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Review

The genetic basis of foliar terpene yield: Implications for breeding and profitability of Australian essential oil crops

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Abstract The family Myrtaceae is known for its high foliar terpene concentrations as well as significant qualitative and quantitative variation in foliar terpenes between taxa, populations and individuals. To date, few studies have investigated the genetic and biochemical processes, which underlie this variation, much of which is known to be under genetic control. Differences in yield are both ecologically and commercially important and a better understanding of its basis will allow a greater understanding of Australian ecosystems as well as improve commercial viability of essential oil industries. Over the past decade a good understanding of the genes involved in terpene biosynthesis has developed in other species and several important regulatory steps have been identified. Much of this work has been done in transgenic plants, so our understanding at a molecular level is strong. Nonetheless, it remains unclear if these processes are transferrable to wild populations, or indeed how ecologically important quantitative variation in terpenoids arise and are maintained in natural ecosystems. In this review we will summarize what is known about terpene biosynthesis and the control of flux through the terpene biosynthetic pathways. We will then argue that this platform of work provides a great resource for Myrtaceae, as well as other plants, to identify candidate genes that control flux through the biosynthetic pathways and how this will inform further studies into the ecological implications of quantitative variation of terpenes. Work into terpene biosynthesis would also provide a framework to improve the profitability of essential oil crops.

Key words: Essential oil, Myrtaceae, *Eucalyptus*, *Melaleuca*, crop improvement.

Introduction

Myrtaceae is characterized by the frequent presence of aromatic essential oils in leaves, buds, fruits and stems and occasionally bark. These oils are mostly complex mixtures of mono- and sesquiterpenes. The family is also known for the significant qualitative and quantitative variations seen between taxa, populations and individuals (Keszei et al. 2008). Previously, there has been significant effort in cataloguing the chemical diversity found within the family (Boland et al. 1991; Brophy et al. 2013; Padovan et al. 2014), but it is only recently that the genetics and biochemical process that underlie this variation have begun to be investigated.

Most work to date has focused on identifying genes coding for terpene synthase enzymes (TPS), which are responsible for much of the chemical diversity seen in the terpene profiles of Myrtaceae (Keszei et al. 2010). Related genomic analysis has shown that the genome of *Eucalyptus grandis* contains more putatively functional TPS enzymes than any other plant that has been sequenced to date (Myburg et al. 2014). TPS enzymes have been characterized in other species of Myrtaceae (mainly *Melaleuca*), of which make both ecologically and commercially important compounds. For example, three terpene synthases, that are responsible for the biosynthesis of the three main terpenes in *Melaleuca alternifolia* have been identified and characterized, including the terpene synthase that makes sabinene hydrate, the precursor to commercially valuable terpinen-4-ol in this species (Keszei et al. 2010). Foliar terpenes give eucalypts and other Myrtaceae their characteristic odour and also act as mediators of many ecological interactions. These include deterrents to insect herbivores (Edwards et al. 1990, 1993; Stone and Bacon 1995), attractants and repellents to vertebrate herbivores (Hume and Esson 1993; Southwell 1978), as cues that indicate the presence of other more toxic secondary metabolites (Lawler et al. 1999) and attractants for parasitoids and pollinators (Giamakis et al. 2001). Although variation in TPS genes can give rise to distinct terpene profiles, they are thought to have limited influence (other than via kinetic constraints) on the quantitative variation in foliar terpene concentrations seen within Myrtaceae (or indeed other plants). Similarly there remains little understanding of how genetic variation interacts with environmental effects to produce variation in foliar terpene concentrations between

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species and individuals (Andrew et al. 2010).

Quantitative variation in foliar terpene concentration is significant to industry as Australia produces essential oils from a number of species of *Eucalyptus* and *Melaleuca*. Currently essential oil industries rely primarily on improved wild plants, because the generation times involved with forest trees make selective breeding a slow and costly process. For example, the tea tree breeding program, which has been running since 1993, only released second-generation seed to the industry in 2007 (Baker et al. 2010). Despite this, the tea tree breeding has made large gains in oil yield, increasing it from 148 kg ha^{-1} to 250 kg ha^{-1} (Doran et al. 2006). However, natural variation in wild tea tree populations suggests that there is potential for far greater gains to be made, with some wild individuals having foliar terpene concentrations almost double that of the mean for improved tea tree stock (Butcher et al. 1994; Homer et al. 2000). In addition to this, terpenes are one of a number of economic products being developed from oil mallees (e.g. *Eucalyptus polybractea* and *E. loxophleba*), which are used to regenerate salt-affected land in Western Australia. Understanding the genetics and biochemical processes that underlie quantitative variation can provide Australian essential oil industries with a significant competitive advantage over overseas competitors. Vast libraries of wild functional variants occur in natural populations that can be exploited by industry to fine-tune and improve essential oil yield and profile. Research and development in this area has the potential to vastly improve gains in oil yields within these industries. Simultaneously, it will help industry achieve those gains in a shorter time and more cost effectively. In the long term, access to these resources will be an important part of maintaining the competitive advantage that Australian essential oil industries have developed over the past 20 years through access to better natural and improved germplasm.

Terpenes are both primary and secondary metabolites

Terpenes are a highly diverse group of plant secondary metabolites with more than 20,000 characterized, unique compounds (Degenhardt and Gershenzon 2003). Although terpenes are primarily known as secondary metabolites, they play a number of roles in primary metabolism as hormones (e.g. abscisic acid), photosynthetic pigments (e.g. carotenoids) and electron carriers (e.g. ubiquinone) (McGarvey and Croteau 1995). Terpenes are involved in mediating a number of important ecological interactions, such as directly in pollinator attraction, alleopathic and antifungal agents which allowing plants to cope with abiotic stressors, as well as indirect defense against herbivores, and quantitative variation in terpene concentration has been shown to have important role, in many of these interactions (Emerick et al. 2008; Hall et al. 2011; King et al. 2004; Latta et al. 2003; Rocchini et al. 2000; Zou and Cates 1997).

