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Article title: The terpene synthase genes of *Melaleuca alternifolia* (tea tree) and comparative gene family analysis among Myrtaceae essential oil crops

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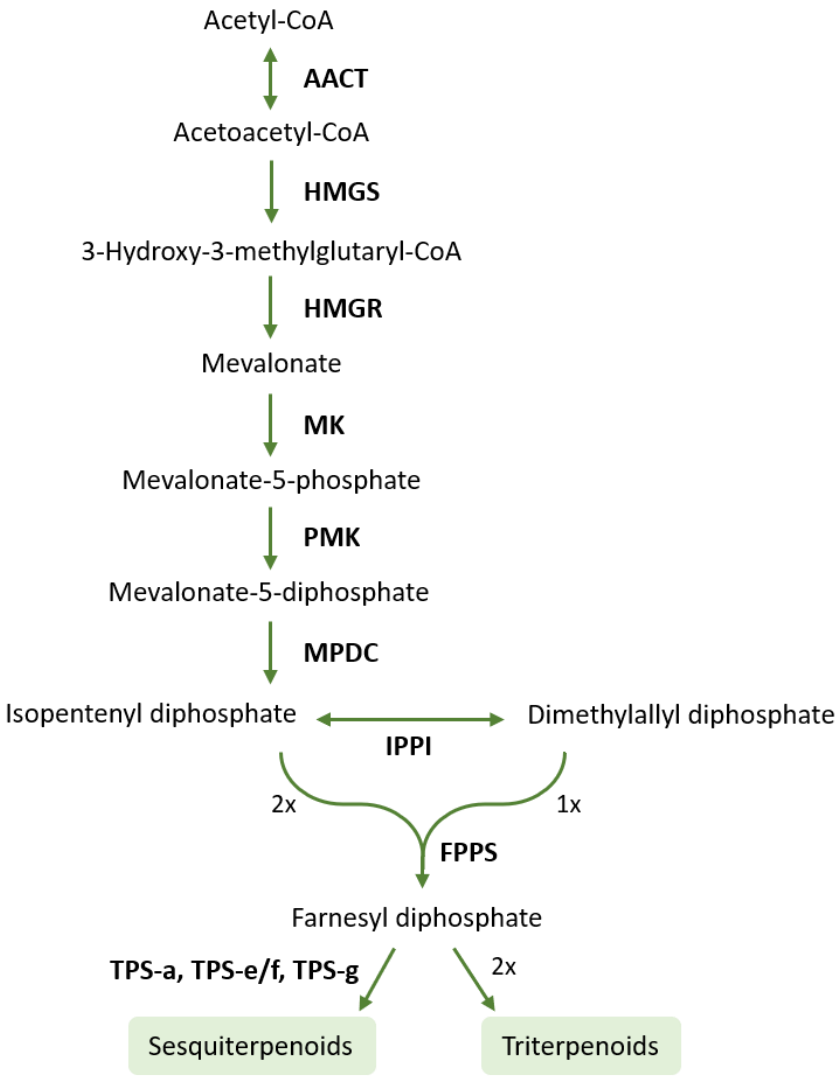
Online Resource 1: Biosynthetic pathway information for terpenoid synthesis

All terpenoids are derived from the isomeric 5-carbon (C5) precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMADP). In plants, these molecules can be synthesised in two distinct pathways. The 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in plastids produces IPP and DMADP from pyruvate and D-glyceraldehyde 3-phosphate (GAP). In contrast, the mevalonate (MVA) pathway is located in the cytosol and generates IDP from acetyl-coenzyme A (CoA), whereas some IDP is transformed into DMADP by the isopentenyl diphosphate isomerase (IPPI) enzyme (Lange et al. 2000).

1.1 The MVA pathway

The biosynthesis of terpenoids in the cytosol starts with the production of precursors in the MVA pathway (Online Resource 1 a). Two molecules of acetyl-CoA are reversibly condensed to acetoacetyl-CoA (AAC) by the AAC thiolase (AACT). Subsequently, the 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) converts AACT to HMG-CoA, which is then reduced to mevalonic acid (MVA). For this reaction, two reduction steps are catalysed by the HMG-CoA reductase (HMGR), which requires two molecules of NADPH. Then, the MVA kinase (MK) phosphorylates MVA to mevalonate-5-phosphate, and a second phosphorylation catalysed by the phospho-MVA kinase (PMK) leads to MVA 5-diphosphate. Finally, MVA 5-disphosphate is decarboxylated in an ATP-dependent reaction catalysed by the MVA diphosphate decarboxylase (MPDC) (Vranová et al. 2013). The product of this reaction, IPP, is partly converted to DMAPP by the IPPI enzyme, and both molecules function as precursors for the synthesis of sesqui- and triterpenoids in the cytosol.



