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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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SUMMARY

The sesquiterpenoid polygodial, which belongs 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 alone. 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 catalyses 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 to be 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 about 200–400 µg g^{-1} fresh weight against both whiteflies and aphids. The putative additional physiological and biochemical requirements for polygodial biosynthesis and stable storage in plant tissues are discussed.

Keywords: drimane sequiterpenes, drimenol, *Persicaria hydropiper*, insect deterrent, cinnamolide, drimendiol, CYP76AJ1.

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 (Kubo and Ganjian, 1981; Escalera *et al.*, 2008). 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*) (Starkenmann *et al.*, 2006; Prota *et al.*, 2014). It was reported that *P. hydropiper* produces and stores polygodial in the leaves and flowerheads in specialized epidermal cavities called valvate or irritant glands (Hagendoorn *et al.*, 1994; Derita *et al.*, 2008; 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*) catalysing 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 liquid chromatography quadrupole time-of-flight mass spectrometry. 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 analysed the chemical profile of three *Persicaria* species, *P. hydropiper*, *Persicaria maculosa* and *Persicaria minor* (Prota *et al.*, 2014). *Persicaria hydropiper* was shown to produce by far the highest amounts of polygodial [6.2 mg g^{-1} fresh weight (FW) 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 expressed sequence tag (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 about 30 times higher abundance in *P. hydropiper* than in *P. maculosa*. The complete open reading frame (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 ORF of 1677 bp, encoding a protein of 559 amino acid residues and a predicted molecular weight of 64 729 Da. 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
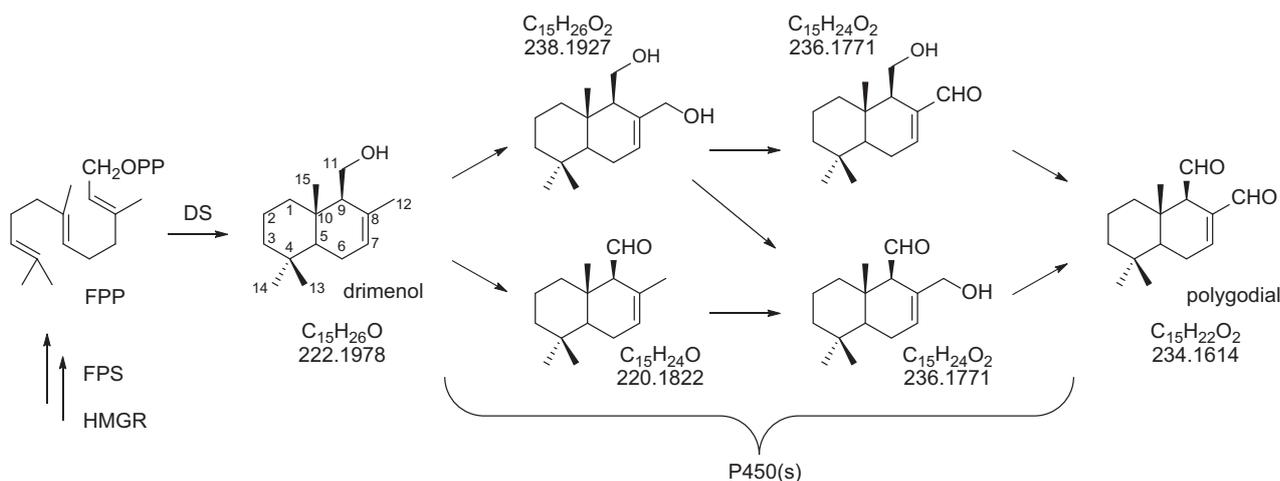
Figure 1. Proposed biosynthetic pathway from farnesyl diphosphate (FPP) to drimenol and polygodial (adapted from Pickett, 1985).
Carbon atoms are numbered in the drimenol structure. Enzymes relevant for this study are indicated. FPS, farnesyl diphosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA 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 FPP, and enzymatically controlled oxidation steps by one or more P450s.
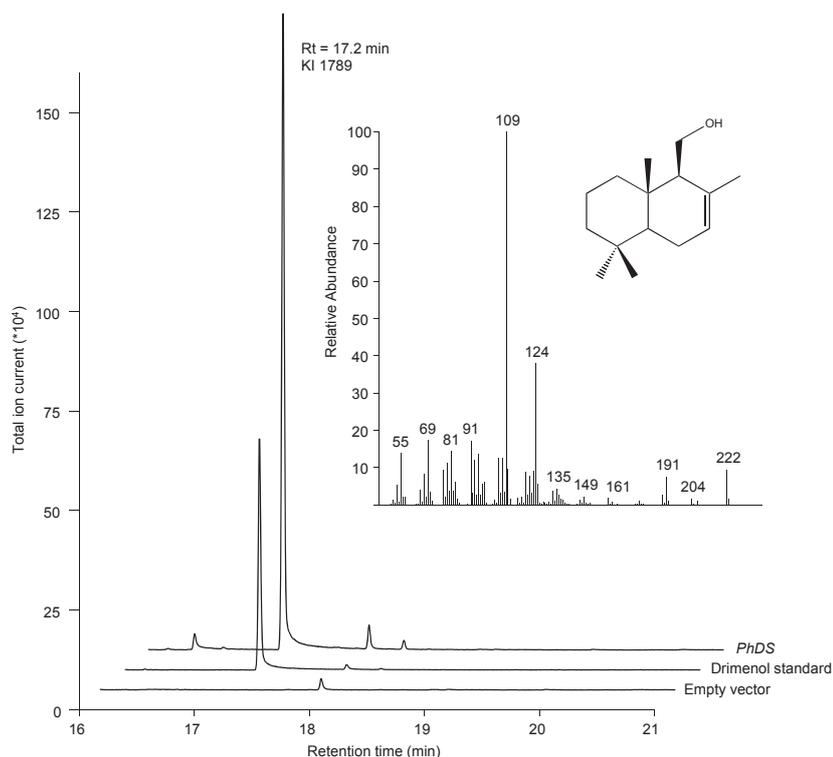
Figure 2. GC-MS chromatogram of the *n*-dodecane layer from the yeast strain WAT11 expressing drimenol synthase from *Persicaria hydropiper* (*PhDS*) and an empty vector as control. The predominant peak was identified as drimenol (Rt = 17.17 min). The mass fragmentation pattern and the structure of drimenol are also shown.

