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Identification of a drimenol synthase and drimenol oxidase from *Persicaria hydropiper*, involved in the biosynthesis of insect deterrent drimanes

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Running head: Biosynthesis of insect deterrent drimanes

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Abstract

The sesquiterpenoid polygodial, belonging to the drimane family, has been shown to be an antifeedant for a number of herbivorous insects. It is presumed to be synthesized from farnesyl diphosphate via drimenol, subsequent C-12 hydroxylation, and further oxidations at both C-11 and C-12 to form a dialdehyde. Here, we have identified a drimenol synthase (*PhDS*) and a cytochrome P450 drimenol oxidase (*PhDOX1*) from *Persicaria hydropiper*. Expression of *PhDS* in yeast and plants resulted in production of drimenol only. Co-expression of *PhDS* with *PhDOX1* in yeast yielded drimendiol, the 12-hydroxylation product of drimenol, as a major product, and cinnamolide. When *PhDS* and *PhDOX1* were transiently expressed by agro-infiltration in *Nicotiana benthamiana* leaves, drimenol was almost completely converted into cinnamolide and several additional drimenol derivatives were observed. *In vitro* assays showed that PhDOX1 only catalyzes the conversion from drimenol to drimendiol, and not the further oxidation into an aldehyde. In yeast and heterologous plant hosts, the C-12 position of drimendiol is therefore likely further oxidized by endogenous

enzymes into an aldehyde and subsequently converted to cinnamolide, presumably by spontaneous hemiacetal formation with the C-11 hydroxyl group followed by oxidation. Purified cinnamolide was confirmed by NMR and shown to be deterrent with an effective deterrent dose (ED_{50}) of ~200-400 µg gFW^{-1} against both whiteflies and aphids. The putative additional physiological and biochemical requirements for polygodial biosynthesis and stable storage in plant tissues are discussed.

Introduction

Sesquiterpenes are a family of C_{15} isoprenoids that are found in both higher and lower plants, microbes and marine organisms. They are predominantly synthesized from farnesyl diphosphate through the mevalonate pathway in the cytosol and many have biological activities, including antimicrobial, anti-tumor, and cytotoxic properties. In plants, they play important roles in the interaction with insects and microbes and can act as attractants, deterrents or antifeedants (Dudareva *et al.* 2005, Sallaud *et al.* 2009). Sesquiterpenes may be acyclic or contain rings in many unique stereochemical configurations. Biochemical modifications such as oxidation may form alcohols, aldehydes, ketones, acids and lactones.

Drimanes are sesquiterpenes that have a bicyclic structure and are widespread in plants, liverworts, fungi and certain marine organisms (sponges) and are also reported to have antibacterial and antifungal properties (Jansen and de Groot 2004). Well-known examples of drimane sesquiterpenes are the dialdehydes warburganal, ugandensidial and muzigadial, which all display insect antifeedant activity against armyworms (Kubo and Ganjian 1981). The best known drimane dialdehyde is polygodial, a compound with a pungent taste to mammals (Escalera *et al.* 2008, Kubo and Ganjian 1981). In plants, polygodial has been shown to act as antifeedant against a number of herbivorous insects (Jansen and de Groot

2004, Prota *et al.* 2013). The most common plant in which polygodial occurs is water-pepper, *Persicaria hydropiper* (formerly *Polygonum hydropiper*) (Prota *et al.* 2014, Starkenmann *et al.* 2006). It was reported that *P. hydropiper* produces and stores polygodial in the leaves and flowerheads in specialized epidermal cavities named valvate or irritant glands (Derita *et al.* 2008, Hagendoorn *et al.* 1994, Prota *et al.* 2014).

Pickett proposed a biosynthetic pathway for the synthesis of polygodial, in which the sesquiterpene alcohol drimenol is a precursor (Pickett 1985) (Figure 1). A drimenol synthase was indeed recently identified in valerian (*Valeriana officinalis*) catalyzing the formation of drimenol from farnesyl diphosphate (FPP) (Kwon *et al.* 2014). The conversion to polygodial was presumed to occur through one or more oxidases belonging to the cytochrome P450 family.

The aim of this study was to identify genes from *P. hydropiper* that encode enzymes involved in the biosynthesis of drimenol and polygodial and to characterize the compounds these enzymes produce in heterologous expression systems such as *Saccharomyces cerevisiae* and *Nicotiana benthamiana*. Enzyme products were evaluated by GC-MS, as well as by untargeted metabolomics using LC-QTOF-MS. The structures of the two major products were determined by NMR and the major one was tested against two phloem-sucking insect pests, namely *Myzus persicae* and *Bemisia tabaci*.

Results

Identification of the drimenol synthase gene *PhDS*

In a previous study, we analyzed the chemical profile of three *Persicaria* spp., *Persicaria hydropiper*, *Persicaria maculosa* and *Persicaria minor* (Prota *et al.* 2014). *P. hydropiper* was shown to produce by far the highest amounts of polygodial (6.2 mg gFW^{-1} in flowers), while in *P. maculosa* only traces of polygodial were detected. To identify genes involved in the biosynthesis of polygodial we made a 454 EST library using both *P. hydropiper* and *P. maculosa* flower material. By sequence assembly, 28,800 and 23,480 contigs were formed for *P. hydropiper* and *P. maculosa*, respectively. Initial comparative screening allowed the identification of a dominant sesquiterpene synthase with ~30 times higher abundance in *P. hydropiper* compared with *P. maculosa*. The complete ORF was cloned and expressed in yeast. GC–MS analysis of the dodecane overlay of the yeast culture showed a product which was identified as drimenol, confirming that this gene encodes a drimenol synthase (*PhDS*) (Figure 2).

The full-length cDNA sequence of *PhDS* (GenBank accession no. KC754968) contained an open reading frame of 1677 bp, encoding a protein of 559 amino acid residues and a predicted molecular weight of 64,729 Daltons. PhDS only shows 30% sequence identity with the previously identified drimenol synthase from *V. officinalis* (VoDS) and sequence analysis revealed two DDxxD motifs for PhDS whereas only one was identified for VoDS (Supplemental Figure S1). For VoDS, the DDxxD motif was shown to be involved in magnesium binding (Kwon *et al.* 2014). Analysis of the deduced amino acid sequence, using SignalP version 4.0 server software, indicated that no signal peptide was apparent in the N-terminal region of PhDS (Bendtsen *et al.* 2004).

Identification of the drimenol oxidase, *PhDOX1*

The *P. hydropiper* cDNA database was also used to search for homologs of the cytochrome P450 sequences of amorpha-4,11-diene oxidase from *Artemisia annua* (Teoh *et al.* 2006), an elicitor-inducible cytochrome P450 from *Nicotiana tabacum* (Ralston *et al.* 2001), a cytochrome P450 hydroxylase from *Hyoscyamus muticus* (Takahashi *et al.* 2007) and GA3 ent-kaurene oxidase from *Arabidopsis thaliana* (Helliwell *et al.* 1999). This search revealed a cytochrome P450 gene in the *P. hydropiper* database that was ~150 times less abundant in *P. maculosa*. This cytochrome P450 gene was cloned into yeast and tested *in vitro*. Microsomal preparations were produced from yeast and activity was tested by adding drimenol. One major additional compound with a mass *m/z* 238 (Figure 3) and a similar base peak (*m/z* 109) as drimenol was detected by GC-MS analysis in the assay with the P450 microsomes but not in the control yeast strain with empty plasmid. However, comparison of the spectra with those of published reference spectra of terpenoids in the National Institute of Standards and Technology (NIST) MS database did not show significant homology to any compound in the database.

To determine the accurate mass of the compound produced, an ethyl acetate extract of yeast co-expressing both *PhDS* and the P450 enzyme was analyzed by LC-Orbitrap-FTMS. The analysis revealed a major compound with a mass of MH+ 203.1780 (Figure 4). This compound was subsequently isolated by preparative HPLC. NMR analysis identified the compound as drimendiol (Supplemental Figure S2), completely in line with the previously published identification data of this compound (Brown 1994, Kuchkova *et al.* 2004). Drimendiol has an *m/z* for its molecular ion MH+ of 239.1927; an explanation for the

observed mass difference to LC-MS may be that upon ionization in the LC-MS detector drimendiol loses two water molecules from the C-11 and C-12 hydroxyl groups.

