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Review article

Terpenoid biosynthesis in trichomes—current status and future opportunities

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Summary

Glandular trichomes are anatomical structures specialized for the synthesis of secreted natural products. In this review we focus on the description of glands that accumulate terpenoid essential oils and oleoresins. We also provide an in-depth account of the current knowledge about the biosynthesis of terpenoids and secretion mechanisms in the highly specialized secretory cells of glandular trichomes, and highlight the implications for metabolic engineering efforts.

Phylogenetic distribution of glandular trichomes

Glandular trichomes (GTs) are epidermal hairs containing cells specialized for particular metabolic functions, usually the biosynthesis and secretion of copious amounts of particular secretory products, such as nectar, mucilage, acyl lipids, digestive enzymes or protective secondary metabolites. Some GTs instead sequester and secrete salts from plant tissues, function as hydathodes (glands associated with guttation), or have roles in the absorption of nutrients. The specialized cells often appear as distinctive globular clusters. GTs can be branched or unbranched, elongated, short, or sessile. Most consist of a group of glandular cells at the apex of a stalk of one or more cells in length (Duke and Paul, 1993; Fahn, 1979, 1988; Uphof, 1962; Werker, 2000). Often a thick cuticle covering the glandular cells separates from the attached cell wall to form a subcuticular pocket in which secretions accumulate (e.g. Ascensão *et al.*, 1999; Bell and Curtis, 1985; Duke and Paul, 1993; Hammond and Mahlberg, 1978; Hanlidou *et al.*, 1991; Turner *et al.*, 2000b) . Figures 1-4 illustrate a variety of GTs. Transverse sections of four common forms of GTs are shown in Figure 1. Figure 2 provides surface views of a range of GT types viewed with scanning electron microscopy (SEM). Figures 3 and 4 illustrate the details of peppermint (*Mentha* × *piperita* L.) monoterpene–secreting GTs with SEM and transmission electron microscopy (TEM).

Glandular trichomes appear to have arisen in plants independently many times. They are common in some genera of ferns (Ogura, 1972; Wollenweber and Schneider, 2000; Wollenweber *et al.*, 1998). They appear to be absent from conifers and are rare in cycads (Napp-Zinn, 1966), but GTs have been found attached to fossil seed fern leaves (Krings *et al.*, 2003). Within the angiosperms, GTs appear to be absent from members of the basal ANITA group (*Amborella*, Nymphaeales, Illiciales, Trimeniaceae and Austrobaileyaceae), except for hydropotes and related GTs of some Nymphaeales (Carpenter, 2006; Warner *et al.*, 2009). GTs are found in a number of monocotyledons, including the members of the Commelinaceae, Eriocaulaceae (Tomlinson 1969), *Dioscorea* (Behnke, 1984), *Cypripedium* (Swanson *et al.*, 1980) and *Sisyrinchium* (Chauveau *et al.*, 2011). GTs are more widespread in the eudicots and are characteristic vegetative epidermal features of many families and genera including, for example, the members of the Lamiales, Solanales, Asterales, Malvales, Caryophyllaceae, Cucurbitaceae, Fabaceae, Rosaceae, Sapindaceae and Saxifragaceae. For a more complete list see the tables provided by Metcalfe and Chalk, (1950, 1979), which present the distributions of GTs of various morphological types within the dicotyledons.

Spatial distribution of glandular trichomes on peppermint leaves

Glandular trichomes are often described as randomly distributed over plant surfaces, although careful observations indicate that this is generally not the case. For instance, in peppermint, peltate GTs are very rarely found as pairs or clusters, but instead appear more or less evenly spaced and separated from each other by a similar number of epidermal cells, but with different and predictable densities in different regions of a leaf (Maffei *et al.*, 1989; Turner *et al.*, 2000a). Furthermore, while they occur abundantly on the epidermis of areoles above regions of mesophyll in peppermint, peltate GTs are nearly absent from the epidermis above the veins, while the large unbranched non-GTs appear to be more abundant there (Figure 3). Colson *et al.* (1993) described a pattern of increasing numbers of GTs found on field-grown peppermint plants, where the total number of GTs produced on leaves increases significantly with each successive leaf pair throughout the growing season, so that each new leaf pair was found to have produced several thousand more glands than leaves of the previously produced node. This heteroblastic series appears to be under genetic control, because very similar progressions have been seen with plants grown under different environmental conditions in growth chambers (Turner *et al.*, 2000a). Because the essential oil yield from peppermint is directly proportional to the number of GTs produced on leaves, even a small shift in this pattern towards a precocious production of the high density of glands found on late season leaves could significantly affect essential oil yields for peppermint (Rios-Estepa *et al.*, 2010). An enhanced understanding of the regulation of GT formation could potentially allow for the engineering of new varieties of essential oil crops with much improved yields.

