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The molecular basis of host plant selection in *Melaleuca quinquenervia* by a successful biological control agent

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ABSTRACT

Melaleuca quinquenervia (Cav) S.T. Blake (broadleaf paperbark) is an Australian tree that has become a serious weed in many places around the world. Two insects *Oxyops vitiosa* (the melaleuca weevil), and *Boreioglycaspis melaleucae* (the melaleuca psyllid), which were introduced to Florida as part of a biological control programme, have been very effective in reducing survival and reproduction of this weed. There are two terpene chemotypes of *M. quinquenervia*; one rich in the sesquiterpene E-nerolidol whereas the other is rich in viridiflorol. Viridiflorol is a strong feeding deterrent for the melaleuca weevil and retards larval development. The larvae therefore avoid the viridiflorol-rich chemotype, in contrast, female melaleuca psyllids prefer to oviposit on these leaves. To identify the molecular basis of these preferences, we isolated and characterised two terpene synthases from the viridiflorol-rich chemotype, both of which utilise farnesyl pyrophosphate and have the same product profile. Chemotypic variation in terpenes in *M. quinquenervia* is under strong genetic control and the reproductive potential of each chemotype is limited by a different insect. These insects could, therefore, be selective agents for the maintenance of chemotypic variation in *M. quinquenervia*.

1. Introduction

Successful biological control programmes depend on there being a high degree of feeding specificity by insect herbivores. This requires extensive testing to provide a detailed understanding of the factors affecting host plant preferences prior to introducing new herbivores. A particularly successful biological control has been established for the woody weed *Melaleuca quinquenervia* (Cav) S.T. Blake (broadleaf paperbark) in Florida.

M. quinquenervia was introduced to southern Florida largely from Australia early in the 20th century and it has become one of the world's worst woody weeds. Herbivory by two Australian insect species significantly reduces the growth and reproduction of *M. quinquenervia* and both have been released as biocontrol agents in Florida (Wheeler and Ordung, 2006). *Oxyops vitiosa* (Pascoe) (Coleoptera: Curculionidae) (the 'melaleuca weevil') larvae feed on young expanding leaves (Wheeler, 2005) whereas *Boreioglycaspis melaleucae* Moore (Hemiptera: Psyllidae) (the 'melaleuca psyllid') feeds on the phloem of *M. quinquenervia* (Wheeler and Ordung, 2005).

M. quinquenervia has been reported to occur in two distinct chemical forms or "chemotypes". Chemotype 1 is characterized by acyclic foliar terpenes in particular high concentrations of the sesquiterpene *E*-nerolidol (74–95% of total oil), and the monoterpene linalool (Fig. 1) (Ireland et al., 2002; Wheeler et al., 2007). Chemotype 2 contains high concentrations of cyclic foliar terpenes especially the sesquiterpene viridiflorol (13–66% of total oil), and the monoterpenes 1,8-cineole and α-terpineol (Fig. 1) (Ireland et al., 2002; Wheeler et al., 2007). A diet rich in viridiflorol reduces the fecundity and larval survival of *O. vitiosa*. Therefore *O. vitiosa* larvae prefer to feed on nerolidol-rich chemotype 1 leaves (Wheeler, 2005). Although chemotypic variation does not affect survival and development of *B. melaleucae*, it does influence adult oviposition. Females prefer to oviposit on the leaves of the viridiflorol-rich chemotype 2 (Wheeler and Ordung, 2005). Thus in *M. quinquenervia*, small changes in the foliar terpene profile of otherwise morphologically identical plants has a profound influence on the success of a targeted biological control programme.

Chemotypic variation in foliar plant terpenes are widely described but poorly understood at the molecular level. The majority of the foliar terpenes found in Australian Myrtaceae are the direct products of terpene synthase enzymes (Keszei et al., 2008). This is thought to be also the case for the dominant foliar terpenes of *M. quinquenervia*, most of which have been shown to be direct products of terpene synthase enzymes in other species. For

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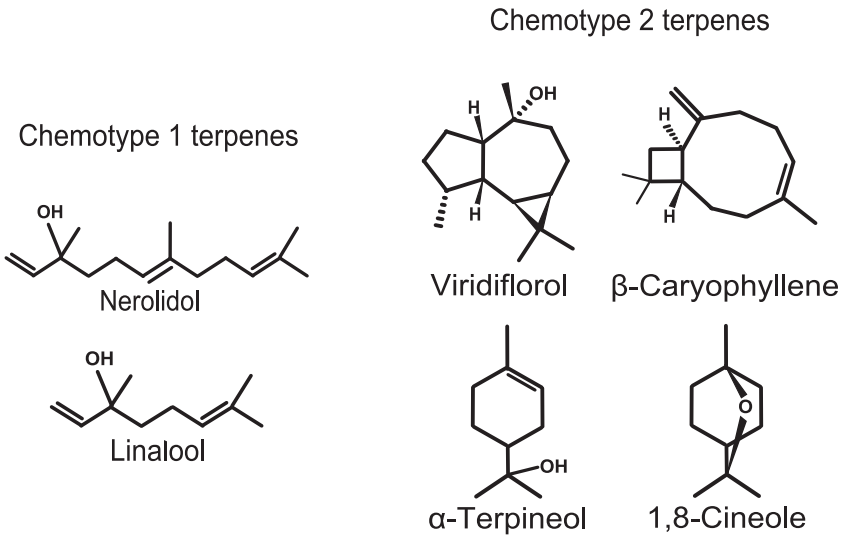