The P450 enzyme was named drimenol oxidase *PhDOX1* (GenBank accession no. KC754969) which encodes a 503-residue protein with a predicted mass of 56,149 Da. The deduced amino acid sequence contains the PFGAGRRICPG motif that corresponds to the highly conserved heme-binding domain PFGxGRRxCxG found in most plant P450s (Durst and O'Keefe 1995). Comparison of the deduced amino acid sequence showed only up to 52% sequence similarity with the CYP76F and CYP76B subfamilies. Therefore, the enzyme was classified by Dr. David Nelson into a new subfamily and designated CYP76AJ1 (Nelson 2009). Kinetic analysis using the yeast microsomal preparations and in the presence of excess NADPH, showed that PhDOX1 efficiently catalyzes the conversion of drimenol into drimendiol, with an estimated K_M = 11 μM.

Characterization of *PhDOX1* products in plants

For analysis of *in planta* activity, *N. benthamiana* leaves were co-infiltrated with *A. tumefaciens* strains harboring plasmids containing *PhDS* and/or *PhDOX1*. *PhDS* agrobacteria were mixed with strains carrying *Arabidopsis* farnesyl diphosphate synthase (*AtFPS2*) and truncated *Arabidopsis* 3-hydroxy-3-methylglutaryl-CoA reductase (*AtHMGR*) to increase terpene production (van Herpen *et al.* 2010). Infiltrated leaves without *PhDOX1* yielded ~392 μg drimenol per gram fresh weight. In contrast to our expectation, targeting AtFPS2 and PhDS both to the plastids or mitochondria reduced the production of drimenol by 40- and 400-fold, respectively.

Co-expression of *PhDOX1* with *PhDS* (targeted to the cytosol) together with *AtFPS2* and *AtHMGR* in *N. benthamiana*, resulted in seven compounds as determined by GC-MS analysis (Figure 5, Supplemental Figure S3). Compound A (*m/z 238)* was also detected in the GC-MS analysis of the yeast *in vitro* assay with PhDOX1 microsomes incubated with drimenol. Besides this compound, four other compounds (B,D,F and G) with a similar base peak (*m/z* 109) as drimenol could not be reliably identified through a NIST library search. The major compound F with a parent ion of *m/z* of 234 showed the highest homology (40.3%) to the sesquiterpene lactone drimenin in a NIST library search but with a different retention index KI 2137 vs 1807.

N. benthamiana leaves transiently expressing *PhDS* and *PhDOX1* were also extracted with methanol and analyzed by LC-MS (Figure 6). A compound with a similar mass and retention time as drimendiol (MH+ 203.1798; i.e. after double water loss, see above) and a major compound with a mass of MH+ 235.1691 were detected. A compound with a similar mass and retention time as the latter was also detected in extracts of yeast expressing *PhDS* and *PhDOX1* (Figure 4). In order to identify this compound it was purified by HPLC and subjected to NMR analysis. The compound was identified as cinnamolide (Supplemental Figure S4). The NMR data corresponded completely to what was reported by Hollinshead and co-workers (Hollinshead *et al.* 1983).

Effects of PhDOX1-generated cinnamolide against whiteflies and aphids

The cinnamolide produced by transient co-expression of *PhDS* and *PhDOX1* in *N. benthamiana* represented the major compound produced *in planta*, and we were interested to test its antifeedant activity against two major insect pests and in relation to the well-known drimane antifeedant polygodial. Cinnamolide was tested in dual-choice assays at three

different concentrations (450, 225 and 113 μg gFW^{-1}). Dose-dependent responses in feeding preference were recorded at regular intervals during a period of 18 or 24 hours post inoculation with the green peach aphid *Myzus persicae* or silverleaf whitefly *Bemisia tabaci* (genotype B), respectively. The 6h-time point was selected as the most reliable as most insects were actively feeding at that time, and the time for induction of potentially confounding secondary responses resulting from the applied compound were kept to a minimum. The feeding preference was assessed using the Antifeedant Index and compared to the published data for polygodial (AI%) (Kutas and Nádasy 2005, Prota *et al.* 2013). The AI% of cinnamolide increased with the increasing concentrations applied, as seen in Figure 7, meaning that there was a clear dose-dependent preference for the control disks over the treated ones, similar as observed for polygodial. The extrapolated effective deterrent dose for 50% feeding deterrence (ED_{50}) was 195 μg gFW^{-1} for whiteflies and 423 μg gFW^{-1} for aphids. For polygodial ED_{50} values of 25 μg gFW^{-1} for whiteflies and 54 μg gFW^{-1} for aphids were reported.

Discussion

In the present study, we identified and characterized a drimenol synthase and drimenol oxidase from *P. hydropiper*, which are involved in the formation and conversion of drimenol. Expression of the drimenol synthase (*PhDS*) showed production of drimenol, while co-expression with the cytochrome P450 oxidase (*PhDOX1*) resulted in production of mainly drimendiol and cinnamolide in both *S. cerevisiae* and *N. benthamiana*.

Production of high levels of sesquiterpenes through the expression of sesquiterpene synthases in *Arabidopsis* or tobacco has generally been difficult when the proteins were targeted to their native cytosolic compartment (Aharoni *et al.* 2005). It was shown for several genes that targeting to the mitochondria or plastids resulted in higher production, presumably because of higher substrate availability in those compartments (Kappers *et al.* 2005, Liu *et al.* 2011, van Herpen *et al.* 2010, Wu *et al.* 2012). Similar to the expression of (*E*)-β-farnesene synthase in *Arabidopsis* (Beale *et al.* 2006), however, for *PhDS* we observed that under the same promoter the native cytosolic enzyme was 40-400 fold more active compared with enzyme targeted to the plastids or mitochondria. Although we cannot rule out that the enzymes were poorly imported into those compartments, the high activity in the cytosol at least suggests that factors other than substrate availability may limit or promote the enzymatic activity of sesquiterpene synthases in plants.

Kwon *et al.* (2014) described a drimenol synthase from *Valeriana officinalis* and speculated about the reaction mechanism. Chemically, the most logical route, the authors argue, would start by protonation at C3 of FPP ("class II mechanism"), followed by two cyclizations, deprotonation and hydrolysis of the diphosphate group to an alcohol. This mechanism would, however, involve a DxDD motif in the protein, which was not found. Instead they found only one aspartate-rich motif DDxxD, involved in binding of the essential magnesium ion needed for diphosphate binding and release, forming an allylic carbocation ("class I mechanism"). As explained by Kwon *et al*. (2004) a reaction sequence towards drimenol in which cleavage of the diphosphate is the first step was then very difficult to envisage from a chemical point of view, but nevertheless seemed to take place. Our *Persicaria hydropiper* drimenol synthase also has no DxDD motif, but two DDxxD motifs, with DDTLD at residues 170-174 and DDIYD at residues 311-315. We assume the latter is the magnesium binding site (similar to the DDTYD sequence in the *V. officinalis* enzyme). The function of the other one located

more upstream is unknown, but could possibly be involved in a protonation type cyclization, which has been proposed by Kwon et al. (2014). The presence of two DDxxD motifs is rare in terpene synthases. Two characterized sesquiterpene synthases from *Abies grandis* (δ-selinene synthase and γ-humulene synthase) by (Steele *et al.* 1998) also have two DDxxD motifs. A second study by Little e*t al*. (2002) took a closer look at the role of the unusual second DDxxD in *A. grandis* sesquiterpene synthases showing a catalytic role for both.

Interestingly, the *V. officinalis* sequence has two glutamic acid residues instead of the aspartic acid residues at that position. Based on blast analysis, the closest homologues to PhDS also only contain the conserved DDxxD motif. However and similar to what was found for VoDS, all have one or two glutamic acid residues instead of the aspartic acid residues for the first DDxxD motif. Glutamic acid could fulfil the same function as the aspartic acid, therefore the role of this region of the protein in protonation induced cyclization is indeed a possibility.