was identified for VoDS (Figure S1 in the Supporting Information). 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 about 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) to drimenol was detected by GC-MS analysis in the assay with the P450 microsomes but not in the control yeast strain with an 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 showed no 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 analysed by LC-Orbitrap-Fourier transform mass spectrometry (FTMS). The analysis revealed a major compound with a mass of MH+ 203.1780 (Figure 4). This compound was subsequently isolated by preparative HPLC. An NMR analysis identified the compound as drimendiol (Figure S2), completely in line with the previously published identification data for 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 from 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 only showed up to 52% sequence similarity with the CYP76F and CYP76B subfamilies. Therefore, the enzyme was classified by Nelson (2009) into a
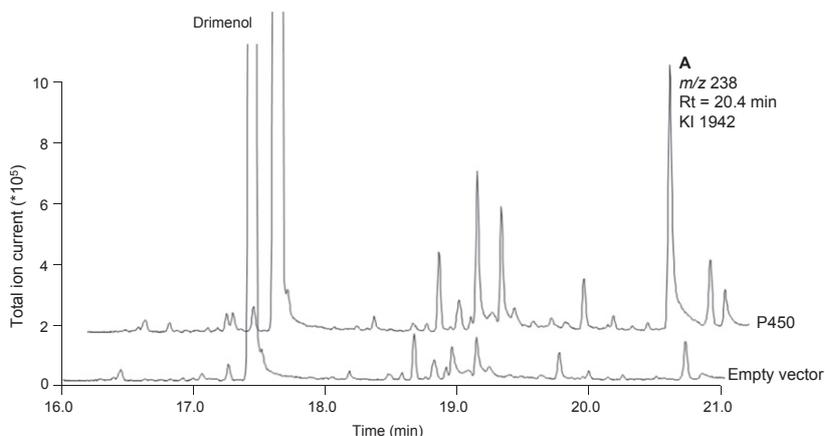
Figure 3. *In vitro* conversion of drimenol.
GC-MS analysis of enzyme reactions employing microsomes of yeast strain WAT11 expressing the selected P450 from *Persicaria hydropiper*, compared with the microsomes prepared from the control yeast strain transformed with an empty pYEDP60 plasmid.
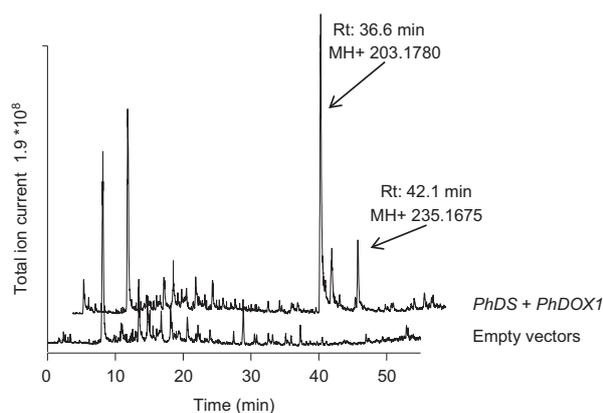