Fig. 1. The major terpenes found in *Melaleuca quinquenervia*. The acyclic compounds on the left are characteristic of chemotype 1, whilst the cyclic compounds on the right are characteristic of chemotype 2.

example, nerolidol synthase from maize (*Zea mays*) (Degenhardt and Gershenzon, 2000) and 1,8-cineole synthase from sage (*Salvia officinalis*) (Wise et al., 1998) have been functionally characterised. Therefore, the likely source of intra-specific foliar terpene variation is variation in terpene synthases. This could occur either through genomic variation, as shown in *Cannabis sativa* (de Meijer et al., 2003) or transcriptomic variations in terpene synthases, as shown in *Ocimum basilicum* (Iijima et al., 2004).

Thus *M. quinquenervia* provides a system in which an important ecological interaction (insect herbivory as a biocontrol agent) has been clearly linked to variation in a specific phenotypic trait (foliar terpene profile) and where there is potentially a direct link to the genes likely to be responsible (terpene synthase genes). This is one of the first eukaryotic systems in which natural variation in foliar terpenes can be linked from the gene through its phenotypic expression to an ecological interaction. Here we describe the isolation, cloning and functional characterisation of two novel genes from chemotype 2 of *Melaleuca quinquenervia* that allow the molecular basis of host pant selection to be explained.

2. Results

2.1. The previously described chemotypes are also present in M. quinquenervia samples studied here

Principal component analysis of the occurrence of sesquiterpenes in the foliage of *M. quinquenervia* (expressed as a proportion of the total peak area of both mono- and sesquiterpenes) resolved two distinct groups using two principal components (Supplementary Fig. S1) which correspond to the previously described nerolidol-rich chemotype 1 and the viridiflorol-rich chemotype 2 (Ireland et al., 2002; Wheeler et al., 2007).

2.2. Isolation and characterisation of terpene synthase cDNA clones from M. quinquenervia

Fragments of terpene synthase-like cDNA sequences were isolated from the leaves of *M. quinquenervia*. A SMART 5′ cDNA library was successfully constructed from chemotype 2 individuals. We isolated 3′ fragments from both chemotype 1 (27 fragments) and chemotype 2 (28), 5′ fragments from chemotype 2 (14) and full-length clones from chemotype 2 (15). From this, four unique full-length terpene synthase-like clones were obtained, designated MqTPS1, MqTPS2, MqTPS4 and MqTPS8 (Table 1). The deduced amino acid sequence of all four unique clones contain the conserved motifs responsible for the terpene synthase function of known terpene synthase enzymes, the RRX_8W, RWW, DDXXD and $(N,D)DX_2(S,T)X_3E$ motifs (seen in Fig. 2). Three of the sequences, MqTPS1, MqTPS2 and MqTPS4 encode proteins of 568 amino acids, whereas MqTPS8 is predicted to encode a 594 amino acid protein. The Signal P algorithm (Emanuelsson et al., 2007) predicts a signalling peptide, most likely a chloroplast targeting peptide, at the N-terminus of MqTPS8. This suggests MqTPS8 is a monoterpene synthase sequence.

2.3. Phylogenetic analysis

Eleven unique 3′ fragments were isolated from chemotype 2 of *M. quinquenervia*, of which seven cluster with angiosperm sesquiterpene synthases in the terpene synthase gene family TPSa and four cluster with angiosperm vegetative monoterpene synthases in the gene family TPSb (Fig. 3). Three unique 3′ sequences were isolated from chemotype 1 and all cluster in TPSa (Fig. 3). Three of the full-length clones isolated from chemotype 2, MqTPS1, MqTPS2 and MqTPS4, cluster in TPSa, whereas the fourth sequence, MqTPS8 clusters in TPSb.

2.4. Two unique sesquiterpene synthases from M. quinquenervia produce the same products

MqTPS1 and MqTPS2 were over-expressed in *E. coli* and the proteins were harvested, and incubated with geranyl pyrophosphate

Table 1
Identity/similarity of the deduced amino acid sequences isolated from chemotype 2 of *M. quinquenervia*. Values were calculated in BioEdit (Hall, 1999) using BLOSUM62. Identity values are above the diagonal (grey) and similarity values are below the diagonal.