The cytochrome P450 that is oxidizing drimenol, PhDOX1, was active when heterologously expressed in both yeast and *N. benthamiana*. LC-MS analysis of purified drimendiol showed one compound of MH+ 203.1798. In the LC-MS, drimendiol apparently loses two water molecules during ionization. Analysis by GC-MS revealed a family of related compounds that shared base peaks of *m/z* 109 and 124 in their mass spectrum, two peaks which are also present in the drimenol spectrum (see Figure 2) and might well be characteristic for drimane type sesquiterpenes. Therefore, we expect that the compounds B, D and G detected by GC-MS (see Supporting information Figure S1) are also drimane type sesquiterpenes. Lack of standards prevented us from further identifying these low abundant compounds. However, compound B (*m/z* 218) and D (*m/z* 236) have masses similar to the calculated masses of intermediates in the putative pathway towards cinnamolide and/or polygodial (Figure 1). Compounds C and E do not show any similarity to drimenol but could be compounds formed by unspecific activities of PhDOX1 or modifications by endogenous enzymes in yeast or *N.*

benthamiana like previously described by others (Hofer *et al.* 2013, Liu *et al.* 2011, van Herpen *et al.* 2010).

The identified *PhDOX1* gene has been classified to belong to the CYP76AJ1 P450 family. The *in vitro* assay showed that PhDOX1 performs the first hydroxylation step to drimendiol, but not the subsequent oxidation to the corresponding aldehyde. Other members of this family were also shown to be involved in terpenoid biosynthesis (Guo *et al.* 2013, Hofer *et al.* 2013). Kinetic analysis of PhDOX1 demonstrated a substrate binding constant (K_M of 11 μM) similar to that reported for other CYP76 subfamily members with K_M values ranging from 2 to 40 μM (Guo *et al.* 2013, Hofer *et al.* 2013, Swaminathan *et al.* 2009, Wang *et al.* 2012).

The proposed pathway to cinnamolide in yeast and *N. benthamiana* starts with the formation of drimenol from farnesyl diphosphate (FPP) by PhDS. In the next step, PhDOX1 hydroxylates drimenol at C-12 to form drimendiol. The resulting C-12 hydroxyl group could subsequently be oxidized into an aldehyde by the action of endogenous *N. benthamiana* or yeast enzymes. The C-12 aldehyde can spontaneously react with the C-11 hydroxyl group into a hemiacetal as depicted in Figure 8, and then be oxidized into cinnamolide. Cyclization through hemiacetal formation has been described before for diterpenes (Yong *et al.* 2008). Sugars also rapidly interconvert between straight-chain and cyclic hemiacetal forms. These sugar cyclization reactions occur spontaneously and reversibly in solution (Alves and Pio 2005). Alternatively, lactone ring formation occurs as reported for the sesquiterpene lactone costunolide through further oxidation of the aldehyde to an acid followed by spontaneous formation of the lactone ring (de Kraker *et al.* 2002).

Interestingly, cinnamolide is not found in *P. hydropiper* despite the fact that we used mRNA of the two most dominant enzymes of that plant to reconstruct this pathway. Apparently, there are conditions in *P. hydropiper* which promote the formation of the dialdehyde (producing polygodial) over the formation of the lactone. *In vitro* assays showed that PhDOX1 only catalyzes the conversion from drimenol to drimendiol, and not the further oxidation into an aldehyde. This indicates that the conversion to polygodial in *P. hydropiper* requires one or more additional P450(s) or dehydrogenases. For example, for the biosynthesis of phytoalexins in rice it has been shown that different cytochrome P450 enzymes act sequentially (Schmelz *et al.* 2014). A "P450" keyword search in the *P. hydropiper* database resulted in 144 hits, some of which show up to 50% sequence similarity with known P450s from the CYP76 family. Comparative screening of the twenty most abundant P450s found in the *P. hydropiper* database search, revealed already 6 potential terpene oxidases with at least 10 times higher abundance in *P. hydropiper* compared with *P. maculosa*. Besides PhDOX1, clearly one or more P450s could be involved in the biosynthesis of polygodial.

Next to this, two other important elements may prevent heterologous expression of polygodial. Firstly, polygodial is a very reactive compound in the presence of proteins. In previous reports, it has been shown that polygodial forms a pyrrole derivative with compounds possessing a primary amine group (Caprioli *et al.* 1987, Kubo *et al.* 2005). In that sense polygodial, if formed, may immediately react inside the cells with a variety of intracellular compounds possessing an amine group, which makes it very difficult to prove production of polygodial in a heterologous expression system like yeast or tobacco. Secondly, in *P. hydropiper* polygodial accumulates in cavities devoid of other cell content (Derita *et al.* 2008, Hagendoorn *et al.* 1994). This indicates that the production of the dialdehyde in plants may require a suit of additional factors and secretory structures to prevent the dialdehyde to spontaneously react to amino-groups of proteins and to prevent that the aldehyde is further

converted to form cinnamolide. Possibly, in *P. hydropiper* the enzymes form a complex such that drimendiol is converted into a dialdehyde in a protected environment, thereby preventing lactone ring formation. Interestingly, polygodial and cinnamolide have been detected together in the fern *Blechnum fluviatile* (Asakawa *et al.* 2001) which supports their close biosynthetic relationship.

Drimendiol has been isolated from *Drimys winteri* and was reported to have anti-feedant activity against the Egyptian cotton leafworm, *Spodoptera littoralis* (Brown 1994, Rodriguez *et al.* 2005, Zapata *et al.* 2009). Furthermore, drimendiol has recently been shown to inhibit quorum sensing in *Chromobacterium violaceum* and *Pseudomonas syringae* (Paza *et al.* 2013). Interestingly, polygodial can be synthesized very efficiently from drimendiol using Swern oxidation as described by Hollinshead and coworkers so that biological production of polygodial, a food ingredient in Japan, would be a possibility using the enzymes described in this paper to produce drimendiol (Hollinshead *et al.* 1983). Cinnamolide has been identified in *Cinnamosma fragrans* and in the stem bark of *Warburgia ugandensis* and was reported to exhibit antifungal activity (Wube *et al.* 2005). Here we demonstrate that cinnamolide can also act as an anti-feedant for both the silverleaf whitefly *Bemisia tabaci* and the green peach aphid *Myzus persicae* (Figure 7), although not as strong as polygodial, with ED_{50} values of 25 vs 195 μg gFW^{-1} and 54 vs 423 μg gFW^{-1}, respectively (Prota *et al.* 2013).

In conclusion, we have isolated and characterized two genes (*PhDS* and *PhDOX1*) involved in the biosynthesis of drimenol and conversion to drimendiol and the sesquiterpene lactone cinnamolide in yeast and plants. The identified drimenol oxidase performs the first hydroxylation step in the biosynthesis pathway of cinnamolide and polygodial. The

subsequent oxidation to its aldehyde is catalysed by endogenous enzymes in yeast and tobacco. We propose that cinnamolide is produced by spontaneous formation of a sesquiterpene hemiacetal which is further oxidized to cinnamolide by endogenous enzymes. In both yeast and *in planta*, several additional drimenol derivatives were observed when *PhDS* and *PhDOX1* were co-expressed, suggesting complex conversions resulting from endogenous enzyme activities in yeast and tobacco.

Experimental procedures

cDNA synthesis and GS FLX Titanium General Library Preparation

cDNA synthesis was performed using the SMART PCR cDNA Synthesis kit (Clontech, USA) according to manufacturer's instructions with minor modifications. An aliquot of 1 μg of total RNA was used as input for the reverse transcriptase reaction using SuperScript II reverse transcriptase (Life Technologies, USA). To facilitate the removal of the poly(A)-tail prior sequencing, first strand cDNA synthesis was carried out with an modified 3' SMART CDS primer IIA containing an rare cutter restriction endonuclease site in the 5' region of the oligo(dT) sequence. cDNA amplification was performed using the Advantage 2 PCR kit (Clontech, USA) following the instructions in the SMART PCR cDNA Synthesis protocol. Double-stranded cDNA was purified using the QiaQuick PCR purification kit (Qiagen, Germany). cDNA quality was determined on a BioAnalyzer using a DNA 7500 chip (Agilent Technologies, Germany). cDNA normalization was carried out using the TRIMMER cDNA Normalization kit (Evrogen, Russia) as per manufacturer's instructions. Amplification of normalized cDNA was performed using the Advantage 2 PCR kit (Clontech Inc., USA) following the instructions in the TRIMMER cDNA Normalization manual. Instead of using the Evrogen's PCR primer M2, a modified biotinylated primer was used for second

amplification of normalized cDNA. The normalization efficiency was determined with a DNA 7500 chip (Agilent Technologies, Germany).