What role do terpenes play within Myrtaceae?

The ecological role of terpenes in Australian Myrtaceae is poorly understood with only a handful of studies showing their significance (see Introduction). Several further ecological roles have been suggested to explain the variation seen across the landscape. These include the suggestion that terpenes enhance fire in eucalypt forests in Australia since foliar concentrations vary latitudinally in relation to fire frequency, with terpene-poor (particularly cineole) species in the fire prone north and terpene-rich species in the less fire prone south (Steinbauer 2010). Although not specifically explored in Myrtaceae, there is evidence from other families and species that terpenes are involved in resistance to heat stress and oxidative damage from other abiotic stressors (Loreto et al. 1998; Sharkey and Singsaas 1995; Singsaas et al. 1997). For example, transgenic poplars showed decreased heat tolerance when the expression of isoprene synthase genes was depressed (Behnke et al. 2007). Similarly, transgenic *Arabidopsis* showed significant increase in heat tolerance when over-expressing an isoprene synthase derived from poplar (Sasaki et al. 2007) and transgenic tobacco modified to emit isoprene showed a small increase in tolerance to heat stress and a large increase in tolerance to oxidative stress and oxidative damage (Vickers et al. 2009). *Eucalyptus* species are some of the highest emitters of isoprene of any plant (He et al. 2000) and *E. grandis* has one of highest number of putative isoprene synthase genes in any sequenced plant (Grattapaglia et al. 2012). Specific experiments are needed to test the hypothesis that the isoprene and monterpene emissions in Myrtaceae have evolved in response to oxidative and heat stress. With such an abundance of terpenoids in Australian environments, this area deserves more study, especially given the potential impacts of climate change on forest industries. For example, with rising global temperatures will plants that emit higher concentrations of volatiles be advantaged? It is here that understanding the role that quantitative variation plays in relation to ecological interactions, herbivory and resilience to abiotic stressors has the potential to provide large economic benefits not just in essential oil industries but in other forest-dependent sectors such as plantation *Eucalyptus*.

Variation in terpene yield and heritability of variation

Recall that quantitative variation in terpene production in Myrtaceae is common and the variation within species can often be large. In blue mallee (*Eucalyptus polybractea*), total foliar terpene concentration varies from 0.7% DM to 13% DM, almost a 20-fold difference (King et al. 2006) and in *Melaleuca alternifolia* there is a 15-fold variation in foliar terpene concentrations (Butcher et al. 1994; Homer et al. 2000). There are many different factors that can produce quantitative variation in terpene concentration in plants such as environmental factors e.g. nutrient availability (Muzika 1993), water stress (Delfine et al. 2005), atmospheric CO_2 concentrations (Peñuelas et al. 1997), seasonality and temperature (Emara and Shalaby 2011; Peñuelas et al. 1997), herbivory (Paré and Tumlinson 1999), mediated by methyl jasmonate (Martin et al. 2003) (but not eucalypts (Henery et al. 2008). However, in all woody species that have been examined to date, the genetic component of variation (expressed as the narrow sense heritability) has, without exception, been high (h^2=0.6–0.9) (Andrew et al. 2005, 2007; Doran and Matheson 1994; Franklin and Snyder 1971; Han and Lincoln 1994; Hanover 1966a, 1966b; O'Reilly-Wapstra et al. 2011; Rockwood 1973; Squillace 1971). High heritability has been observed for specific terpenes e.g. foliar 1,8-cineole concentrations in *Eucalyptus kochii* (Barton et al. 1991); and *Eucalyptus melliodora* (Andrew et al. 2005), as well as total foliar terpene concentrations in *Eucalyptus camaldulensis* (Doran and Matheson 1994) and *Melaleuca alternifolia* (Doran et al. 2006). The high heritability and large variation of foliar terpene concentrations in Myrtaceae raises a number of questions: How and why are foliar terpene concentrations so variable? What is the genetic architecture underlying this variation and how is it maintained in natural and improved populations? What evolutionary forces (if any) maintain the variation we see within populations? What impacts does the variation in terpene concentrations have in Australian ecosystems? Answering these questions will only be possible once we have an understanding of the genetic processes that underlie the variation in foliar terpene concentrations within Myratceae.

Myrtaceae is also interesting because to date, induction, a process that has been shown to be important in influencing terpene concentration in other species of woody plants (Banchio et al. 2009; Mumm et al. 2003; Phillips et al. 2007) has not been demonstrated in the family (Henery et al. 2008). Although Henery argued that induction might not be expected in an evergreen woody plant like *Eucalyptus*, there is scope for studies with other stimuli that have induced terpenes in other plants. These include fungal treatment (Phillips et al. 2007), methyl salicylate (Phillips et al. 2007) and UV light (Zavala and Ravetta 2002). This may provide greater clarity about the ecological roles that terpenes play in Myrtaceae and also provide insights into how to potentially boost terpene yield in Myrtaceae based essential oil crops.

Recent studies by Goodger and Woodrow have raised the possibility that foliar terpene concentrations in Myrtaceae may be constrained by the volume of sub-dermal secretory cavities (Goodger et al. 2010; Goodger and Woodrow 2010, 2012). By isolating these cavities in *Eucalyptus* species they have shown that the volume of secretory cavities is strongly correlated with foliar terpene concentrations. For example, the correlation between total cavity volume and foliar terpene concentration in *E. polybractea* was r^2=0.96 (Goodger and Woodrow 2012). Interestingly, there was no relationship between the density of secretory cavities and foliar terpene concentrations, suggesting that the volume of the cavities is limiting. It is likely that the secretory cavity volume is under genetic control and that it may play a role in determining foliar terpene concentrations in Myrtaceae. While the remainder of this review will focus on the terpene biosynthetic pathways, constraints around the storage of synthesized terpenes must be kept in mind.