Figure 4. LC-MS chromatogram of the ethyl acetate extract of yeast co-transformed with *PhDS* and *PhDOX1* compared with the empty vector control. The scale of the *Y*-axis is identical in both chromatograms.

new subfamily and designated CYP76AJ1. Kinetic analysis using the yeast microsomal preparations and in the presence of excess NADPH showed that PhDOX1 efficiently catalyses 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 *Agrobacterium 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 about 392 μg of drimenol per gFW. In contrast to our expectation, targeting both AtFPS2 and PhDS 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 (Figures 5 and 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) to 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 of 2137 versus 1807.

Nicotiana benthamiana leaves transiently expressing *PhDS* and *PhDOX1* were also extracted with methanol and analysed by LC-MS (Figure 6). A compound with a similar mass and retention time to 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 to 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 (Figure S4). The NMR data corresponded completely to what was reported by 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
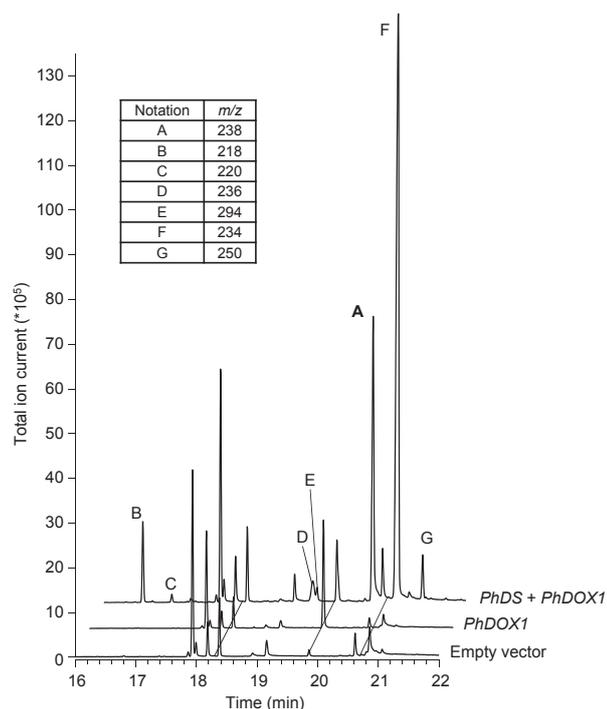
Figure 5. GC-MS chromatograms of dichloromethane extracts of *Nicotiana benthamiana* leaves transiently expressing *PhDS* and *PhDOX1* and an empty vector pBinPlus control construct.