	MqTPS1	MqTPS2	MqTPS4	MqTPS8
MqTPS1		0.98	0.99	0.07
MqTPS2	0.99		0.98	0.07
MqTPS4	0.99	0.98		0.07
MqTPS8	0.17	0.18	0.17	

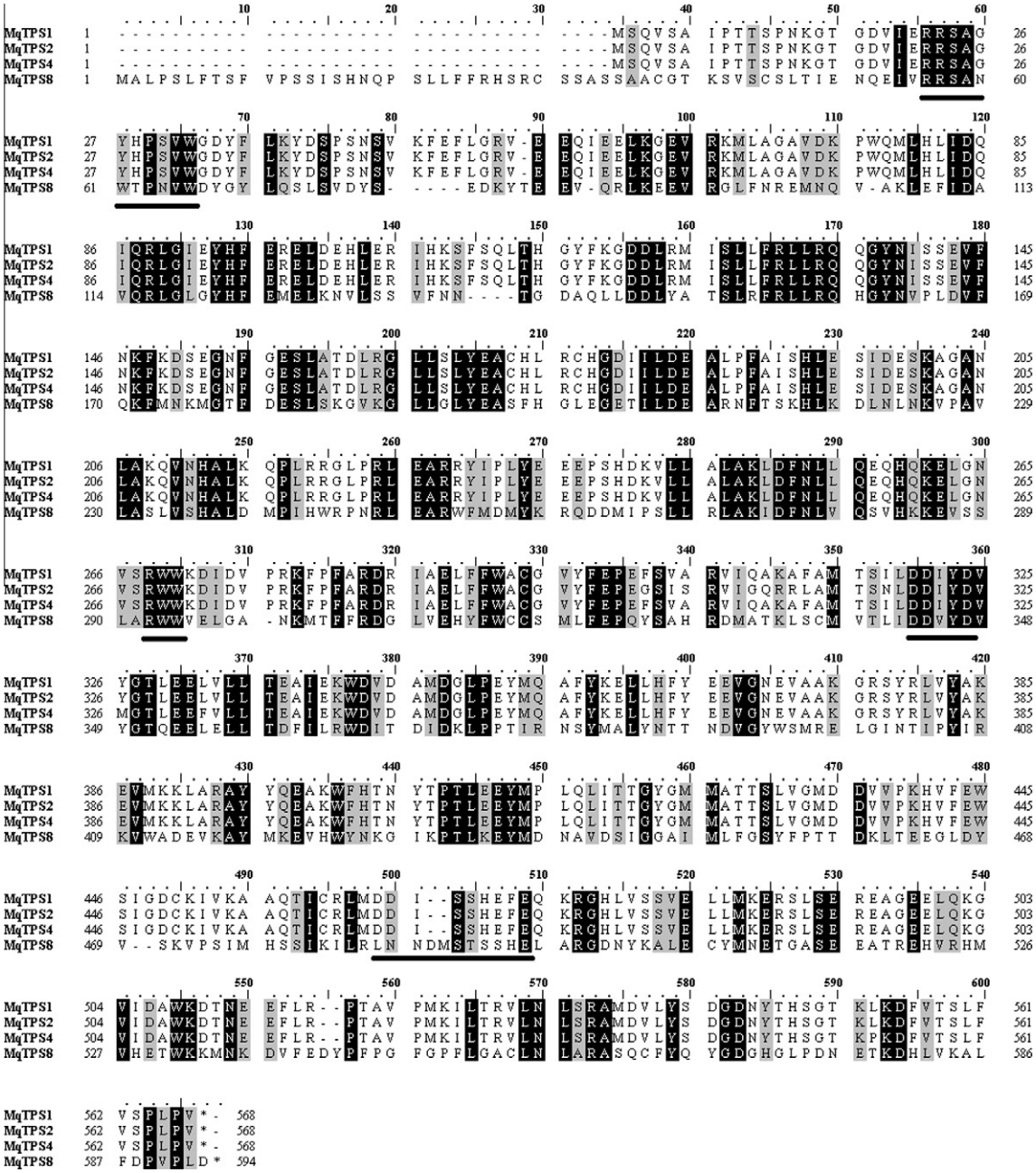

Fig. 2. The deduced amino acid sequences of four terpene synthases isolated from chemotype 2 of *M. quinquenervia* aligned in BioEdit (Hall, 1999). Residues highlighted in black are the same across all sequences and those in grey are similar in chemical properties across all sequences. The conserved motifs responsible for terpene synthase function are underlined.

(GPP) and farnesyl pyrophosphate (FPP). No terpenes were detected when incubated with GPP. The terpene product profiles of MqTPS1 and MqTPS2, when incubated with FPP, contained several sesquiterpenes dominated by viridiflorol, with small amounts of 1,6-germacradien-5-ol (Fig. 4). The major components of the enzyme product profile are also present in the profile from the foliar terpene extract (Fig. 4). MqTPS4 and MqTPS8 could not be subcloned into the expression vector.

2.5. Genomic and foliar transcriptomic presence of terpene synthases

Primers were designed based on cDNA sequences of the characterised viridiflorol synthases (MqTPS1 and MqTPS2). The sequences of MqTPS1 and MqTPS2 amplify from the genomic DNA of individuals from both chemotypes (1200 bp fragments) but they only amplify in the cDNA of chemotype 2 individuals (600 bp fragments) (Fig. 5).