The terpene biosynthetic pathways in plants

Variation in foliar terpene concentrations is most likely to be caused by the expression of genes and copy number variation in several distinct biosynthetic pathways. Terpenes all share the same precursor molecule, isopentenyl pyrophosphate (IPP). The biosynthesis of isopentenyl pyrophosphate occurs via two spatially separated pathways, which both synthesize IPP (Figure 1) (Eisenreich et al. 1998). The mevalonate (MVA) pathway is located in the cytosol and the deoxyxylulose phosphate pathway (DXP)/2-*C*-methyl-D-erythritol 4-phosphate pathway (MEP) is located in the plastid (hereafter called MEP pathway). The MVA pathway obtains its precursor molecule, acetyl-CoA, from the Krebs cycle (McGarvey and Croteau 1995) and is found in all eukaryotes and some bacteria. The MEP pathway utilizes glyceraldehyde phosphate from the Calvin cycle as its precursor molecule (Rohmer and Rohmer 1999) and is only found in plants, some bacteria and protozoa. As well as synthesizing IPP, the MEP pathway also produces dimethylallyl pyrophosphate (DMAPP) at an 85 : 15 ratio (IPP : DMAPP) (Rohdich et al. 2003). For some time, it was thought that these two pathways were functionally separate with the MVA pathway providing substrate for the synthesis of sesqui- and tri-terpenes (McGarvey and Croteau 1995) and the MEP pathway

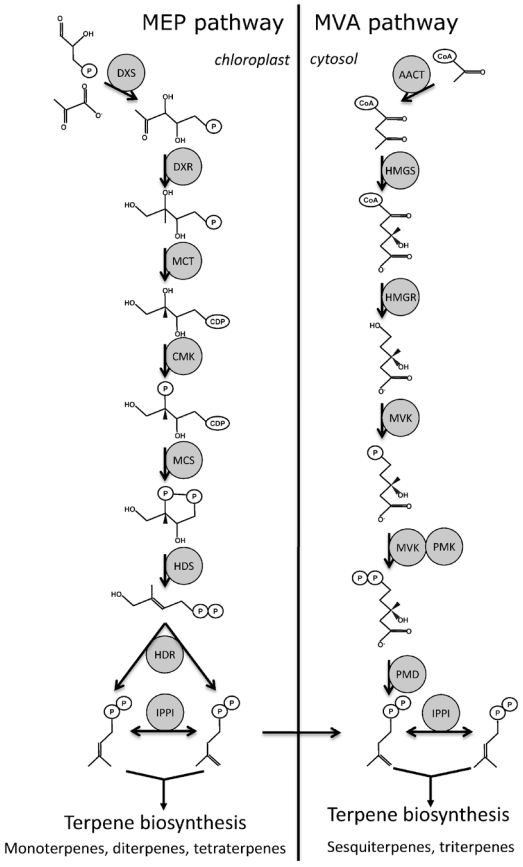

Figure 1. The terpene biosynthesis pathways in plants. *The Methylerythritol pathway* (*MEP*); 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase (MCT), 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase (CMK), 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS), 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (HDS) and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (HDR) and the *Mevalonate pathway* (*MVA*); acetoacetyl-CoA thiolase (AACT), hydroxymethylglutaryl CoA synthase (HMGS), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), mevalonate kinase (MVK), phosphomevalonate kinase (PMK), mevalonate diphosphate decarboxylase (PMD). Both pathways synthesise isopentenyl pyrophosphate, which is converted to Dimethylallyl pyrophosphate by isopentyl pyrophosphate isomerase (IPPI).

providing substrate for the synthesis of monoterpenes, carotenoids, abscisic acid, gibberellic acid and diterpenes (Rohmer and Rohmer 1999). However, this view has been modified by the finding of uni-directional transport of isopentenyl units from the plastid to the cytosol across the plastid double membrane, suggesting that substrates are shared between the cytosol and the plastid (Kappers et al. 2008; Laule et al. 2003). The work shows that in some species the extent of transport of precursors across the plastid double membrane is significant. For example, in snapdragon (*Antirrhinum majus*) precursors from the MEP pathway are primarily responsible for the biosynthesis of sesquiterpenes in the cytosol (Dudareva et al. 2005). Nonetheless, it has been suggested that under normal physiological conditions transport of precursors is likely to generally be low (below 1%) (Eisenreich et al. 2001). There is indirect evidence that transport across the plastid double membrane may be significant within Myrtaceae. Webb et al. (2013) showed significant correlation between the expression of MEP pathway genes and sesquiterpene concentrations. However, the extent to which this occurs and its importance remains uncertain.

The MEP pathway

The MEP pathway consists of seven consecutive enzymatic steps, the first of which is the condensation of pyruvate and glyceraldehyde 3-phosphate to form 1-deoxy-d-xylulose 5- phosphate (DOXP) by DXP synthase (DXS) (Figure 1). DOXP is then converted to 2-*C*-methyl-D-erythritol 4-phosphate (MEP) by DOXP reductoisomerase (DXR). MEP is then converted into 1-hydroxy-2-methyl-2-(*E*)-butenyl 4 diphosphate (HMBPP) by 2-*C*-methyl-D-erithritol 4-phospahte cytidyltransferase (MCS), 4-(cytidine 5′diphospho)-2*C*-methyl-D-erithritol kinase (CMK), 2-*C*-methyl-D-erithritol 2,4-cyclodiphospahte (MCS) and (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS) (Figure 1). The last step in the MEP pathway is the conversion of HMBPP into IPP and DMAPP by (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) at approximately a 5:1 ratio (Rohdich et al. 2002). Many of the genes within the pathway have been shown to have more than one copy in some species. For example, multiple copies of *dxs* have been found in *Medicago truncatula* (Walter et al. 2002), *Arabidopsis thaliana* (Araki et al. 2000; Estévez et al. 2000), *Oryza sativa* (Kim et al. 2005), *Picea abies* (Phillips et al. 2007), *Ginkgo biloba* (Kim et al. 2008a), *Eucalyptus grandis* (Külheim et al. 2011) and *Melaleuca alternifolia* (Webb et al. 2013). Other genes including *cmk* (Kim et al. 2008a), *hdr* (Kim et al. 2008b) and *dxr* (Seetang-Nun et al. 2008) can occur as multi-copy genes (in *Ginkgo biloba, Pinus taeda* and *Hevea brasiliensis*, respectively), although all are present as single copies in *Arabidopsis thaliana* (Cordoba et al. 2009), so unlike *dxs*, multiple copies of these genes are not universal amongst plants (Table 1). Very little work has been done on copy number variation within the Myrtaceae family. The work that has been done suggest that there are three copies of *dxs* for some species within the family (Külheim et al. 2011; Webb et al. 2013).