Seven novel peaks (A–G) were detected. Mass fragmentation spectra of these peaks are shown in Figure S1. Compound A was identified as drimendiol and compound F was identified as cinnamolide (Figures 6 and S4); none of the other peaks could be identified.

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 h post-inoculation with the green peach aphid *Myzus persicae* or silverleaf whitefly *Bemisia tabaci* (genotype B), respectively. The 6-h time point was selected as the most reliable because most insects were actively feeding at that time, and the time for induction of potentially confounding secondary responses resulting from the applied compound was kept to a minimum. The feeding

preference was assessed using the antifeedant index (AI) and compared with the published data for polygodial (AI%) (Kutas and Nádasy, 2005; Prota *et al.*, 2013). The AI% of cinnamolide increased with increasing concentration applied, as seen in Figure 7, meaning that there was a clear dose-dependent preference for the control discs over the treated ones, similar to what was 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 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 is generally difficult when the proteins are 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; van Herpen *et al.*, 2010; Liu *et al.*, 2011; Wu *et al.*, 2012). However, similar to the expression of (*E*)-β-farnesene synthase in Arabidopsis (Beale *et al.*, 2006), we observed for *PhDS* that under the same promoter the native cytosolic enzyme was 40–400 times more active than 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.
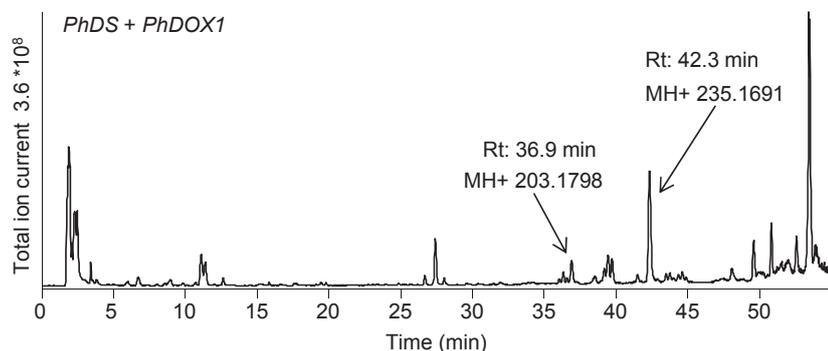

Figure 6. LC-MS chromatogram of a methanolic extract of *Nicotiana benthamiana* leaves transiently expressing *PhDS* and *PhDOX1*.

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 with published results for polygodial (Prota *et al.*, 2013).