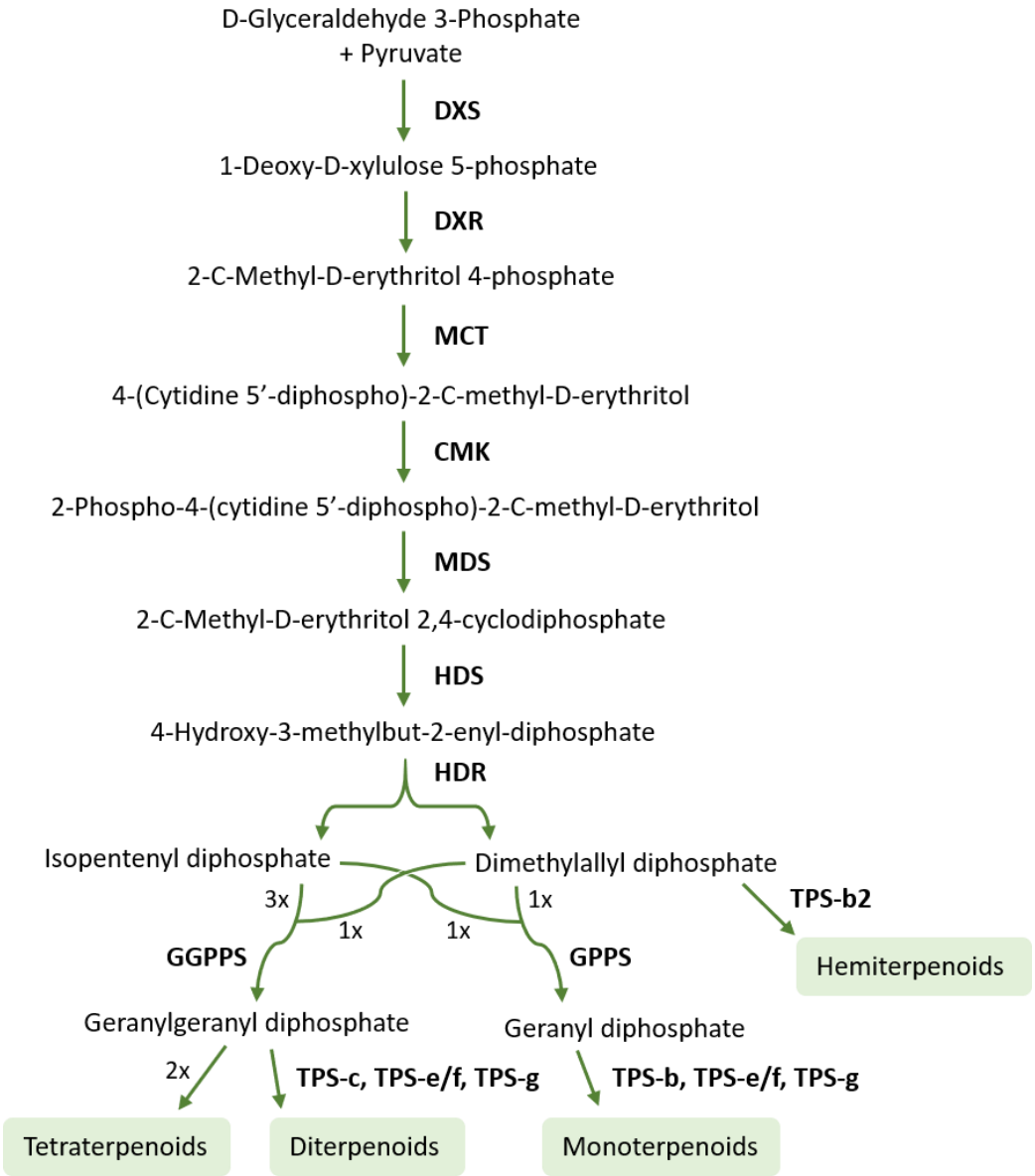
Online Resource 1 a Terpenoid synthesis in the cytosol, where Isopentenyl diphosphate and Dimethylallyl diphosphate are produced via the MVA pathway. TPS classification according to Chen et al. (2011). For a more detailed literature review on the MVA biosynthetic pathway, including molecular structures and enzyme classifications, readers are directed to Vranová et al. (2013).

1.2 The MEP pathway

The MEP pathway is independent of the MVA pathway, but leads to the same product, IPP. While the MVA pathway exists in most organisms, like archaebacteria, yeasts, animals and some gram-positive bacteria, the MEP pathway is restricted to gram-negative bacteria, green algae and cyanobacteria (Vranová et al. 2013). The only exception is constituted by higher plants, which are able to use both pathways.