There is speculation that some of these multi-copy genes, in particular *dxs*, may be involved in the biosynthesis of different terpenoid products and possibly subject to independent regulation. Phillips et al. (2007) showed in Norway spruce (*Picea abies*), that when oleoresin production is induced via wounding and fungal treatment, the expression of *dxs2a*, *dxs2b*, *dxr*

Table 1. Putative terpene biosynthesis gene numbers within a range of different plant species.

Gene ID	*M. truncatula*	*G. max*	*A. thaliana*	C. clementina	*E. grandis*	*V. vinifera*	*Z. mays*	*O. sativa*
Methylerythritol pathway								
1-Deoxy-D-xylulose 5-phosphate synthase	3	8	1	2	2	5	2	2
1-Deoxy-D-xylulose 5-phosphate reductoisomerase	1	3	1	2	1	1	3	1
2-*C*-Methyl-D-erythritol 4-phosphate cytidylyltransferase	—	—	1	1	1	1	2	1
4-Diphosphocytidyl-2*C*-methyl-D-erythritol kinase	—	2	1	1	1	1	1	1
2*C*-Methyl-D-erythritol 2,4-cyclodiphosphate synthase	1	1	1	1	2	1	2	—
4-Hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	1	2	1	1	1	1	1	1
1-Hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase	—	2	1	3	2	1	1	1
Isopentyl diphosphate isomerase	1	2	2	1	2	1	3	2
Mevalonate pathway								
Hydroxymethylglutaryl CoA synthase	1	5	1	2	2	2	4	3
3-Hydroxy-3-methylglutaryl coenzyme A reductase	3	7	2	2	5	3	9	2
Mevalonate kinase	1	2	1	1	1	1	5	1
Mevalonate diphosphate decarboxylase	—	2	2	1	2	1	1	1
Terpene pathway								
Geranyl diphosphate synthase	—	2	1	2	1	1	2	2
Farnesyl diphosphate synthase	—	1	—	—	1	—	—	—
Geranylgeranyl diphosphate synthase	2	4	9	8	3	1	3	2
TPS (a, b, c, e, f, g, h)	38	29	33	76	140	73	35	43

Medicago truncatula (Barrel clover), *Glycine max* (Soybean), *Arabidopsis thaliana, Citrus clementina* (Clementine), *Eucalyptus grandis* (Rose gum), *Vits vinfera* (Grape), *Zea mays* (Maize) and *Oryza sativa* (rice). Source: www.phytozome.com with domain search for each protein. Copy numbers may be exaggerated due to the presence and inclusion of pseudogenes or underestimated due to restricted search parameters.

and *hdr* were all upregulated but *dxs*1 was not affected. When cell cultures of the same species were treated with methyl salicylate, chitosan and a beetle-associated fungal pathogen, *Ceratocystis polonica*, *dxs2a* was upregulated, but it was not upregulated after methyl jasmonate treatment. In contrast, *dxs*2b was upregulated by methyl jasmonate and chitosan treatments but not the other treatments (Phillips et al. 2007). In *Medicago truncatula*, transcripts of *dxs* type1 accumulate to high levels in photosynthetic tissues, whereas *dxs* type 2 accumulates in roots upon colonization with mycorrhizal fungi (Walter et al. 2002). This same pattern is seen in other plants (Kim et al. 2006; Walter et al. 2000, 2002) including *Pinus abies* (Phillips et al. 2007) and *Pinus densiflora* (Kim et al. 2009). These results suggest that *dxs* type 1 genes have a role in providing the various terpenoids vital for photosynthesis, while *dxs* type 2 genes may be involved in the biosynthesis of specific terpene secondary metabolites that are induced by specific stimuli. It also suggests that tissue-specific regulation may be important in the MEP pathway. Whether these same patterns hold for Myrtaceae is not known but we can expect that the multi-copy genes within the family are likely to be performing similar specialized functions.

Over the last decade a better understanding of how the MEP pathway is regulated and elements that regulate flux through the pathway, has been developed in model and crop species. However, very little work has been done with wild plants. Work with transgenic plants has shown that when *dxs* and *dxr* are upregulated there is an associated increase in terpene concentrations. For example, over-expression of *dxr* and *dxs* increased foliar terpene concentrations by over 100% in transgenic peppermint (*Mentha piperita*) (Croteau et al. 2005). Similar results were obtained in transgenic *A. thaliana* plants over-expressing *dxs* (Carretero-Paulet et al. 2002, 2006). Adding to this work, differential expression of both these genes is strongly correlated with differences in terpene concentration between different cultivars of grape (Battilana et al. 2009), basil (Xie et al. 2008) and tomato (Enfissi et al. 2005). In the case of grape, there was also a significant QTL observed for terpene concentration that co-located with one of the three copies of the *dxs* gene (Battilana et al. 2009). This suggests that both these genes may be involved in controlling variation in terpene production between individuals in a number of species.

HDR has also been identified as a potential rate-limiting step within the pathway. Transgenic *Arabidopsis*, over-expressing *hdr*, as well as taxadiene synthase, resulted in a 13 fold increase in taxadiene compared to plants just over-expressing taxadiene synthase (Botella-

Pavía et al. 2004). The importance *hdr* may play in controlling yield in Myrtaceae is supported by work in *E. globulus*, which identified QTLs for both *hdr* and *hds* that are correlated with increased foliar terpene concentration (Külheim et al. 2011).