3. Discussion

This study has identified terpene synthases responsible for one of the chemotypes of *Melaleuca quinquenervia* and thus the study provides a direct molecular link between herbivory by insect biocontrol agents and the chemical profile of the plants. There are few examples where the molecular basis of differential host plant selection has been demonstrated in such a major weed species.

Two well-defined chemotypes are present in the natural (Ireland et al., 2002) and naturalised range (Wheeler et al., 2007) of *M. quinquenervia* and both were found during this study.

Fig. 3. Maximum likelihood bootstrap phylogeny constructed on deduced amino acid sequences of the C-terminal domain of representative terpene synthases from all subfamilies of terpene synthase genes, including sequences from *Eucalyptus* (*E.*) and *Melaleuca* (*M.*). The numbers on the branches are bootstrap values of 100 replicates. The bold grey sequences were isolated from chemotype 1 and the bold black sequences were isolated from chemotype 2. Supplementary Table S2 contains the source and, if possible, GenBank accession numbers of all sequences used in this analysis.

Chemotype 1 consistently has a high concentration of *E*-nerolidol (54–99% total oil) and linalool (14–30%) and chemotype 2 consistently has a high concentration of viridiflorol (10–75%), 1,8-cineole (0.7–55%) and α-terpineol (0.3–14%). β-caryophyllene was found in significant proportions (0.4–28%) in all samples regardless of chemotype in both the natural and naturalised range (Ireland et al., 2002; Wheeler et al., 2007). These data suggest that *M. quinquenervia* in Florida (Wheeler et al., 2007) are a true subset of those found in Australia as analysed by Ireland et al. (2002) and in this study.

3.1. A new class of terpene synthases in Myrtaceae

All *M. quinquenervia* sequences isolated in this study are more closely related to *Melaleuca alternifolia* or *Callistemon citrinus* sequences than to any *Eucalyptus* sequences (Fig. 3). This is consistent with the conclusions of Bohlmann et al. (1998) who suggested that terpene synthases are more similar to other terpene synthases from the same species, than terpene synthases with the same function in different species. The majority of the sequences isolated from chemotype 2 of *M. quinquenervia* form a distinct group within clade 5 in Myrtaceae (Keszei et al., 2010a). The two characterised viridiflorol synthases form part of this group and represent the first characterised terpene synthases that produce tricyclic sesquiterpenes containing a seven, five and three membered ring. This further supports the hypothesis that clade 5 in Fig. 3 consists of terpene synthases that make tricyclic sesquiterpenes with a viridiflorane skeleton.

The three sequences isolated from chemotype 1 cluster in a clade known to contain β-caryophyllene synthases from Myrtaceae (Keszei et al., 2010a). These sequences were also found in the foliar transcriptome of chemotype 2 individuals. As β-caryophyllene is the only compound found in both chemotypes of *M. quinquenervia*, these sequences are most likely β-caryophyllene synthases.

3.2. Molecular basis of chemotypic variation

We have isolated the most abundant unique full-length terpene synthase clones from leaves of *M. quinquenervia* and two clones were confirmed to encode viridiflorol synthases following functional characterisation. The enzyme product profile closely resembles the foliar sesquiterpene profile of a chemotype 2 individual (Fig. 4). Chemotype 2 is characterised by the presence of high levels of viridiflorol, whereas this sesquiterpene could not be detected in chemotype 1 individuals. The characterised viridiflorol synthase sequences are present in the genome of all individuals irrespective of chemotype. However, they are absent from the foliar transcriptome of chemotype 1 individuals. Thus, the differential expression of terpene synthases explains why some individuals of *M. quinquenervia* have a significant foliar concentration of viridiflorol and others have no detectable foliar viridiflorol.

Nerolidol is the most abundant terpene in foliar terpene extracts from chemotype 1 individuals; however we were unable to identify an enzyme that produced nerolidol. There is evidence in yeast (*Saccharomyces cerevisiae*) that prenyl transferases can produce acyclic terpenes (Blanchard and Karst, 1993) such as nerolidol and, given the difficulty in isolating a nerolidol synthase from chemotype 1 individuals, it is likely that nerolidol in *M. quinquenervia* is not produced by a terpene synthase enzyme, but instead by a prenyl transferase.