Random cDNA shearing prior FLX Titanium library was performed by nebulization of 15 to 20 µg of normalized cDNA according the instructions in the GS FLX Titanium General Library Preparation manual (Roche Applied Science, Germany). After nebulization, the randomly sheared cDNA was purified using the QiaQuick PCR purification kit (Qiagen, Germany) and the size distribution was determined with a DNA 1000 chip (Agilent Technologies, Germany). Removal of biotinylated 3' cDNA ends comprising the poly(A)-tail was performed as follow. First, the nebulized cDNA was digested with a restriction endonuclease which specifically recognizes the restriction site that was previously introduced in the 5' region of the poly(A) tail. After digestion, the biotinylated ends were bound to M-270 streptavidin Dynabeads according to manufacturer's instructions (Life Technologies, USA) and the supernatant containing the digested cDNA without biotinylated ends was collected and purified using the MinElute PCR purification kit (Qiagen, Germany). Exclusion of smaller-sized fragments was performed using the double SPRI method as described in the Roche GS FLX Titanium General Library Preparation protocol (Roche Applied Science, Germany). Further library preparation was performed according to the standard GS FLX Titanium General Library Preparation Method protocol as supplied by Roche Applied Science (Germany). The cDNA samples were analyzed by massive parallel pyrosequencing using a Genome Sequencer FLX Titanium sequencing platform (Roche Applied Science, Germany).

Isolation and cloning of drimenol synthase and *cytochrome P450* genes from *P. hydropiper*

The identification of polygodial biosynthetic genes relied first on obtaining a 454 EST library from both *P. hydropiper* (water-pepper) and *P. maculosa* (lady's thumb). Good quality mRNA was obtained from young flowers from both species and the normalized cDNA sequenced. Comparative screening of the libraries allowed the identification of a sesquiterpene synthase with a much higher apparent abundance in *P. hydropiper* compared to *P. maculosa*. The full-length putative drimenol synthase (*PhDS*) gene was amplified using high fidelity Phusion polymerase (Finnzymes) and the primers PhDS-F (BsaI) 5'GTGACGGTCTCCCATGTCTACTGCCGTTAACGTCC'3 and PhDS-R (NotI) 5' GTGACGCGGCCGCCTAAATCGGAATGGGATCGGTGA'3 with the addition of *BsaI* restriction site (with *NcoI* overhang) and *Not*I restriction sites which allowed subsequent cloning into the pYES3/CT yeast expression vector (Invitrogen), containing the TRP1 auxotrophic selection marker. The *PhDS* gene was transformed into yeast strain WAT11, expressing the *Arabidopsis thaliana* ATR1 NADPH-cytochrome P450 reductase (Urban *et al.* 1997). The sequence of the gene was deposited in the NCBI GenBank Nucleotide database under accession number KC754968.

A BLAST search using already known sesquiterpene cytochrome P450 sequences from literature, revealed several potential terpene hydroxylases with, once again, a much higher abundance in *P. hydropiper* compared to *P. maculosa*. The most abundant full-length *P450* gene was cloned, using the *NotI/PacI* restriction sites, into the yeast expression vector pYEDP60 (Pompon *et al.* 1996) modified to contain the *Pac*I and *Not*I sites at the polylinker. The candidate P450 was co-transformed with the *PhDS* from *P. hydropiper* into the yeast strain WAT11. After transformation, yeast clones expressing the *PhDS* alone or *PhDS* combined with the P450 were selected on Synthetic Dextrose minimal medium (0.67% Difco

yeast nitrogen base medium without amino acids, 2% D-glucose, 2% agar) supplemented with amino acids, but omitting L-tryptophan or uracil, adenine sulphate and L-tryptophan, respectively, for auxotrophic selection of transformants. The sequence of this drimenol oxidase (PhDOX1) gene was deposited in the NCBI GenBank Nucleotide database under accession number KC754969. The sequence was also submitted to David Nelson's cytochrome P450 homepage (http://drnelson.uthsc.edu/cytochromeP450.html) and was assigned the name CYP76AJ1 (Nelson 2009).

Co-expression of *PhDS* with putative P450 in yeast

A starter yeast culture was grown overnight at 30 °C in 5 ml of Synthetic Galactose minimal medium (0.67% Difco yeast nitrogen base medium without amino acids, 2% D-galactose, amino acids, but omitting either L-tryptophan or uracil, adenine sulphate and L-tryptophan). The starter culture was diluted to OD_{600} of 0.05 in 50 ml of Synthetic Galactose minimal medium and incubated at 200 rpm at 30 °C. The culture was grown for 3 days and the metabolites from the medium were extracted with 10 mL ethyl acetate. From this, a sample was analyzed by GC-MS.

Plasmid construction for expression in *Nicotiana benthamiana*

The *PhDS* and *PhDOX1* genes were cloned into ImpactVector1.1 (www.impactvector.com/) to express them under the control of the Rubisco small subunit (RbcS) promoter for expression in *N. benthamiana*. The *PhDS* gene was also cloned into ImpactVector 1.4 and 1.5 to fuse it with the RbcS promoter and the plastid or CoxIV mitochondrial targeting sequence, respectively. To clone each gene into the pBinPlus binary vector between the right and left

borders of the T-DNA for plant transformation, a recombination reaction was carried out, using the Gateway-LR Clonase TM II (Invitrogen).

Transient expression in leaves of *Nicotiana benthamiana*

A. tumefaciens infiltration (agro-infiltration) was performed as described previously (van Herpen *et al.* 2010). Briefly, *A. tumefaciens* strains were grown at 28 °C at 220 rpm for 24 hours in LB media with kanamycin (50 mg L^{-1}) and rifampicin (100 mg L^{-1}). Cells were harvested by centrifugation for 20 min at 4000 g and 20 °C, and then resuspended in 10 mM MES buffer containing 10 mM $MgCl_2$ and 100 µM acetosyringone (4′-hydroxy-3′,5′-dimethoxyacetophenone, Sigma) to a final OD_{600} of 0.5, followed by incubation at room temperature with shaking at 50 rpm for 3 hours. For co-infiltration, equal volumes of the *A. tumefaciens* strains were mixed. *N. benthamiana* plants were grown from seeds on soil in the greenhouse with a minimum of 16 hour light. Day temperatures were approximately 28 ºC, night temperatures 25 ºC. Strain mixtures were infiltrated into leaves of four-week-old *N. benthamiana* plants using a 1 mL syringe without needle. The bacteria were slowly injected into the intercellular space through the abaxial side of the leaf. The plants were grown and the infiltrated leaves were collected 4 or 5 days after infiltration.

To analyze the compounds accumulated in the leaves, the harvested plant material was snap frozen and ground in liquid nitrogen. 500 mg of powder was extracted with 2 ml dichloromethane. The extracts were briefly vortexed and submerged in a sonication bath for 5 min. They were then centrifuged for 10 min at 1200 rpm and the clear part of the solution was transferred to a fresh vial. Finally, the extracts were dehydrated using anhydrous Na_2SO_4 and concentrated by evaporating the solvent to a volume of about 0.5 mL. Analysis of the samples was performed by GC-MS (Agilent GC-MS, Agilent technologies).

GC-MS analysis

Analytes from 1 µL samples were separated using a gas chromatograph (5890 series II, Hewlett-Packard) equipped with a 30 m × 0.25 mm, 0.25 µm film thickness column (ZB-5, Phenomenex) using helium as carrier gas at flow rate of 1 ml/min. The injector was used in splitless mode with the inlet temperature set to 250 °C. The initial oven temperature of 45 °C was increased after 1 min to 300 °C at a rate of 10 °C/min and held for 5 min at 300 °C. The GC was coupled to a mass-selective detector (model 5972A, Hewlett-Packard). Compounds were identified by comparison of mass spectra and retention times (rt) with those of the authentic standards, when available, or with known retention indices (R.I.) from literature. Drimenol and polygodial were kindly provided by John Pickett (Rothamsted Research, UK). Putative identification was achieved by comparing mass spectra with the NIST mass spectra library.