Antifeedant index (AI%) = $[(C - T)/(C + T)] \times 100$. Positive values indicate an antifeedant effect, negative values a phagostimulant effect. Error bars indicate the standard error, six replicates for aphids and eight for whiteflies with an average of 98 whiteflies and 20 aphids per replicate.
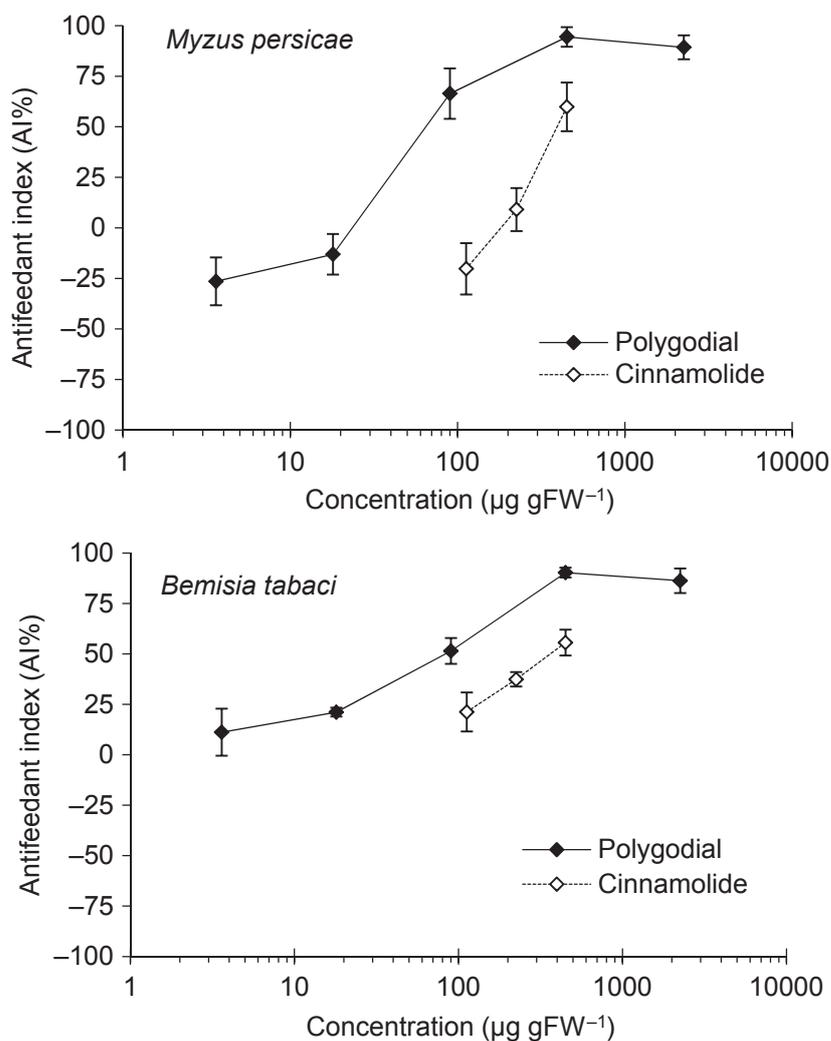
Kwon *et al.* (2014) described a drimenol synthase from *V. officinalis* and speculated about the reaction mechanism. Chemically, the most logical route, the authors argued, would start by protonation at C3 of FPP ('class II mechanism'), followed by two cyclizations, deprotonation and hydrolysis of the diphosphate group to an alcohol. This mechanism, however, would involve a DxDD motif in the protein, which was not found. Instead only one aspartate-rich motif, DDxxD, was found, which is 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.* (2014), a reaction sequence towards drimenol in which cleavage of the diphosphate is the first step is then very difficult to envisage from a chemical point of view, but nevertheless seems to take place. Our *P. 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 further 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 sesquiterpene synthases from *Abies grandis* (δ-selinene synthase and γ-humulene synthase) characterized by Steele *et al.* (1998) also have two DDxxD motifs. A second study by Little and Croteau (2002) took a closer look at the role of the unusual second DDxxD in *A. grandis* sesquiterpene synthases and showed 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 a role for 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 Figure S1) are also drimane-type sesquiterpenes. Lack of standards prevented us from further identifying these low-abundance compounds. However, compounds 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*, as previously described by others (van Herpen *et al.*, 2010; Liu *et al.*, 2011; Hofer *et al.*, 2013).

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 (Swaminathan *et al.*, 2009; Wang *et al.*, 2012; Guo *et al.*, 2013; Hofer *et al.*, 2013).

The proposed pathway to cinnamolide in yeast and *N. benthamiana* starts with the formation of drimenol from 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 to give a hemiacetal, as depicted in Figure 8, and then be oxidized into cinnamolide. Cyclization through hemiacetal formation has been described previously 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 catalyses 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 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%