3.3. How do multiple chemotypes persist in natural populations?

In most species of Myrtaceae where chemotypes have been described, the foliar terpene profile amongst the different

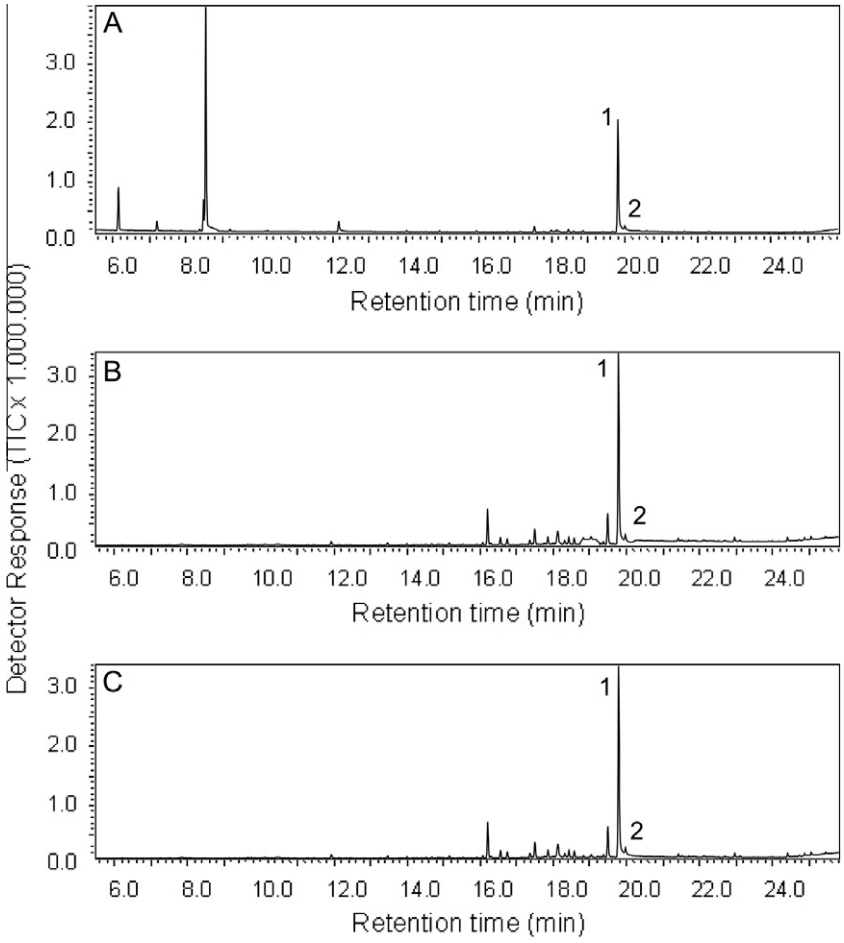

Fig. 4. Comparison of the foliar terpene extract from *Melaleuca quinquenervia* sample MQ001 (A), the chemotype 2 individual whose RNA was used in gene isolation; the product profile of MqTPS1 (B) and MqTPS2 (C) when incubated with farnesyl diphosphate (FPP). Viridiflorol is the dominant sesquiterpene in both enzyme profiles and the foliar extract, however there are other significant peaks in both enzyme product profiles that are also present in much lower concentrations in the foliar terpene extract, viridiflorol (1) and globulol (2).

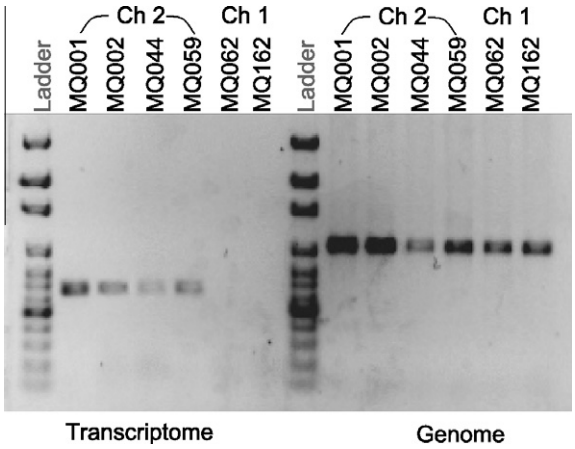

Fig. 5. Genomic (right) and foliar transcriptomic (left) amplification profile of viridiflorol synthase (MqTPS1) in foliar extracts of *M. quinquenervia*. Samples MQ001, MQ002, MQ044 and MQ059 are chemotype 2, whereas samples MQ062 and MQ164 are chemotype 1. The primers were designed across introns two and three, thus, 1200 bp fragments amplify in genomic DNA, whereas a 600 bp fragment amplifies in cDNA.

chemotypes is very similar but with large differences between the relative abundance of just a few compounds (Keszei, 2006). For example, there are six chemotypes described for *Melaleuca alternifolia*, each of which has a similar foliar terpene profile, but differ in the presence and concentration of three key terpenes (Butcher et al., 1994; Keszei et al., 2010a).

M. quinquenervia is unique in that the two chemotypes share only a single compound, β-caryophyllene, in otherwise different terpene profiles. This level of difference in foliar terpene profiles is most often found in leaves at different ontogenetic stages. For example in the lemon-scented tea tree (*Leptospermum petersonii*), young leaves contain high concentrations of bicyclogermacrene and germacrene D, whereas the mature leaves contain high concentrations of the acyclic compounds neral, citronellal and geraniol (Brophy et al., 2000). Brophy et al. (2000) found leaves at the fifth node had the juvenile acyclic chemotype and those at the sixth node had the mature chemotype. Therefore, there must be a switch operating that changes the foliar terpene chemistry from the juvenile state to the mature state. If this switch was rendered non-functional, then mature leaves would maintain the juvenile leaf terpene profile. These individuals could persist in the population, under natural selection, if the young chemotype was advantageous.