LC-Orbitrap-FTMS analysis

High-resolution mass spectrometry was performed on an LC‑LTQ-Orbitrap FTMS system (Thermo Scientific) operating in positive ionization mode. The instrument consisted of an Accela HPLC, an Accela photodiode array detector, connected to an LTQ/Orbitrap hybrid mass spectrometer equipped with an ESI source. Chromatographic separation took place on a Phenomenex Luna C18 analytical column (150 × 2.0 mm, 3 µm particle size), using H_2O and acetonitrile, both containing 0.1% v/v formic acid, at a flow rate of 0.19 mL/min and a column temperature of 40 °C. A linear gradient from 5 to 75% acetonitrile in 45 min was applied, which was followed by 15 min of washing and equilibration before the next injection. The injection volume was 5 µl.

Drimendiol purification

Ethyl acetate extract of yeast expressing *PhDS* and *PhDOX1* was evaporated with N_2 flow and redissolved and concentrated in 100% MeOH. The HPLC separation was performed using a water:acetonitrile gradient from 40% to 75% acetonitrile. All solutions contained 0.1% v/v formic acid. The flow rate was 1 mL min^{-1} and 1-min fractions were automatically collected. The fractions of interest were freeze-dried and analyzed by NMR.

Microsome preparation and in vitro enzyme assay

pYEDP60 plasmid containing PhDOX1 was transformed into WAT11 strain and colonies were selected on SD medium omitting adenine sulphate and uracil for auxotrophic selection. Microsomes from yeast cultures were prepared as previously published (Bertea *et al.* 2001, Pompon *et al.* 1996). Microsomal preparations from WAT11 cultures containing empty pYEDP60 plasmid were used as negative controls. Enzyme assays were conducted in a total volume of 490 µl, containing 40 mM KP_i buffer (pH = 7.5), 3 - 200 µM drimenol or 200 µM drimendiol, 2% DMSO and 72 µl of microsomal preparation in a 1.5 mL glass vial. The enzymatic reaction was started by the addition of 2 mM NADPH. The reactions were incubated for 2.5 h at 25 °C at 250 rpm. Terpenes were extracted with 1.5 mL of ethyl acetate and ten-fold concentrated under nitrogen flow. The samples were dried using anhydrous Na_2SO_4 and used for GC–MS analysis. Peak area of the product formed were used to calculate the K_M.

NMR spectroscopy

NMR analysis of the plant extract was performed at Spinnovation Analytical BV (Nijmegen, the Netherlands) on a Bruker Avance III 500 MHz spectrometer equipped with a 5-mm CPTCI cryo probe (^{1}H-^{13}C/^{15}N/^{2}H + Z-gradients) operating at 303 K. The structure identification of polygodial, used as a reference compound is based on a 1D ^{1}H, ^{1}H-^{1}H-DQF-COSY, ^{1}H-^{1}H-TOCSY, ^{1}H-^{1}H-NOESY, ^{1}H-^{13}C-HSQC and ^{1}H-^{13}CHMBC spectra. The structure identification of the purified compound is based on the same selection of NMR experiments with the exception of the ^{1}H-^{13}C-HMBC spectrum due to the limited amount of material. The proton and carbon chemical shifts were referenced to the internal reference TMS (proton, δ = 0.00 ppm; carbon, δ =0.00 ppm). The data were processed using Topspin 2.1 pl5.

NMR analysis of the isolated yeast compound was performed at Biqualys/Wageningen UR (Wageningen, the Netherlands) on a Bruker Advance III 600 MHz spectrometer equipped with a cryo-probe. 1D-^{1}H NMR measurements of 128 scans and a d1 of 4 s (i.e., almost 15 min) were obtained at a receiver gain of 128 in MeOD using standard pulse sequences. Two-dimensional (2D) ^{1}H measurements (COSY, HSQC, HMBC; using standard pulse sequences) were conducted for structural confirmation.

Cinnamolide purification

Cinnamolide was purified from a plant methanolic extract, obtained from transiently transformed *N. benthamiana* plants expressing the *AtHMGR*, *AtFPS*, *PhDS* and the *PhDOX1* P450. The extract was evaporated and injected onto a C18 analytical column. The HPLC separation was performed using a water:acetonitrile gradient from 40% to 75% acetonitrile.

After identification of the desired fraction by UV absorbance, it was collected multiple times until a sufficient amount was obtained. The resulting fractions were pooled and evaporated using a SpeedVac and the crystalline compound was weighed and finally dissolved in 96% ethanol.

Insects

The insects used in this study were the silverleaf whitefly *Bemisia tabaci* (genotype B) and the green peach aphid *Myzus persicae*. Whiteflies were reared on tomato (cv. Moneymaker) in a greenhouse at 26 °C and 60% relative humidity with a photoperiod of 16h light and 8h dark. To perform the assays, adult flies of both genders were collected from leaves with an aspirator. They were cold-anesthetized for 5 min at 7 °C in a 3 cm diameter plastic cylinder covered with Parafilm®, before being released into the Petri dish at the start of the dual-choice assay, to ensure they would not fly out before the dishes were sealed. Apterous aphid adults (*Myzus persicae*) were collected from Chinese cabbage (*Brassica rapa* L. subspecies *pekinensis*), on which they had been reared at room temperature in ambient light. They were placed in 2-ml-test tubes and starved for about 30 min before inoculating them in the dishes.

Insect choice assays

In the dual-choice assays, insects were presented with four circular tomato leaf areas coated with either 50% ethanol in water (control) or a solution of cinnamolide in 50% ethanol in water (treatment; two disks with each solution). Three cinnamolide concentrations were used; 1 mg mL^{-1}, 500 µg mL^{-1} and 250 µg mL^{-1} in 50% ethanol. Fresh Moneymaker tomato leaves collected from 5 or 6 weeks old plants were cut in half longitudinally and placed abaxial-side

up in a 9-cm-Petri dish on 45 ml of 8 g L^{-1} water agar substrate. The leaves were then covered with a Petri dish bottom through which four holes of 16 mm diameter were drilled, making sure that each half leaf had two circular areas available for insects to feed upon. On each circular area (disk), 15 µL of solution were applied and spread using a size 3-watercolour brush. The control and treatment solutions were each spread on two diametrically opposed disks to control for potential environmental cues, such as light. The insects were introduced into the dishes after the solvent had evaporated. Insects were put onto the lid of the dishes and then covered with the bottom of the Petri dish containing the leaves. On average, 98 whiteflies and 20 aphids per plate were used for each assay. For each combination of insect species and compound concentration, eight biological replicates (eight separate arenas) were used. The plates were sealed with Parafilm® and kept upside down. The assays with aphids were carried out at room temperature on a lab bench (uncontrolled conditions). The whiteflies assays were performed in a climate chamber at 25 °C, 60% humidity and 16/8h light/dark photoperiod. The assays were carried on for either 18 or 24 hours and the feeding insects counted at 30 min, 1h, 2h, 6h, and 18h for whiteflies and at 30 min, 1h, 2h, 6h, and 24h for aphids. Insects were considered to be feeding when they were immobile on one spot on a leaf disk. Five leaf disks were weighed to be able to determine the concentration of the insect repellent compounds in relationship to fresh weight (FW) of the leaf area treated. The average mass of each disk was found to be 34 mg. It was estimated that the concentrations of coated cinnamolide correspond to approximately 450, 225 and 113 µg gFW^{-1}.

Data analysis

To express the repellence potency of cinnamolide towards the two insect species tested, the Antifeedant Index (AI%) was calculated according to Kutas and Nádasy, as follows: AI%=[(C-T)/(C+T)]x100, where C indicates the number of insects feeding on the control, and T the number of insects feeding on the treatment (Kutas and Nádasy 2005). The AI% assumes positive values when the tested compound is an antifeedant, and negative values when the compound is a phagostimulant.

To determine the ED_{50}, the effective dose at which 50% of the insects are deterred (i.e. the concentration at which twice as many insects feed on the control compared to the treatment), a Probit analysis was carried out using the PASW statistics 18 (SPSS Inc.) package.

Acknowledgments

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Short Supporting Information legends

Supplemental Figure S1. Alignment of drimenol synthase amino acid sequences from *P. hydropiper* and *V. officinalis.*

Supplemental Figure S2. Mass fragmentation spectra of compounds detected by GCMS on extracts from *PhDS* and *PhDOX1* agro-infiltrated *N. benthamiana* leaves.