$C_{15}H_{26}O_2$
238.1927

$C_{15}H_{24}O_2$
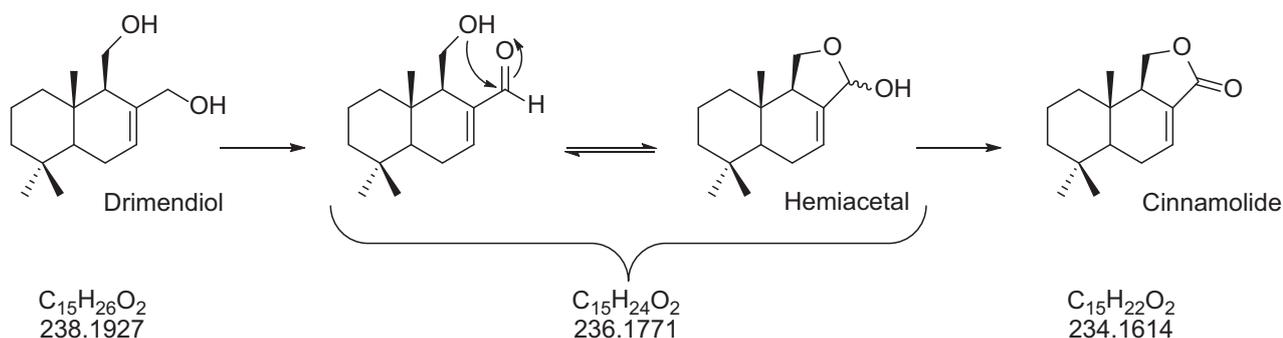236.1771

$C_{15}H_{22}O_2$
234.1614

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.

sequence similarity with known P450s from the CYP76 family. Comparative screening of the 20 most abundant P450s found in the *P. hydropiper* database search revealed already six potential terpene oxidases with at least ten times higher abundance in *P. hydropiper* than in *P. maculosa*. Besides PhDOX1, one or more P450s could clearly 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 was 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 that possess an amine group, which makes it very difficult to prove production of polygodial in a heterologous expression system such as yeast or tobacco. Secondly, in *P. hydropiper* polygodial accumulates in cavities that are devoid of other cell content (Hagendoorn *et al.*, 1994; Derita *et al.*, 2008). This indicates that the production of the dialdehyde in plants may require a suit of additional factors and secretory structures to prevent the dialdehyde from spontaneously reacting to amino-groups of proteins and to prevent that aldehyde being further converted to form cinnamolide. It is possible that 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), supporting their close biosynthetic relationship.

Drimendiol has been isolated from *Drimys winteri* and was reported to have antifeedant 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 *et al.* (1983), 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. 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 antifeedant for both the silverleaf whitefly *Bemisia tabaci* and the green peach aphid *Myzus persicae* (Figure 7), although it is not as strong as polygodial, with ED$_{50}$ values of 25 versus 195 µg gFW^{-1} and 54 vesus 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

Synthesis of cDNA and GS FLX Titanium General Library Preparation

Synthesis of cDNA was performed using a SMART PCR cDNA Synthesis kit (Clontech, http://www.clontech.com/) according to the 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 (Thermo Fisher Scientific, http://www.thermofisher.com/). To facilitate the removal of the poly(A)-tail prior to sequencing, first-strand cDNA synthesis was carried out with a modified 3′ SMART CDS primer IIA containing a rare cutter restriction endonuclease site in the 5′ region of the oligo(dT) sequence. Amplification of cDNA was performed using the Advantage 2 PCR kit (Clontech) following the instructions in the SMART PCR cDNA Synthesis protocol. Double-stranded cDNA was purified using the QiaQuick PCR purification kit (Qiagen, http://www.qiagen.com/). The quality of the cDNA was determined on a BioAnalyzer using a DNA 7500 chip (Agilent Technologies, http://www.agilent.com/). Normalization of cDNA was carried out using the TRIMMER cDNA Normalization kit (Evrogen, http://evrogen.com/) as per the manufacturer's instructions. Amplification of normalized cDNA was performed using the Advantage 2 PCR kit (Clontech) following the instructions in the TRIMMER cDNA Normalization manual. Instead of using 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).