Such a system could have lead to the two very different chemotypes found in *M. quinquenervia*. Chemotype 2 is much more common and widespread than chemotype 1 (Ireland et al., 2002), thus chemotype 1 is most likely the juvenile leaf chemotype which has been retained in adult leaves. If individuals arose with a non-functional switch such that all their leaves remained as chemotype 1, then in the most simple case, the psyllid *B. melaleucae* in high

abundance would decrease the reproductive potential of individuals with chemotype 2 leaves, giving individuals with a non-functional switch a selective advantage. Thus, these individuals can persist in the population. These possibilities could be tested by examining the ontogeny of terpene chemistry in *M. quinquenervia*. *M. quinquenervia* is a weed that has spread throughout the world and is found growing in the presence and absence of herbivores (Dray et al., 2006; Greenway, 1994; Turner et al., 1998; Trilles et al., 2006) that can act as selective agents on terpene chemistry. *M. quinquenervia*, therefore, provides a unique opportunity to investigate how multiple chemotypes are maintained in natural populations.

4. Experimental

4.1. Sample collection

Melaleuca quinquenervia leaf samples were collected from sites throughout the natural distribution from Sydney to the Queensland border (Supplementary Table S1). Foliar samples were collected from 15 trees at each site and for DNA extraction samples of these leaves were stored in sealed polythene bags. A separate sample was taken for analysis of foliar terpenes following extraction in ethanol and water content was estimated after drying a third sample at 60 °C for 48 h. A final sample (15–20 leaves) of leaves were flash frozen in liquid nitrogen and stored at −80 °C for extraction of RNA.

4.2. Foliar terpene analysis

Foliar terpenes were extracted and analysed by GC–MS as described by Southwell and Russell (2002) using *n*-tridecane as an internal standard. 1.5 g of mature leaf was taken from a branch and put into a sealed vial, containing 10 ml of ethanol and 0.25 g l^{-1} tridecane (the internal standard used for quantification of chemical compounds) for oil extraction. Chemical analysis of the oil extract was performed using capillary gas chromatography and mass spectrometry (GC–MS) on an Agilent 6890 GC using an Alltech AT-35 (35% phenyl, 65% dimethylpolyoxylane) column (Alltech, Wilmington, DE). The column was 60 m long with an internal diameter of 0.25 mm with a stationary phase film thickness of 0.25 μm. Helium was used as a carrier gas. The ethanol extract was filtered through a 0.45 μm filter, and 1 μl was injected at 250 °C at a 1:25 split ratio. The temperature program was as follows: 100 °C for 5 min, ramping to 200 °C at 20 °C min^{-1} followed by a ramp to 250 °C at 5 °C min^{-1}, and held at 250 °C for 4 min. The total elution time was 25 min. The components of the solvent extract were detected using an Agilent 5973 Mass Spectrometer. Seven peaks were identified by comparison to authentic standards (α-pinene, β-pinene, β-myrcene, terpinene-4-ol, 1,8-cineole, α-terpineol and β-caryophyllene, kindly made available by Mike Lacey and Thomas Wallenius, CSIRO Canberra).

The remaining peaks were identified by comparisons of mass spectra and retention times to compounds isolated from *M. quinquenervia* and *M. alternifolia* using the same AT35 chromatographic column and run parameters (Southwell and Stiff, 1990; Southwell and Lowe, 1999). Peaks were quantified based on the MS trace in MSD Chemstation Data Analysis (Agilent Technologies, Deerfield, IL) and converted to concentrations on a dry weight basis by comparison with the internal standard. The relative abundance of each compound was determined and GenStat 12th Edition (Payne et al., 2008) was used to perform principal component analysis on this data.

4.3. RNA extraction

Total RNA was extracted from the leaves using the QIAGEN RNeasy Plant Mini Kit (QIAGEN, Valencia CA) but with the addition of 10.4 mg·ml^{-1} polyvinylpyrrolidone (PVP) and 112.5 mg·ml^{-1} sodium isoascorbate (Na-iASC) to the lysis buffer to reduce interference by polyphenols (Suzuki et al., 2003; Daohong et al., 2004).