Supplemental Figure S3. 1H-NMR and 13C-NMR parameters of drimendiol NMR analysis and the structure of drimendiol. Atom numbers are referred to in the table.

Supplemental Figure S4. 1H-NMR and 13C-NMR parameters of cinnamolide NMR analysis and the structure of cinnamolide. Atom numbers are referred to in the table.

References

Aharoni, A., Jongsma, M.A. and Bouwmeester, H.J. (2005) Volatile science? Metabolic engineering of terpenoids in plants. *Trends in plant science*, **10**, 594-602.

Alves, C.A. and Pio, C.A. (2005) Secondary organic compounds in atmospheric aerosols: Speciation and formation mechanisms. *J Brazil Chem Soc*, **16**, 1017-1029.

Asakawa, Y., Toyota, M., Oiso, Y. and Braggins, J.E. (2001) Occurrence of polygodial and 1-(2,4,6-trimethoxyphenyl)-but-2-en-1-one from some ferns and liverworts: Role of pungent components in bryophytes and pteridophytes evolution. *Chemical & pharmaceutical bulletin*, **49**, 1380-1381.

Beale, M.H., Birkett, M.A., Bruce, T.J., Chamberlain, K., Field, L.M., Huttly, A.K., Martin, J.L., Parker, R., Phillips, A.L., Pickett, J.A., Prosser, I.M., Shewry, P.R., Smart, L.E., Wadhams, L.J., Woodcock, C.M. and Zhang, Y. (2006) Aphid alarm pheromone produced by transgenic plants affects aphid and parasitoid behavior. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 10509-10513.

Bendtsen, J.D., Nielsen, H., von Heijne, G. and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. *Journal of molecular biology*, **340**, 783-795.

Bertea, C.M., Schalk, M., Karp, F., Maffei, M. and Croteau, R. (2001) Demonstration that menthofuran synthase of mint (Mentha) is a cytochrome P450 monooxygenase: cloning, functional expression, and characterization of the responsible gene. *Archives of biochemistry and biophysics*, **390**, 279-286.

Brown, G.D. (1994) Drimendiol, a Sesquiterpene from Drymis-Winterii. *Phytochemistry*, **35**, 975-977.

Caprioli, V., Cimino, G., Colle, R., Gavagnin, M., Sodano, G. and Spinella, A. (1987) Insect Antifeedant Activity and Hot Taste for Humans of Selected Natural and Synthetic 1,4-Dialdehydes. *Journal of Natural Products*, **50**, 146-151.

Chang, G.Y., Park, S.H., Youn, Y.C. and Kwon, O.S. (2004) Neuroimaging findings in scleroderma en coup de sabre. *Neurology*, **63**, 2197-2198; author reply 2197-2198.

de Kraker, J.W., Franssen, M.C.R., Joerink, M., de Groot, A. and Bouwmeester, H.J. (2002) Biosynthesis of costunolide, dihydrocostunolide, and leucodin. Demonstration of cytochrome p450-catalyzed formation of the lactone ring present in sesquiterpene lactones of chicory. *Plant physiology*, **129**, 257-268.

Derita, M.G., Gattuso, S.J. and Zacchino, S.A. (2008) Occurrence of polygodial in species of Polygonum genus belonging to Persicaria section. *Biochemical Systematics and Ecology*, **36**, 55-58.

Dudareva, N., Andersson, S., Orlova, I., Gatto, N., Reichelt, M., Rhodes, D., Boland, W. and Gershenzon, J. (2005) The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 933-938.

Durst, F. and O'Keefe, D.P. (1995) Plant cytochromes P450: an overview. *Drug metabolism and drug interactions*, **12**, 171-187.

Escalera, J., von Hehn, C.A., Bessac, B.F., Sivula, M. and Jordt, S.-E. (2008) TRPA1 Mediates the Noxious Effects of Natural Sesquiterpene Deterrents. *Journal of Biological Chemistry*, **283**, 24136-24144.

Guo, J., Zhou, Y.J.J., Hillwigc, M.L., Shen, Y., Yang, L., Wang, Y.J., Zhang, X.A., Liu, W.J., Peters, R.J., Chen, X.Y., Zhao, Z.B.K. and Huang, L.Q. (2013) CYP76AH1 catalyzes turnover of miltiradiene in tanshinones biosynthesis and enables heterologous production of ferruginol in yeasts. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 12108-12113.

Hagendoorn, M.J.M., Geelen, T.A.M., Vanbeek, T.A., Jamar, D.C.L., Tetteroo, F.A.A. and Vanderplas, L.H.W. (1994) Occurrence of Polygodial in Plant Organs and Tissue-Culture of Polygonum Hydropiper. *Physiologia Plantarum*, **92**, 595-600.

Helliwell, C.A., Poole, A., Peacock, W.J. and Dennis, E.S. (1999) Arabidopsis ent-kaurene oxidase catalyzes three steps of gibberellin biosynthesis. *Plant physiology*, **119**, 507-510.

Hofer, R., Dong, L., Andre, F., Ginglinger, J.F., Lugan, R., Gavira, C., Grec, S., Lang, G., Memelink, J., Van der Krol, S., Bouwmeester, H. and Werck-Reichhart, D. (2013) Geraniol hydroxylase and hydroxygeraniol oxidase activities of the CYP76 family of cytochrome P450 enzymes and potential for engineering the early steps of the (seco)iridoid pathway. *Metabolic engineering*, **20**, 221-232.

Hollinshead, D.M., Howell, S.C., Ley, S.V., Mahon, M., Ratcliffe, N.M. and Worthington, P.A. (1983) The Diels-Alder Route to Drimane Related Sesquiterpenes - Synthesis of Cinnamolide, Polygodial, Isodrimeninol, Drimenin and Warburganal. *J Chem Soc Perk T 1*, 1579-1586.

Jansen, B.J.M. and de Groot, A. (2004) Occurrence, biological activity and synthesis of drimane sesquiterpenoids. *Natural Product Reports*, **21**, 449-477.

Kappers, I.F., Aharoni, A., van Herpen, T.W., Luckerhoff, L.L., Dicke, M. and Bouwmeester, H.J. (2005) Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis. *Science*, **309**, 2070-2072.

Kubo, I., Fujita, K., Lee, S.H. and Ha, T.J. (2005) Antibacterial activity of polygodial. *Phytother Res*, **19**, 1013-1017.

Kubo, I. and Ganjian, I. (1981) Insect antifeedant terpenes, hot-tasting to humans. *Cellular and Molecular Life Sciences*, **37**, 1063-1064.

Kuchkova, K.I., Aricu, A.N., Dragalin, I.P. and Vlad, P.F. (2004) Convenient synthesis of drimenol and its oxidation with selenium dioxide. *Russ Chem B+*, **53**, 2862-2865.

Kutas, J. and Nádasy, M. (2005) Antifeedant effects of several plant extracts on Colorado potato beetle larvae. *Acta Phytopathologica et Entomologica Hungarica*, **40**, 355-365.

Kwon, M., Cochrane, S.A., Vederas, J.C. and Ro, D.K. (2014) Molecular cloning and characterization of drimenol synthase from valerian plant (Valeriana officinalis). *FEBS letters*, **588**, 4597-4603.

Little, D.B. and Croteau, R.B. (2002) Alteration of product formation by directed mutagenesis and truncation of the multiple-product sesquiterpene synthases delta-selinene synthase and gamma-humulene synthase. *Archives of biochemistry and biophysics*, **402**, 120-135.

Liu, Q., Majdi, M., Cankar, K., Goedbloed, M., Charnikhova, T., Verstappen, F.W., de Vos, R.C., Beekwilder, J., van der Krol, S. and Bouwmeester, H.J. (2011) Reconstitution of the costunolide biosynthetic pathway in yeast and Nicotiana benthamiana. *PloS one*, **6**, e23255.

Nelson, D.R. (2009) The cytochrome p450 homepage. *Human genomics*, **4**, 59-65.

Paza, C., Carcamo, G., Silva, M., Becerra, J., Urrutia, H. and Sossa, K. (2013) Drimendiol, a drimane sesquiterpene with quorum sensing inhibition activity. *Natural product communications*, **8**, 147-148.

Pickett, J.A. (1985) PRODUCTION OF BEHAVIOUR-CONTROLLING CHEMICALS BY CROP PLANTS. *Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci.*, **310**, 235-239.