Studies in *Melaleuca alternifolia* suggest that rather than *dxs a*nd *dxr* being of primary importance, the coordinated upregulation of the entire pathway shows significant correlation to oil yield (Webb et al. 2013). While *dxs* and *dxr* clearly play a role, as indicated by the evidence cited above, Webb et al. (2013) was one of the few studies to have investigated gene expression in the entire pathway and their impact on oil concentration. It demonstrated coordinated expression of the genes within the MEP pathway (*dxs, dxr, mcs, cmk* and *hds)* and strongly supported the hypothesis that the genes within the entire pathway are likely to be upregulated when flux through the pathway increases. A different study on rice found coordinated induction of the first three steps of the MEP pathway upon application of the elicitor *N*-acetylchitooctaose (Okada et al. 2007). Given the lack of further similar studies it is likely that this pattern of coordinated upregulation may also be seen in many other plant species. The role that intermediate steps within the pathway (*cmk, mcs, mct, hds*) play in controlling concentration is an area that deserves more attention not just in Myrtaceae but more broadly in other species.

Studies in Myrtaceae to date have largely confirmed findings in model and crop species. However, studies in Myrtaceae emphasize the benefits of a broader approach in elucidating some as yet poorly explored detail of how terpene concentration is controlled. Work in Myrtaceae has so far been successful in identifying single nucleotide polymorphisms (SNPs) that affect oil concentration, but functional characterization of the SNPs has not yet been achieved. Knowing whether SNPs are affecting phenotype by changing enzyme kinetics, gene expression or via some other mechanism would be interesting. Nonetheless, from the perspective of a plant breeder it would have limited practical utility. Given that the driving force behind research into quantitative traits in the family is likely to be the needs of industry, future research would probably be better served building on the work done so far and focusing on a more holistic approach to optimizing multiple traits (e.g. a genomic selection approach) rather than on determining exactly how individual SNPs affect phenotype.

What regulates metabolite flux through the MEP pathway?

Transcript abundance of genes within the MEP pathway is affected by a number of different factors. In *Arabidopsis*, all genes within the MEP pathway accumulate upon exposure to light and show circadian patterns of expression, peaking in the early morning and being lowest at night. Regulation by light of MEP pathway genes has been reported in a number of plant species (Carretero-Paulet et al. 2002; Guevara-García et al. 2005; Hans et al. 2004; Hsieh et al. 2008; Kim et al. 2005) and may be a universal response in plants. Phillips et al. (2007) showed that *dxs*, *dxr* and *hdr* were upregulated upon wounding and induction via a number of different stimuli. Nutritional cues have also been shown to change transcript abundance e.g. sugars increase the accumulation of MEP pathway transcripts in dark-grown plants (Hsieh and Goodman 2005). Given that the MEP pathway uses D-glyceraldehype 3-phosphate and pyruvate as precursors, which are derived from photosynthesis and glycolysis, the sensitivity to sugars should not be surprising. In all these cases, coordinated regulation of most or all of the genes in the pathway has been demonstrated after exposure to different stimuli. This raises the possibility that the genes in the MEP pathway could be under the control of an unidentified master regulatory factor. Given that much of the work to date in relation to yield has focused on just three genes, *dxs*, *dxr* and *hdr*, it will be important to determine whether the pattern seen in *M. alternifolia* of the coordinated regulation of all the transcripts within the MEP pathway between individuals that vary in oil yield is something that holds for plants more broadly. Similar studies that tested whether transgenic plants that over express *dxs*, *dxr* and *hdr*, also display enhanced expression of other MEP pathway genes would be instructive.

Work on mutant plants has provided additional evidence of various other mechanisms that regulate flux through the MEP pathway. There is evidence that retrograde signaling may play a part in regulation of flux through the pathway. For example, mutants of *Arabidopsis* with altered chloroplast development, including MEP pathways mutants, show reduced levels of MEP transcripts (Guevara-García et al. 2005). Similarly, *Aribidopsis* plants that are treated with norflurazon, a carotenoid biosynthesis inhibitor, show the same pattern of reduced MEP transcripts, which is thought to be the result of retrograde chloroplast to nuclear signaling (Jarvis 2003; Pogson et al. 2008). These results suggest that the genes in the MEP pathway are regulated by retrograde chloroplast to nuclear signaling when chloroplast development is arrested or compromised. Further work in this area has the potential to enhance our understanding of how metabolite flux through the MEP pathway is controlled.

Work looking at the expression and protein accumulation of *Arabidopsis clb6-1* mutants vs wildtype plants suggest that post-transcriptional regulation may also play an important role in regulating flux through the MEP pathway. For the majority of genes within the MEP

pathway, low transcript levels in mutants correspond to low protein levels. However, this is not necessarily the case for *dxs* and *hdr*, two genes shown to affect oil yield in other studies. The *clb6-1* mutants show low levels of *hdr* and *dxs* transcripts, yet the HDR and DXS proteins accumulate at levels greater than those found in wild type plants (Guevara-García et al. 2005). Similarly, when wild-type plants are treated with an inhibitor that blocks DXR, they accumulate high concentrations of DXS protein (Guevara-García et al. 2005). This suggests that post-transcriptional regulation plays a role in the regulation of the MEP pathway. Due to the post-transcriptional regulation of the MEP pathway, relying on evidence of transcript abundance only may not be sufficient in Myrtaceae or other species.