Random cDNA shearing prior to the FLX Titanium library was performed by nebulization of 15–20 µg of normalized cDNA according the instructions in the GS FLX Titanium General Library Preparation manual (Roche Applied Science, http://www.roche.com/). After nebulization, the randomly sheared cDNA was purified using the QiaQuick PCR purification kit (Qiagen) and the size distribution was determined with a DNA 1000 chip (Agilent Technologies). 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 the manufacturer's instructions (Thermo Fisher Scientific) and the supernatant containing the digested cDNA without biotinylated ends was collected and purified using the MinElute PCR

purification kit (Qiagen). Exclusion of smaller-sized fragments was performed using the double solid-phase reverse immobilization method as described in the Roche GS FLX Titanium General Library Preparation protocol (Roche Applied Science). Further library preparation was performed according to the standard GS FLX Titanium General Library Preparation Method protocol as supplied by Roche Applied Science. The cDNA samples were analysed by massive parallel pyrosequencing using a Genome Sequencer FLX Titanium sequencing platform (Roche Applied Science).

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 was sequenced. Comparative screening of the libraries allowed the identification of a sesquiterpene synthase with a much higher apparent abundance in *P. hydropiper* than *P. maculosa*. The full-length putative drimenol synthase (*PhDS*) gene was amplified using high-fidelity Phusion polymerase (Thermo Fisher Scientific, http://www.thermofisher.com/) and the primers PhDS-F (*Bsa*I) 5′-GTGACGGTCTCCCATGTCTACTGCCGTTAACGTCC-3′ and PhDS-R (*Not*I) 5′-GTGACGCGGCCGCCTAAATCGGAATGGGATCGG TGA-3′ with the addition of the *Bsa*I restriction site (with *Nco*I overhang) and *Not*I restriction sites, allowing subsequent cloning into the pYES3/CT yeast expression vector (Invitrogen, http://www.invitrogen.com/), containing the TRP1 auxotrophic selection marker. The *PhDS* gene was transformed into yeast strain WAT11, expressing the *A. 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 the literature, revealed several potential terpene hydroxylases with, once again, a much higher abundance in *P. hydropiper* than *P. maculosa*. The most abundant full-length *P450* gene was cloned, using the *Not*I/*Pac*I 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 into the yeast strain WAT11 with the *PhDS* from *P. hydropiper*. After transformation, yeast clones expressing *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 or 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. A sample of this was analysed by GC-MS.

Plasmid construction for expression in *N. benthamiana*

The *PhDS* and *PhDOX1* genes were cloned into ImpactVector1.1 (http://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 *N. benthamiana*

Agrobacterium 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 h in LB medium 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 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer containing 10 mM MgCl$_2$ and 100 μM acetosyringone (4′-hydroxy-3′,5′-dimethoxyacetophenone, Sigma, http://www.sigmaaldrich.com/) to a final OD_{600} of 0.5, followed by incubation at 20°C with shaking at 50 rpm for 3 h. For co-infiltration, equal volumes of the *A. tumefaciens* strains were mixed. *Nicotiana benthamiana* plants were grown from seeds on soil in a greenhouse with a minimum of 16-h light. Day temperatures were approximately 28°C and night temperatures 25°C. Strain mixtures were infiltrated into leaves of 4-week-old *N. benthamiana* plants using a 1 ml syringe without a 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 analyse the compounds accumulated in the leaves, the harvested plant material was snap frozen and ground in liquid nitrogen. Five hundred milligrams 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 1000 *g* and the clear part of the solution was transferred to a fresh vial. Finally, the extracts were dehydrated using anhydrous Na$_2$SO$_4$ and concentrated by evaporating the solvent to a volume of about 0.5 ml. Analysis of the samples was performed by GC-MS (Agilent Technologies).