Pompon, D., Louerat, B., Bronine, A. and Urban, P. (1996) Yeast expression of animal and plant P450s in optimized redox environments. *Method Enzymol*, **272**, 51-64.

Prota, N., Bouwmeester, H.J. and Jongsma, M.A. (2013) Comparative antifeedant activities of polygodial and pyrethrins against whiteflies (Bemisia tabaci) and aphids (Myzus persicae). *Pest management science*.

Prota, N., Mumm, R., Bouwmeester, H.J. and Jongsma, M.A. (2014) Comparison of the chemical composition of three species of smartweed (genus Persicaria) with a focus on drimane sesquiterpenoids. *Phytochemistry*, **108**, 129-136.

Ralston, L., Kwon, S.T., Schoenbeck, M., Ralston, J., Schenk, D.J., Coates, R.M. and Chappell, J. (2001) Cloning, heterologous expression, and functional characterization of 5-epi-aristolochene-1,3-dihydroxylase from tobacco (Nicotiana tabacum). *Archives of biochemistry and biophysics*, **393**, 222-235.

Rodriguez, B., Zapata, N., Medina, P. and Vinuela, E. (2005) A complete 1H and 13C NMR data assignment for four drimane sesquiterpenoids isolated from Drimys winterii. *Magnetic resonance in chemistry : MRC*, **43**, 82-84.

Sallaud, C., Rontein, D., Onillon, S., Jabes, F., Duffe, P., Giacalone, C., Thoraval, S., Escoffier, C., Herbette, G., Leonhardt, N., Causse, M. and Tissier, A. (2009) A novel pathway for sesquiterpene biosynthesis from Z,Z-farnesyl pyrophosphate in the wild tomato Solanum habrochaites. *The Plant cell*, **21**, 301-317.

Schmelz, E.A., Huffaker, A., Sims, J.W., Christensen, S.A., Lu, X., Okada, K. and Peters, R.J. (2014) Biosynthesis, elicitation and roles of monocot terpenoid phytoalexins. *The Plant journal : for cell and molecular biology*, **79**, 659-678.

Starkenmann, C., Luca, L., Niclass, Y., Praz, E. and Roguet, D. (2006) Comparison of volatile constituents of Persicaria odorata(Lour.) Sojak (Polygonum odoratum Lour.) and Persicaria hydropiper L. Spach (Polygonum hydropiper L.). *J Agric Food Chem*, **54**, 3067-3071.

Steele, C.L., Crock, J., Bohlmann, J. and Croteau, R. (1998) Sesquiterpene synthases from grand fir (Abies grandis). Comparison of constitutive and wound-induced activities, and cDNA isolation, characterization, and bacterial expression of delta-selinene synthase and gamma-humulene synthase. *The Journal of biological chemistry*, **273**, 2078-2089.

Swaminathan, S., Morrone, D., Wang, Q., Fulton, D.B. and Peters, R.J. (2009) CYP76M7 is an ent-cassadiene C11alpha-hydroxylase defining a second multifunctional diterpenoid biosynthetic gene cluster in rice. *The Plant cell*, **21**, 3315-3325.

Takahashi, S., Yeo, Y.S., Zhao, Y., O'Maille, P.E., Greenhagen, B.T., Noel, J.P., Coates, R.M. and Chappell, J. (2007) Functional characterization of premnaspirodiene oxygenase, a cytochrome P450 catalyzing regio- and stereo-specific hydroxylations of diverse sesquiterpene substrates. *The Journal of biological chemistry*, **282**, 31744-31754.

Teoh, K.H., Polichuk, D.R., Reed, D.W., Nowak, G. and Covello, P.S. (2006) Artemisia annua L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. *FEBS letters*, **580**, 1411-1416.

Urban, P., Mignotte, C., Kazmaier, M., Delorme, F. and Pompon, D. (1997) Cloning, yeast expression, and characterization of the coupling of two distantly related Arabidopsis thaliana NADPH-Cytochrome P450 reductases with P450 CYP73A5. *Journal of Biological Chemistry*, **272**, 19176-19186.

van Herpen, T.W., Cankar, K., Nogueira, M., Bosch, D., Bouwmeester, H.J. and Beekwilder, J. (2010) Nicotiana benthamiana as a production platform for artemisinin precursors. *PloS one*, **5**, e14222.

Wang, Q., Hillwig, M.L., Okada, K., Yamazaki, K., Wu, Y., Swaminathan, S., Yamane, H. and Peters, R.J. (2012) Characterization of CYP76M5-8 indicates metabolic plasticity within a plant biosynthetic gene cluster. *The Journal of biological chemistry*, **287**, 6159-6168.

Wu, S., Jiang, Z., Kempinski, C., Eric Nybo, S., Husodo, S., Williams, R. and Chappell, J. (2012) Engineering triterpene metabolism in tobacco. *Planta*, **236**, 867-877.

Wube, A.A., Bucar, F., Gibbons, S. and Asres, K. (2005) Sesquiterpenes from Warburgia ugandensis and their antimycobacterial activity. *Phytochemistry*, **66**, 2309-2315.

Yong, K.W.L., Salim, A.A. and Garson, M.J. (2008) New oxygenated diterpenes from an Australian nudibranch of the genus Chromodoris. *Tetrahedron*, **64**, 6733-6738.

Zapata, N., Budia, F., Vinuela, E. and Medina, P. (2009) Antifeedant and growth inhibitory effects of extracts and drimanes of Drimys winteri stem bark against Spodoptera littoralis (Lep., Noctuidae). *Ind Crop Prod*, **30**, 119-125.

Figure legends

Figure 1. Proposed biosynthetic pathway from farnesyl diphosphate (FPP) to drimenol and polygodial (adapted from Pickett, 1985). Carbon atoms are numbered in the drimenol structure. Enzymes relevant for this study have been indicated. FPS: farnesyl diphosphate synthase; HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase; DS: drimenol synthase. The biosynthesis of polygodial is believed to involve the oxidation of drimenol, which may be obtained in one step by the cyclization of farnesyl diphosphate, and enzymatically controlled oxidation steps by one or more P450s.

Figure 2. GC–MS chromatogram of the n-dodecane layer from the yeast strain WAT11 expressing drimenol synthase from *Persicaria hydropiper* (*PhDS*) and an empty vector as control. The predominant peak was identified as drimenol (Rt = 17.17 min). Mass fragmentation pattern and the structure of drimenol are also shown.

Figure 3. *In vitro* conversion of drimenol. GC-MS analysis of enzyme reactions employing microsomes of WAT11 yeast strain expressing the selected P450 from *Persicaria hydropiper*, compared to the microsomes prepared from the control yeast strain transformed with an empty pYEDP60 plasmid.

Figure 4. LC-MS chromatogram of the ethyl acetate extract of yeast co-transformed with *PhDS* and *PhDOX1* compared with the empty vector control. The scale of the Y-axis is identical in both chromatograms.

Figure 5. GC-MS chromatograms of dichloromethane extracts of *N. benthamiana* leaves transiently expressing *PhDS* and *PhDOX1* and an empty vector pBinPlus control construct. Seven novel peaks (A-G) were detected. Mass fragmentation spectra of these peaks are shown in supplemental Figure S1. Compound A was identified as drimendiol and compound F was identified as cinnamolide (Figure 6, supplemental Figure S4), all other peaks could not be identified.

Figure 6. LC-MS chromatogram of a methanolic extract of *N. benthamiana* leaves transiently expressing *PhDS* and *PhDOX1*.

Figure 7. Effect of increasing concentrations of cinnamolide on the feeding behaviour of *Bemisia tabaci* and *Myzus persicae* at 6 h from the start of the experiment compared to published results for polygodial (Prota *et al.* 2013).

Antifeedant index (AI%) = [(C-T)/(C+T)] x 100. Positive values indicate an antifeedant effect, negative values a phagostimulant effect. Error bars indicate the standard error, 6 replicates for aphids and 8 for whiteflies, average of 98 whiteflies and 20 aphids per replicate.

Figure 8. Putative pathway from drimendiol to cinnamolide. PhDOX1 hydroxylates drimenol to form drimendiol. In the next step, this hydroxyl group is oxidized into an aldehyde by an endogenous plant or yeast enzyme. This aldehyde reacts with the other hydroxyl group to form a hemiacetal that is further oxidized to cinnamolide.