The MEP pathway in plants is located in chloroplasts, and starts with a reaction catalysed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS) (Online Resource 1 b). DXS condensates D-glyceraldehyde 3-phosphate (GA-3-P) with pyruvate, which leads to the production of 1-deoxy-D-xylulose 5-phosphate (DXP), releases one CO_2 molecule per reaction and is irreversible. Thereafter, DXP is reduced to 2-C-methyl-D-erythritol 4-phosphate (MEP) by the DXP reductoisomerase (DXR) enzyme. The transformation of MEP into 4-(cytidine 5'diphospho)-2-C-methyl-D-erythritol (CDP-ME) is catalysed

by the 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase (MCT). Then, CDP-ME is further phosphorylated to 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-ME2P) by 4-(cytidine 5'-diphospho)-2-C-methyl-D-eryhtritol kinase (CMK). Thereupon, the 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS) converts CDP-ME2P to 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP), which is subsequently reduced to 4-hydroxy-3-methylbut-2-enyldiphosphate (HMBPP) in a reaction catalysed by HMBPP synthase (HDS). The last reaction of the MEP pathway is catalysed by HMBPP reductase (HDR), which produces a mixture of IPP and DMAPP (Vranová et al. 2013).



Online Resource 1 b Terpenoid synthesis in chloroplasts, where the 5-carbon precursors Isopentenyl diphosphate and Dimethylallyl diphosphate are produced via the MEP pathway. TPS classification according to Chen et al. (2011), showing only TPS clades that are present in angiosperms. For a more detailed literature review on the MEP biosynthetic pathway, including molecular structures and enzyme classifications, readers are directed to Vranová et al. (2013).

1.3 Terpenoid synthesis

Both, MVA and MEP pathway, result in the production of IPP and DMAPP. These isomers are the universal building blocks of terpenoids, hence all terpenoids are multiples of 5-carbon units that are coupled in head-to-tail reactions (Sell 2010). This coupling of IPP and DMAPP is catalysed by the activity of prenyl transferases to produce prenyl diphosphate intermediates (Wang and Ohnuma 2000). In the cytosol and mitochondria, the farnesyl pyrophosphate synthase (FPPS) catalyses the condensation of two molecules IPP and one molecule DMAPP to produce the 15-carbon molecule farnesyl diphosphate (FPP), which serves as precursor for sterol, sesquiterpenoid and triterpenoid synthesis (Online Resource 1 a) (Henry et al. 2015).

In plastids, geranyl diphosphate (GPP) synthases condensate one molecule IPP and one molecule DMAPP to produce GPP (C10), which is the precursor for monoterpenoid synthesis (Vranová et al. 2013). DMAPP can also act as a direct substrate for hemiterpenoid synthesis, which includes the hemiterpene isoprene and oxygen-containing derivates of isoprene (Jan and Abbas 2018). Furthermore, geranylgeranyl diphosphate (GGPP, C20) can be produced from one molecule DMAPP and three molecules IPP, and acts as precursor for di- and tetraterpenoid synthesis in plastids (Online Resource 1 b). GGPP synthases can be located in plastids, mitochondria and the endoplasmic reticulum (Henry et al. 2015).

The final step of terpenoid synthesis is carried out by terpene synthases (TPS), and the resulting molecules are classified based on the number of carbon atoms they contain. From the few common prenyl diphosphate substrates DMADP (C5), geranyl diphosphate (GDP, C10), farnesyl diphosphate (FDP, C15), and geranylgeranyl diphosphate (GGDP, C20), terpene synthases can produce an extensive variety of terpenoids, whereby each enzyme is capable of generating different products from a single substrate (Schwab 2003). TPS enzymes have been divided into 3 classes, whereby class I and II TPS are involved in primary metabolic and regulatory pathways, and class III TPS are associated with secondary metabolic processes (Keszei et al. 2010). Furthermore, TPS can be classified into seven clades. Class I TPS contain the clades TPS-c (diterpenoids), TPS-e/f (mono-, sesqui- and diterpenoids) and TPS-h (microbial TPS-like). TPS-d (mono-, sesqui-, diterpenoids) belong to class II and are gymnosperm-specific, and class III contains TPS-a (sesquiterpenoids), TPS-b (cyclic monoterpenoids and hemiterpenoids), and TPS-g (acyclic mono-, sesqui-, diterpenoids) (Chen et al. 2011). With exception of TPS-h and TPS-d, all remaining TPS clades are present in Myrtaceae (Külheim et al. 2015; Calvert et al. 2018). The proteins of the TPS class I usually have a length of around 800-850 amino acids (aa), whereby class III TPS are 500-650 aa long (Bohlmann et al. 1998; Aubourg et al. 2002).

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