The work outlined above suggests that control of metabolite flux through the MEP pathway is likely to be complex. While transgenic studies have identified putative bottlenecks in the pathway, other work suggests all genes within the MEP pathway may need to be regulated in a coordinated fashion to increase flux though the pathway at least in some instances. The lack of work on the intermediate genes within the pathway and the regulation of the pathway as a whole represent a gap in our knowledge. Given the number of stimuli that induce terpene biosynthesis and flux through the pathway and the complex ecological interactions that terpenes mediate, it is likely that there will be many levels of control on the MEP pathway. More work is needed to further elucidate how flux through the pathway is controlled, but the work on chloroplast to nuclear signaling and posttranscriptional control represents a significant step towards an understanding of this complex issue.

MVA pathway

The MVA pathway is responsible for the biosynthesis of sesquiterpenes and triterpenes (e.g. sterols) and occurs in all animals and plants. The first step in the MVA pathway is the condensation of two acetyl-CoA molecules into AcAc-CoA by AcAc-CoA thiolase (AACT), the second step is the condensation of AcAc-CoA and one acetyl-CoA molecule into 3-hydroxy-3-methylglutaryl (HMG)-CoA by the 3-hydroxy-3-methylglutaryl synthase (HMGS) protein (Figure 1). It was thought that this process was carried out solely by HMGS but recent work has shown that it is carried out by two different proteins AACT and HMGS (Nagegowda 2010). The next step in the pathway is the catalysis of a double reduction reaction resulting in the formation of mevalonate utilized by 3-hydroxy-3-methylglutaryl reductase (HMGR). This is followed by two phosphorylation reaction carried out either both by mevalonate kinase or in some species phosphomevalonate is phosphorylated by phosphomevalonate kinase (Figure 1). The final step of the MEV pathway is a decarboxylation of diphosphomevalonate, catalyzed by diphosphomevalonate decarboxylase (PMD). The sole product of this pathway is IPP unlike the MEP pathway, which also produces a small amount of DMAPP. Of the genes in the MVA pathway, all genes can occur as multi-copy genes (Table 1). In *Hevea brasiliensis* there are two copies of *hmgs* and four copies of *hmgr* while the other genes in the pathway are single copy (Kim et al. 2008b), in *Medicago truncatula* there are five copies of *hmgr* (Kevei et al. 2007). All the reactions within the pathway have been characterized in plants and some work has focused on the control of metabolite flux through the MVA pathway in plants. For example, *Arabidopsis* transformed to express an additional copy of *hmgr* showed a large increase in phytosterols (2.4 fold) (Enfissi et al. 2005). The importance of *hmgr* is confirmed by other studies in *Arabidopsis* which demonstrate that transforming plants to express a different version of *hmgr* can lead to large increases in phytosterols as well as cycloartenol by 2–10 fold (Chappell et al. 1995; Harker et al. 2003). This suggests that *hmgr* is a key regulatory step in the MVA pathway. Given the predominance of monoterpene-dominated oils in commercially important essential oil producing Myrtaceae it is not surprising that no work has been done on the relationship between gene expression and terpene yield in Myrtaceae.

Downstream of IPP

The two upstream terpene biosynthetic pathways in plants, the MEP and MVA pathways, both primarily produce the same compound, IPP, in the plastid and cytosol, respectively. It is thought that the most important rate controlling steps are likely to be early on in both pathways, when substrates from the Krebs and Calvin cycle are allocated between primary and secondary metabolism. Downstream, the genes are not as likely to have as large an effect on yield, although there may be feedback regulatory mechanisms that are important.

The first enzymatic step downstream of the two pathways is isopentenyl pyrophosphate isomerase (IPPI), which catalyses the conversion of isopentenyl pyrophosphate (IPP) to its structural isomer dimethylallyl pyrophosphate (DMAPP) (Figure 2). Most plants have two copies of *ippi* (Table 1), which are thought to have specialised but overlapping functions (Cunningham and Gantt 2000; Nakamura et al. 2001; Okada et al. 2008; Phillips et al. 2008). In *Arabidopsis*, one copy is expressed more strongly in the cytosol (termed *idi2*) and the other more strongly in the plastid (termed *idi1*). However, knockouts mutants of each gene in *Arabidopsis* have shown that each *idi* gene can

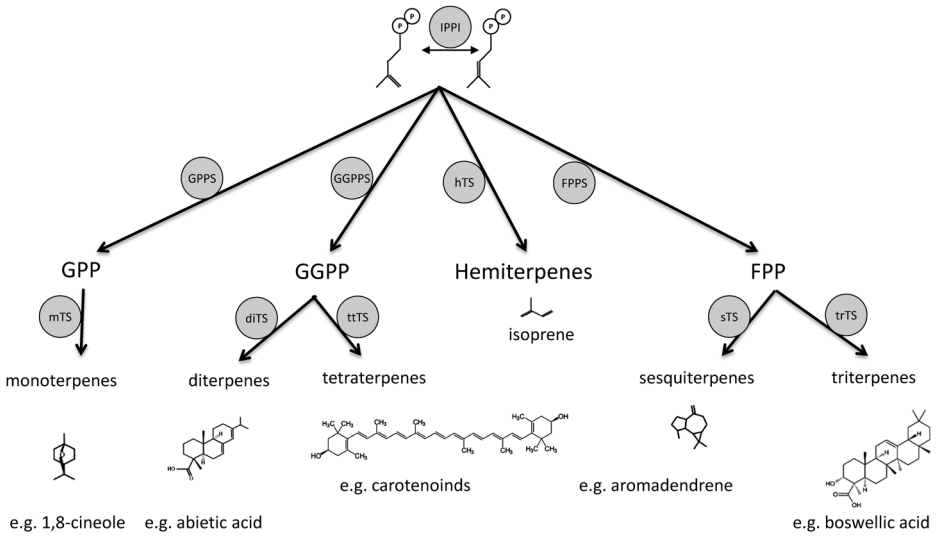

Figure 2. Biosynthesis of terpenes downstream of the MEP and MVA pathways. Showing the conversion of IPP into geranyl pyrophosphate (GPP) by geranyl pyrophosphate synthase (GPPS), geranylgeranyl pyrophosphate (GGPP) by geranylgeranyl pyrophosphate synthase (GGPPS) and farnesyl pyrophosphate (FPP) by farnesyl pyrophosphate synthase as well as the conversion of IPP, GPP, GGPP and FPP into terpenes by terpenes synthases (monoterpene synthases (mTS), diterpene synthases (diTS), triterpene synthases (diTS), tertraterpene synthases (ttTS), sesquiterpene synthases (sTS) and hemiterpene synthases (hTS).