4.4. Isolation of terpene synthase-like fragments from chemotype 2 of *M. quinquenervia*

117.6 ng of total RNA was used to synthesis the first strand of cDNA in a lock-docking reverse-transcription PCR (LD-RTPCR) (Borson et al., 1992), using Moloney Murine Leukemia Virus (H^- Point Mutant) Reverse Transcriptase (M-MLV H^- RT-Promega Corporation, Madison, WI) and a tagged T_{30}VN primer (5′-gcnaaygaymgngcnwsnttttttttttttttttttttttttttttttvn-3′). The second cDNA strand was synthesised using a terpene synthase specific primer (Keszei et al., 2010a,b) (DDXYDFX-5′-tbgaygayrthtaygatvymtatgg-3′). Gel electrophoresis was used to visualise the products and to select fragments of an appropriate size for purification using Wizard SV Gel and PCR Clean Up System (Promega Corporation, Madison, WI) as per the manufacturer's instructions, eluting in 30 μl of nuclease free water. A Nanodrop 1000 Spectrophotometer (ThermoFisher Scientific, Scoresby, VIC) was used to determine the DNA concentration of the purified amplicon.

The SMART 5′-RACE cDNA Amplification System (Clontech Mountain View, CA) was used to generate a SMART 5′ cDNA library from the previously extracted RNA. Full 5′ ends of the transcripts were obtained using the gene specific 5′ RACE primer designed on the 3′ fragments (MqSt1ufl-5′-gtctgacgtccgcaaacggaacaccaa-3′). These fragments were purified, cloned and sequenced in the same way the 3′ RACE. The sequence traces were verified and reviewed in FinchTV v1.4.0 (Geospiza Inc, Seattle, WA) and the contigs were manually assembled in BioEdit (Hall, 1999).

4.5. Full-length clone isolation of terpene synthase genes from chemotype 2 of *M. quinquenervia*

Full-length primers were designed using the 5′ and 3′ sequence information obtained (MqSt1ufl-5′-gtctgacgtccgcaaacggaacaccaa-3′ and MqSte7R2-5′-ttcacaccggcaaggggctaacg-3′), and were used in second strand synthesis reactions, containing *Taq* Ti DNA polymerase (Fisher Biotec, Wembley, WA) to amplify full-length putative terpene synthase transcripts. The products were ligated, cloned and sequenced using the methods described below for 3′ RACE fragments. The sequence information was reviewed and verified in FinchTV v1.4.0 (Geospiza Inc., Seattle, WA) and the contigs were manually assembled in BioEdit (Hall, 1999).

4.6. Cloning and sequencing of *M. quinquenervia* terpene synthase fragments

The 3′ RACE fragments were ligated into the pGEM-T vector (Promega Corporation, Madison, WI) following the manufacturer's instructions and transformed into JM109 chemically competent *E. coli* cells (10^8 cfu g^{-1} – Promega Corporation, Madison, WI) as per the manufacturer's instructions. After a 19 h incubation at 37 °C, blue–white screening was used to choose positive colonies for colony PCR with M13F and M13R primers (Messing et al., 1983). Gel electrophoresis was used to visualise the products and fragments of an appropriate size were selected for purification using Wizard SV Gel and PCR Clean Up System (Promega Corporation, Madison, WI) as per the manufacturer's instructions, eluting in 30 μl of nuclease free water. A Nanodrop 1000 Spectrophotometer (ThermoFisher Scientific, Scoresby, VIC) was used to determine the DNA concentration.

The STeP sequencing protocol, described by Platt et al. (2007), was applied to the purified fragments, from which the DNA was precipitated using the ABI sodium acetate DNA precipitation

protocol (Applied Biosystems, Foster City CA). Sequencing was performed on the ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City California). The sequence information was verified and reviewed in FinchTV v1.4.0 (Geospiza Inc., Seattle, WA) and the contigs were assembled manually in BioEdit (Hall, 1999).

4.7. Phylogenetic analysis of unique M. quinquenervia terpene synthase fragments

A phylogenetic tree was generated using the RAxML Blackbox online interface (http://phylobench.vital-it.ch/raxml-bb/index.php) containing the C-terminal domain of terpene synthases from 20 species of Myrtaceae (Keszei et al., 2010a), representative sequences from all terpene synthase subfamilies as described by Bohlmann et al. (1998) and all unique sequences isolated from *M. quinquenervia*. Deduced amino acid sequences were used in a maximum likelihood search using the Dayhoff substitution matrix and empirical base frequencies.

4.8. Expression and functional characterisation unique full-length terpene synthase clones isolated from chemotype 2 of M. quinquenervia

The full-length clones were subcloned into the expression vector pASK-IBA7 (IBA GmbH, Göttingen, Germany) as *Bsp*MI fragments. These constructs were introduced into the *E. coli* strain TOP10 (Invitrogen, Carlsbad, CA) and sequenced. Colonies containing the expression construct were grown in a liquid culture to an OD_{600} of 0.6 at 28 °C. 200 μg l^{-1} anhydrotetracycline (IBA, Gottingen, Germany) was used to induce expression. After incubation at 18 °C for 20 h, cells were collected by centrifugation. The cells were disrupted four times by sonication at 36 W for 30 s in a chilled extraction buffer (50 mM 3-(*N*)-2-hydroxypropane sulfonic acid (Mopso), pH 7.0, with 5 mM $MgCl_2$, 5 mM sodium ascorbate, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM DTT, and 10% (v/v) glycerol). Cell fragments were collected by centrifugation and the supernatant was desalted into an assay buffer (10 mM Mopso, pH 7.0, 1 mM DTT, and 10% (v/v) glycerol) through an Econopac 10DG Column (Bio-Rad, Hercules, CA). The assay buffer (200 μl), containing the bacterial extract, was incubated in a Teflon sealed 7 ml glass screw cap test tube with 10 μM GPP or (*E*,*E*)-FPP, 20 mM $MgCl_2$, 0.2 mM $MnCl_2$, 0.2 mM $NaWO_4$ and 0.1 mM NaF. A 100 μm polydimethylsiloxane solid phase microextraction fibre was placed in the headspace during a 60 min incubation at 30 °C and a 15 min incubation at 40 °C. The fibre was then inserted directly into the injector of a gas chromatograph to analyse the enzyme products.

