 g/L D-glucose monohydrate; 0.15 g/L $CaCl_2.2H_2O$; 10 mg/L $FeCl_3.6H_2O$; 4 mg/L vitamin C; 0.4 mg/L $NiSO_4.6H_2O$; 4 mg/L $MnSO_4.H_2O$; 12 mg/L $ZnSO_4.7H_2O$; 0.16 g/L $(NH_4)_2Fe(SO_4)_2.6H_2O$; pH = 7.4) with 50 mg/L kanamycine at 220 rpm and 30 °C for 24 h. The culture was diluted 1:10 in 22.5 mL of RS102 medium and 2.5 mL of n-dodecane in 250-mL baffled Erlenmeyer flasks, and cultivation was continued for 72 h at 30 °C and 220 rpm. Valencene content was analysed as described earlier.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Tissues used for expression analysis of CnVS.
Figure S2 Open reading frames for Citrus VS (A) and CnVS (B) after codon optimization for expression in *R. sphaeroides*.
Figure S3 Western blot analysis of valencene synthase expression in *R. sphaeroides*.