compensate for the other in both parts of the cell and that there is no significant difference in terpenes produced or growth phenotypes by either *idi1* or *idi2* knockouts when compared to the wild type (Okada et al. 2008; Phillips et al. 2008). IPP and DMAPP form the backbone of all plant terpenes, apart from isoprene which is synthesized directly from IPP and the ratio of IPP : DMAPP is thought to be important in allocating resources to the synthesis of the different classes of terpene. Prenyl pyrophosphate synthases catalyze the conversion of IPP and DMAPP to prenyl pyrophosphates that are the substrate for terpene synthases. The three prenyl pyrophosphates used in terpene biosynthesis are geranyl pyrophosphate (GPP), synthesized by geranyl pyrophosphate synthase (*gpps*), that is used for monoterpene biosynthesis, geranylgeranyl pyrophosphate (GGPP), synthesized by geranylgeranyl pyrophosphate synthase (*ggpps*) that is used for di- and tetraterpene biosynthesis and farnesyl pyrophosphate (FPP), synthesized by farnesyl pyrophosphate synthase (*fpps*) that is used for sesqui- and triterpene synthesis (McGarvey and Croteau 1995). The ratio of IPP to DMAPP determines which of these prenyl synthases will be most active; *gpps* works most efficiently with a IPP : DMAPP ratio of 1 : 1 (Bouvier et al. 2000), *ggpps* works most efficiently with a ratio of 3 : 1 (Allen and Banthorpe 1981; Ohnuma et al. 1989) and *fpps* works most efficiently with a ratio of 2 : 1 (Hugueney and Camara 1990). The key intermediate gene *ippi* is responsible for controlling the IPP : DMAPP ratio and may have an important role in regulating the proportion of the different classes of terpene that are synthesised (Phillips et al. 2008). Wildung and Croteau (2005) showed that when both copies of *ippi* were down-regulated in peppermint, (which primarily produces monoterpenes), there was a large increase in the concentration of foliar sesquiterpenes. In Myrtaceae, the expression of *ippi2* is correlated with total sesquiterpene concentrations as well as the ratio of mono- to sesquiterpenes (Webb et al. 2013), providing some evidence that genes within this part of the pathway may play a role in resource allocation and may affect the ratio of different classes of terpenes present in the leaf oils.

The role that prenyl pyrophosphates play in determining flux through the terpene biosynthetic pathways has not been investigated in depth. However, studies with peppermint have suggested that GPPS can act as a bottleneck to terpene biosynthesis. When *gpps* was over-expressed in peppermint, there was an increase in total foliar terpene concentrations (Croteau et al. 2005). To date only a limited number of studies within Myrtaceae have examined the role of prenyl pyrophosphates in relation to terpene yield but none have been supportive. More work is needed to determine the potential role prenyl pyrophosphates play as bottlenecks and whether there are feedback loops that help direct precursors for the biosynthesis of specific sub-classes or products.

Biosynthesis of specific terpenes

Specific terpene compounds are produced by the action of a large family of enzymes known as terpene synthases (TPS) that convert prenyl pyrophosphates into terpenes. A large number of TPS genes have been characterized to date (Chen et al. 2011; Tholl 2006) and they are

broadly classified based on their primary substrate (GPP, GGPP or FPP). Most terpene synthase enzymes are capable of producing multiple products from a single precursor e.g. TPS1, a sesquiterpene synthase in *Zea mays* (maize) produces a mix of (*E*)-farnesene, (*E*,*E*)-farnesol, and (3*R*)-(*E*)-nerolidol when incubated with FPP and also linalool and geraniol when incubated with GPP (although far less efficiently) (Schnee et al. 2002). Other terpene synthases, e.g. geraniol synthase from *Cinnamomum tenuipilum* (Yang et al. 2005) produce a single product. In *Melaleuca alternifolia*, three terpene synthase genes have been shown to be responsible for the biosynthesis of the majority of monoterpenes in the plant. Variation in the expression of cineole and terpinolene synthase and presence/absence of sabinene hydrate synthase has been shown to be responsible for the six different chemotypes in the plant (Keszei et al. 2010). The ability of TPS enzymes to produce multiple products as well as the diversity of terpene synthases in many plants are some of the reasons there is so much diversity in terpene structures across the plant kingdom.

Whereas many plant terpenes are direct products of TPS enzymes, e.g. 1,8-cineole (Chen et al. 2004; Keszei et al. 2010), other terpenes are modified from the primary products of terpene synthases. The most common class of enzymes responsible for modifying terpenes are cytochrome P450 enzymes (Keeling and Bohlmann 2006) e.g. in peppermint six further steps catalysed by CYP450 enzymes are required for the biosynthesis of menthol from the monoterpene limonene (Croteau et al. 2005), adding further to the diversity of terpenes seen in plants.

The conversion of prenyl pyrophosphates into terpenes by TPS genes is unlikely to be rate limiting in itself, however, work on carotenoid biosynthesis suggest that in some cases, genes responsible for the creation of terpenes could play a role in regulation by reinforcing feedback loops. For example, phytoene synthase is thought to be an important rate limiting step in carotenoid biosynthesis and may play a role in controlling flux through the pathway by feedback regulation of *dxs* (Rodríguez-Villalón et al. 2009). Whether feedback loops also exist with other *tps* genes is unknown. Given the large number of *tps* genes in Myrtaceae, there is the possibility of feedback loops between these genes and the MEP and MVA pathway. For example, a QTL containing phytoene synthase is correlated with sesquiterpene yield in *Eucalyptus globulus* (O'Reilly-Wapstra et al. 2011), suggesting the existence of a possible feedback loop. This is an area that deserves more study to better understand how the entire terpene biosynthesis pathways are regulated and how that regulation affects oil yield. Understanding the interplay between the different parts of the pathway and their interactions could lead to significant improvements in the yield of essential oil crops.