GC-MS analysis

Analytes from 1-μl samples were separated using a gas chromatograph (5890 series II, Hewlett-Packard, http://www.hp.com/) equipped with a 30 m × 0.25 mm column with 0.25 μm film thickness (ZB-5, Phenomenex, http://www.phenomenex.com/) using helium as the carrier gas at flow rate of 1 ml min^{-1}. 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^{-1} 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 the 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, https://www.thermofisher.com/) 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 electrospray ionization 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^{-1} 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% methanol. 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 analysed by NMR.

Microsome preparation and *in vitro* enzyme assay

The YEDP60 plasmid containing PhDOX1 was transformed into the 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 (Pompon *et al.*, 1996; Bertea *et al.*, 2001). 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 mм potassium phosphate buffer (pH = 7.5), 3–200 μм drimenol or 200 μм 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 mм 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 10-fold concentrated under nitrogen flow. The samples were dried using anhydrous Na_2SO_4 and used for GC-MS analysis. The peak area of the product formed was used to calculate K_M.

NMR spectroscopy

NMR analysis of the plant extract was performed at Spinnovation Analytical BV (http://www.spinnovation-analytical.com/) on a Bruker Avance III 500 MHz spectrometer equipped with a 5-mm CPTCI cryo probe (^1H–^{13}C/^{15}N/^2H + Z-gradients) operating at 303 K. The structural identification of polygodial, used as a reference compound, is based on One-dimensional (1D)-^1H, ^1H-^1H-DQF-COSY, ^1H-^1H-TOCSY, ^1H-^1H-NOESY, ^1H-^{13}C-HSQC and ^1H-^{13}CHMBC spectra. The structural identification of the purified compound is based on the same selection of NMR experiments, with the exception of the ^1H-^{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 (http://www.biqualys.nl/en) on a Bruker

Advance III 600 MHz spectrometer equipped with a cryo-probe. 1D-^1H NMR measurements of 128 scans and a delay time 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) ^1H measurements (COSY, HSQC and 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 *AtHMGR*, *AtFPS*, *PhDS* and *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 *B. tabaci* (genotype B) and the green peach aphid *M. persicae*. Whiteflies were reared on tomato (cv. Moneymaker) in a greenhouse at 26°C and 60% relative humidity with a photoperiod of 16-h light and 8-h dark. To perform the assays, adult flies of both genders were collected from leaves with an aspirator. They were cold-anaesthetized 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 (*M. 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 discs 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-week-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 the bottom of a Petri dish 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 (disc), 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 discs 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 whitefly assays were performed in a climate chamber at 25°C, 60% humidity and 16-h/8-h light/dark photoperiod. The assays were continued for either 18 or 24 h and the feeding insects

counted at 30 min, 1, 2, 6 and 18 h for whiteflies and at 30 min, 1, 2, 6 and 24 h for aphids. Insects were considered to be feeding when they were immobile on one spot on a leaf disc. Five leaf discs were weighed to be able to determine the concentration of the insect repellent compounds in relationship to the FW of the leaf area treated. The average mass of each disc was found to be 34 mg. It was estimated that the concentrations of coated cinnamolide corresponded 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 AI% was calculated according to Kutas and Nádasy, as follows: AI% = $[(C - T)/(C + T)] \times 100$, where C is the number of insects feeding on the control plant and T the number of insects feeding on the treatment plant (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 with the treatment), a Probit analysis was carried out using the PASW statistics 18 (SPSS Inc. http://www.spss.com.hk/) package.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.
Figure S1. Alignment of drimenol synthase amino acid sequences from *Persicaria hydropiper* and *Valeriana officinalis.*
Figure S2. Mass fragmentation spectra of compounds detected by GC-MS on extracts from *PhDS* and *PhDOX1* agro-infiltrated *Nicotiana benthamiana* leaves.
Figure S3. ^{1}H-NMR and ^{13}C-NMR parameters of drimendiol NMR analysis and the structure of drimendiol. Atom numbers are referred to in the table.
Figure S4. ^{1}H-NMR and ^{13}C-NMR parameters of cinnamolide NMR analysis and the structure of cinnamolide. Atom numbers are referred to in the table.

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