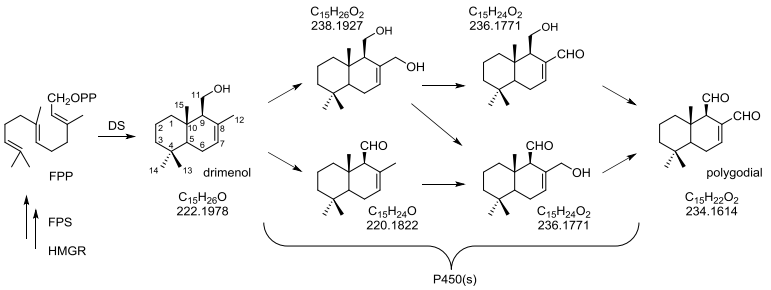

Figure 1. Proposed biosynthetic pathway from farnesyl diphosphate (FPP) to drimenol and polygodial (adapted from Pickett, 1985). Carbon atoms are numbered in the drimenol structure. Enzymes relevant for this study have been indicated. FPS: farnesyl diphosphate synthase; HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase; DS: drimenol synthase. The biosynthesis of polygodial is believed to involve the oxidation of drimenol, which may be obtained in one step by the cyclization of farnesyl diphosphate, and enzymatically controlled oxidation steps by one or more P450s.

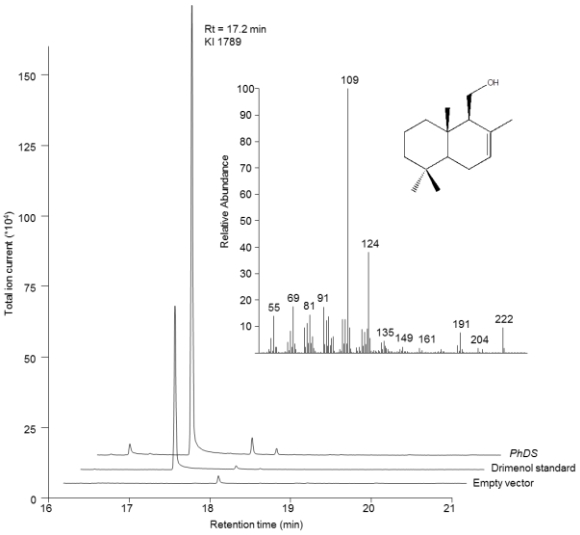

Figure 2. GC–MS chromatogram of the n-dodecane layer from the yeast strain WAT11 expressing drimenol synthase from *Persicaria hydropiper* (*PhDS*) and an empty vector as control. The predominant peak was identified as drimenol (Rt = 17.17 min). Mass fragmentation pattern and the structure of drimenol are also shown.

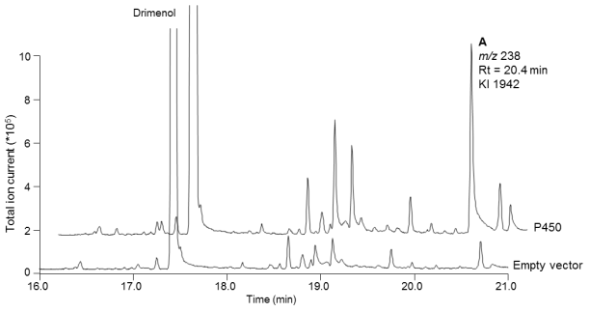

Figure 3. In vitro conversion of drimenol. GC-MS analysis of enzyme reactions employing microsomes of WAT11 yeast strain expressing the selected P450 from Persicaria hydropiper, compared to the microsomes prepared from the control yeast strain transformed with an empty pYEDP60 plasmid.

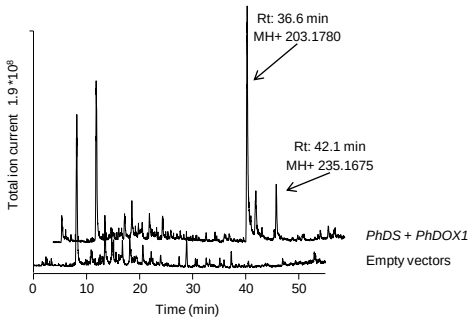

Figure 4. LC-MS chromatogram of the ethyl acetate extract of yeast co-transformed with PhDS and PhDOX1 compared with the empty vector control. The scale of the Y-axis is identical in both chromatograms.

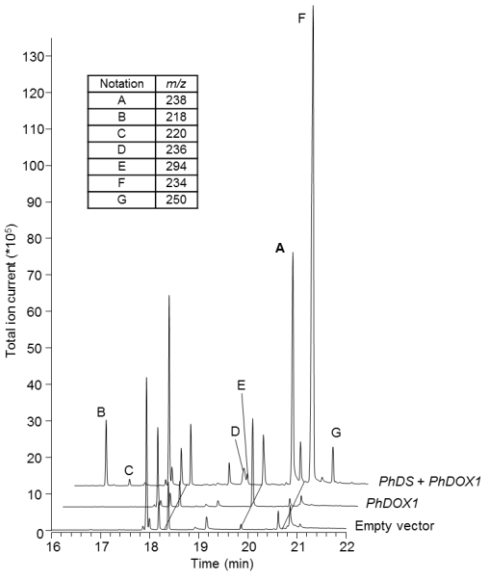

Figure 5. GC-MS chromatograms of dichloromethane extracts of N. benthamiana leaves transiently expressing PhDS and PhDOX1 and an empty vector pBinPlus control construct. Seven novel peaks (A-G) were detected. Mass fragmentation spectra of these peaks are shown in supplemental Figure S1.

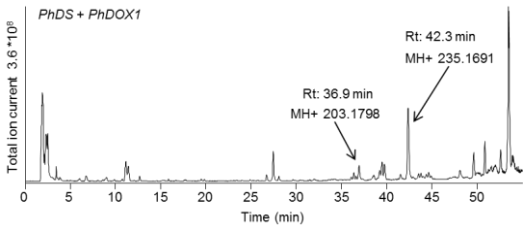

Figure 6. LC-MS chromatogram of a methanolic extract of N. benthamiana leaves transiently expressing PhDS and PhDOX1.

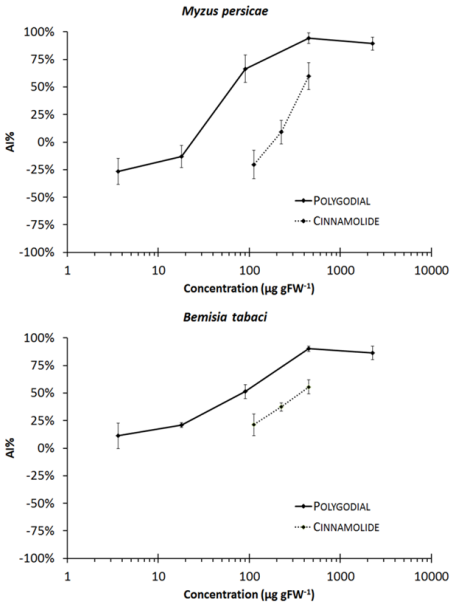

Figure 7. Effect of increasing concentrations of cinnamolide on the feeding behaviour of Bemisia tabaci and Myzus persicae at 6 h from the start of the experiment compared to published results for polygodial (Prota et al. 2013).
Antifeedant index (AI%) = [(C-T)/(C+T)] x 100. Positive values indicate an antifeedant effect, negative values a phagostimulant effect. Error bars indicate the standard error, 6 replicates for aphids and 8 for whiteflies, average of 98 whiteflies and 20 aphids per replicate.

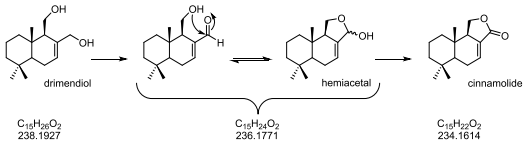

Figure 8. Putative pathway from drimendiol to cinnamolide. PhDOX1 hydroxylates drimenol to form drimendiol. In the next step, this hydroxyl group is oxidized into an aldehyde by an endogenous plant or yeast enzyme. This aldehyde reacts with the other hydroxyl group to form a hemiacetal that is further oxidized to cinnamolide.