The studies outlined above pose many open questions as to the exact mechanisms by which flux though the terpene biosynthetic pathways is regulated, suggesting complex multiple levels of control are responsible for controlling metabolite flux through the pathways. Work on transgenic plants shows that upregulation of single genes within the pathways can increase terpene yield. However, the upregulation of all genes in the MEP pathway in *Melaleuca alternifolia* and upon exposure to light in *Arabidopsis*, suggests that at least in some cases, a coordinated upregulation of all genes in the pathway is necessary for increased flux through the pathway. Similarly, induction studies show specific regulation of different copies of *dxs* in a number of different species, suggesting that control of flux through the MEP pathway is tightly controlled in response to varying stimuli, at least in some species. Post-transcriptional regulation and retrograde chloroplast to nuclear signaling are two examples of mechanisms that may underlie the coordinated responses in gene expression and yield through the MEP pathway in different conditions. The consensus of studies on phytoene synthase suggests that feedback from genes lower in the terpene biosynthetic pathways may also play an important role in allocating the optimal amount of resources to different terpene classes to respond to the changing terpenoid needs of plants.

Overview and conclusions

Unlike the model or inbred crop species used in most studies of terpene biosynthesis, Myrtaceae exhibits large amounts of quantitative variation in oil concentration in wild populations, which we know is under strong genetic control. The molecular work done to date in the family has shown good success in identifying genes and regions within those genes that may affect oil concentration. This confirms the potential of molecular genetics in understanding how variation in oil concentration in Myrtaceae is maintained. Recent publications and annotations of genome sequences such as that of *Eucalyptus grandis* (Myburg et al. 2014), other forest trees such as Black Cottonwood (Tuskan et al. 2006) and plant species with high levels of variation in terpenes such as grape (Jaillon et al. 2007) provide great genomic resources, which can be used to develop new strategies for molecular breeding of essential oil crop species within Myrtaceae and beyond. Re-sequencing individuals of the same or related species that show variation in terpene yield is now easily achievable and will provide great insight into the genomic basis of oil yield. This will include the candidate genes discussed in this review, but also transcriptional regulators and other, as yet unknown genes and genomic regions.

Recall that Australian essential oil crops are generally reliant on improved wild material. The long generation times make traditional selective breeding costly and time consuming. Molecular techniques have the potential to vastly improve oil yields in these industries by identifying the genes and alleles that underlie quantitative variation in oil yield, making more targeted selective breeding possible. It remains to be seen whether practical molecular breeding will focus on specific SNPs or broader approaches such as genomic selection (Kainer, Foley and Kulheim, unpublished). Given that most oil-bearing Myrtaceae have only just started to be improved, it is possible for industry to more fully utilize the natural variation in oil yield across the range of the respective species. A better understanding of the biosynthesis of terpenes and the bottlenecks to oil yield may allow for this range wide genetic variation to be exploited for economic gain. The broader implications are considerable and range from selecting particular oil profiles to create more desirable or profitable products through to larger gains in oil yield.

An understanding of the genetics of oil yield has further, wide-reaching consequences. Newly emerging diseases have the potential to decimate essential oil industries. The Introduction of Myrtle rust (*Puccinia psidii*) to Australia in April 2010 (Carnegie and Cooper 2011) has already compromised the production of lemon myrtle (*Backhousia citriodora*). The genetic variation in wild populations of commercial species as well as within existing commercial material provides the best defense against such threats. However, there remain significant challenges such as maintaining yields in resistant material. Under particular threat is the tea tree oil industry because *Melaleuca alternifolia* shows high levels of susceptibility to the rust (Morin et al. 2012). Although the full effect of myrtle rust infection is not yet known, it has the potential to cause enormous losses. In the future, the continued health of these industries mean that germplasm that is resistant to rust must be identified. If resistant material is found among germplasm that has already been improved to provide greater yield, there may be a significant loss of genetic variation within the breeding population and potentially a decrease in oil yield as well as potential future gains of oil yield from breeding programmes. The potential of genetic screening tools in helping overcome such constraints is significant. If markers for oil yield can be identified, seedlings can be screened for rust resistance and then resistant trees screened for high yielding genetic variants. This will help maintain the gains in oil yield made by the existing tea tree breeding programme and protect the significant investment that has already been made in improving tea tree oil.

Identifying markers for oil yield, opens up other potential avenues to improve profit. In the case of essential oil industries, it may be possible to incorporate high yielding markers from "non commercial" material (e.g. non-commercial chemotypes) into commercial material. In the case of *Melaleuca alternifolia*, the highest oil yields in the species are not in the commercial chemotype (Homer et al. 2000). Why yield is higher in the other chemotypes is unknown at this stage. However, it may be that alleles associated with high yield are more common in non-commercial chemotypes and rare in the commercial one. Understanding the genetic basis of yield in the non-commercial chemotypes may provide a previously unexplored avenue for increasing yield in commercial material.

Understanding the genetic basis that underlie yield of essential oils should make these industries more resilient and profitable. New resources such as the *Eucalyptus* genome sequence that was recently published (Myburg et al. 2014) provide extraordinary tools to continue this work. In the long-term, genetic variation in native Myrtaceae populations across the country provides Australia with a significant advantage over international competitors, not just in essential oil crops but also other forest products. This variation can be utilized to improve foliar concentration, biomass or other commercially important traits such as disease resistance. However, to date there has been very little investment in tree genetics within Australia and only haphazard attempts to conserve the wild genetic resources that underlie some of these industries. This together with loss of genetic diversity through land clearing is a serious and on-going problem that should receive urgent attention from industry and government.

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