4.9. Genomic and foliar transcriptomic presence of two characterised terpene synthases isolated from M. quinquenervia

Primers were designed on the differences between all terpene synthase fragments isolated from *M. quinquenervia*. These primers were applied to the gDNA, extracted from ground leaf using the QIAGEN DNeasy Plant Mini Kit (QIAGEN, Valencia, California) and cDNA of individuals from both chemotypes in a series of PCR and second strand synthesis reactions (reaction conditions in Appendix 1).

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Appendix A. 1: Optimised reaction conditions

A.1. 3′ RACE

The reactions on chemotype 1 (MQ062), a total volume of 20 μl, contained 2.75 mmol $MgCl_2$, 0.55 mmol of each dNTP, 0.5 μmol DDXYDF1 (5′-ttgatgacatatatgatgtctcatgg-3′) or DDXYDF2 (5′-tsgacgatrtctaygatctctatgg-3′), 0.5 μmol T_{30}VN-Tag and 2 units of *Taq Ti*. The thermocycle consisted of a 30 s denaturation at 94 °C, a 30 s annealing at 52 °C and a 2 min extension at 72 °C for 30 cycles. The reactions on chemotype 2 (MQ001), a total volume of 20 μl, contained 4 mmol $MgCl_2$, 0.55 mmol of each dNTP, 0.5 μmol DDXYDF1 or DDXYDF2, 0.5 μmol T_{30}VN-Tag and 2 units of *Taq Ti*. The thermocycle consisted of a 30 s denaturation at 94 °C, a 40 s annealing at 62 °C and a 2:30 extension at 72 °C for 5 cycles, followed by 25 cycles where the annealing temperature was decreased 0.2 °C per cycle, from 62 °C to 57 °C.

A.2. Full-length clone isolation

The reactions on chemotype 2 (MQ001) had a total volume of 20 μl and contained 5 mmol $MgCl_2$, 5.5 mmol of each dNTP, 0.5 μmol MqSt1uf1 (5′-gtctgacgtccgcaaacggaacaccaa-3′), 0.5 μmol MqSte7R2 (5′-ttcacaccggcaaggggctaacg-3′) and 2 units of *Taq Ti*. The thermocycle consisted of a 30 s denaturation at 94 °C, a 30 s annealing at 52 °C and a 2 min extension at 72 °C for 5 cycles, followed by 25 cycles where the annealing temperature was decreased 0.2 °C per cycle, from 62 °C to 57 °C.

A.3. Colony PCR

These colonies served as templates for 20 μl colony PCR containing 850 μmol $MgCl_2$, 80 μmol of each dNTP, 48 pmol M13F (Messing et al., 1983), 48 pmol M13R (Messing et al., 1983) and 0.4 units of *Taq Ti*. The thermocycle consisted of a 30 s denaturation at 94 °C, a 40 s annealing at 52 °C and a 2:30 extension at 72 °C for 30 cycles.

A.4. Genomic presence

Twenty microliter reactions contained 55.6 ng gDNA, 44 mmol $MgCl_2$, 8.8 mmol of each dNTP, 12 μmol MqStVf1 (5′-cgtgaactagatgagcacttagaacg-3′) or MqStVF2 (5′-agcctctatgaagcttgtcatttg-3′), 12 μmol MqStVR1 (5′-cctgtataaccctggccacagagaac-3′) or MqStVR2 (5′-gcctttggcctataaccctggat-3′) and 2 units of *Taq Ti*. The thermocycle consisted of a 40 s denaturation at 94 °C, a 1 min annealing at 55 °C or 56 °C, respectively and a 1:15 extension at 72 °C for 20 cycles.

A.5. Foliar transcriptomic presence

Twenty microliter reactions contained 2 μl first strand synthesis reaction, 44 mmol $MgCl_2$, 8.8 mmol of each dNTP, 12 μmol MqStVf1 or MqStVF2, 12 μmol MqStVR1 or MqStVR2 and 2 units of *Taq Ti*. The thermocycle consisted of a 40 s denaturation at 94 °C, a 30 s annealing at 55 °C or 56 °C, respectively and a 20 s extension at 72 °C for 20 cycles.

Appendix B. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.05.013.

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