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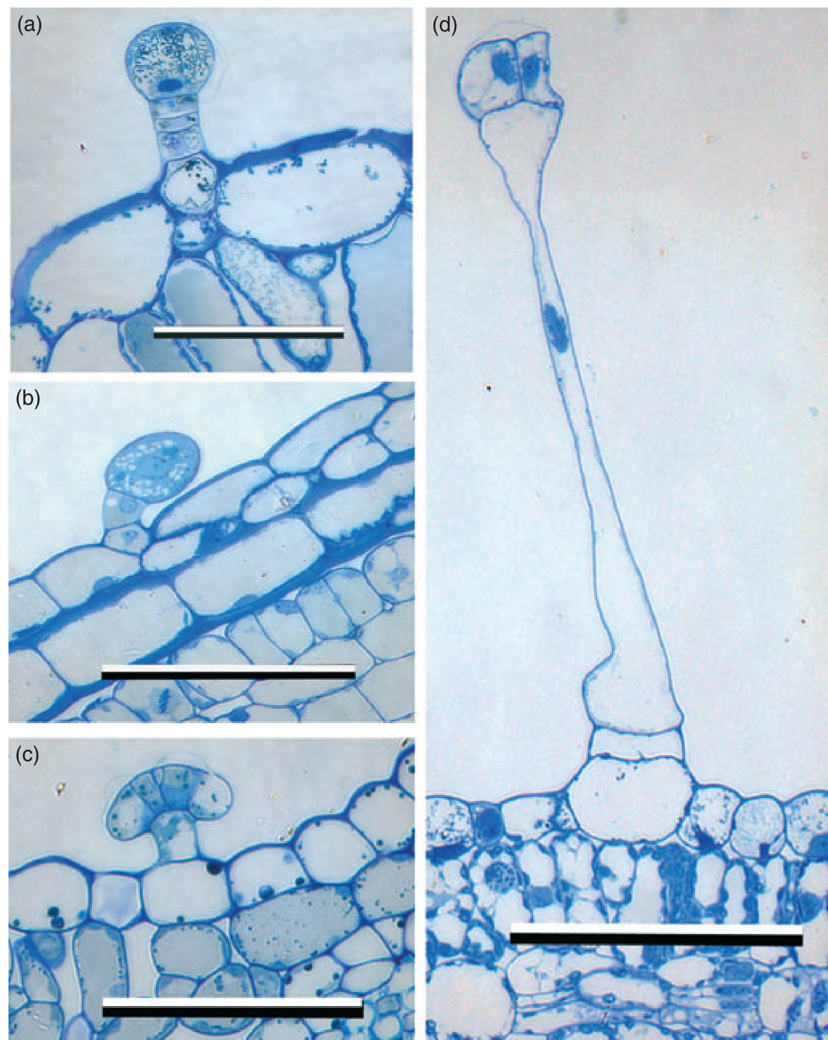

Figure 1 Examples of eudicot glandular trichomes shown in bright field micrographs of 1 μm thick sections, stained with toluidine blue. (a) Leaf cross-section of lime-scented *Pelargonium grossularioides* (L.) L'Hér. ex Aiton (Geraniaceae) with a capitate glandular trichome in medial section showing a basal cell at the epidermis, three stalk cells and a larger glandular cell at the apex (bar = 50 μm). (b) Small capitate glandular trichome on a young leaf of peppermint *Mentha* × *piperita* L. (Lamiaceae) consisting of one basal cell, one stalk cell and one glandular apical cell (bar = 50 μm). (c) Resin-secreting peltate glandular trichome from the leaf surface of *Diplacus aurantiacus* (Curtis) Jepson (Phrymaceae). This small trichome consists of a disc of cells at the apex of a single stalk cell (bar = 50 μm). (d) Elongated glandular trichome from a leaf of *Nicotiana benthamiana* Domin (Solanaceae). This trichome consists of four glandular cells at the apex of an elongated stalk cell, which is subtended by a short stalk cell and a basal cell (bar = 50 μm).

Epidermal pattern formation is an active field of plant molecular biological research in model plants, which has the potential to provide profound insights into the regulation of the distribution of trichomes and stomata on the surfaces of plant leaves and stems. The epidermis provides a two-dimensional system that is potentially less complicated than the patterning of three-dimensional tissues within plant organs. Recent studies have identified networks of transcription factors that appear to act together as activators or inhibitors of trichome initiation and maturation in protodermal cells. We will not discuss this topic further and instead refer the interested reader to recent reviews on trichome patterning (Balkunde *et al.*, 2010; Grebe, 2012; Pesch and Hülskamp, 2009).

Ultrastructure of glandular trichome stalk cells

Glandular trichomes stalks can consist of one, two or more rows of cells. They often contain one or more sets of barrier cells that have evidently suberized lateral walls, much like the Casparian strip of root endodermal cells. It has been suggested that the suberized cell walls form a barrier to apoplastic flow of aqueous metabolites, allowing the stalk cells to regulate directional transport of metabolites to the glandular cells above (Dell and McComb, 1978; Werker, 2000). Although this is a reasonable hypothesis, we know of no experimental investigations testing it. Some stalk cells can appear quite specialized. Stalk cells of peppermint peltate GTs have strongly suberized lateral walls, but their ultrastructure suggests additional unknown functions (Figure 4a). At secretory phase, the peppermint peltate gland stalk cells appear to be metabolically active and contain numerous large, non-green, spherical plastids that differ considerably in form from the leucoplasts of the glandular cells. They also contain relatively small vacuoles, numerous mitochondria and a relatively large number of microbodies (Turner *et al.*, 2000b). Such features were also reported for stalk cells of *Nepeta racimosa* L. (Bourett *et al.*, 1994). These specializations are associated with the secretory phase of GT development, but in peppermint the stalk cells do not appear to contain any of the enzymes of p-menthane monoterpene biosynthesis, and the functions of its unusual plastids and the abundant microbodies remain unexplored (Turner and Croteau, 2004; Turner *et al.*, 1999, 2012).

Golgi-type versus smooth endoplasmic reticulum-type secretion mechanisms

By the mid 1970s, it had been recognized that plant glandular cells secreting mucilage, nectar or proteolytic enzymes often

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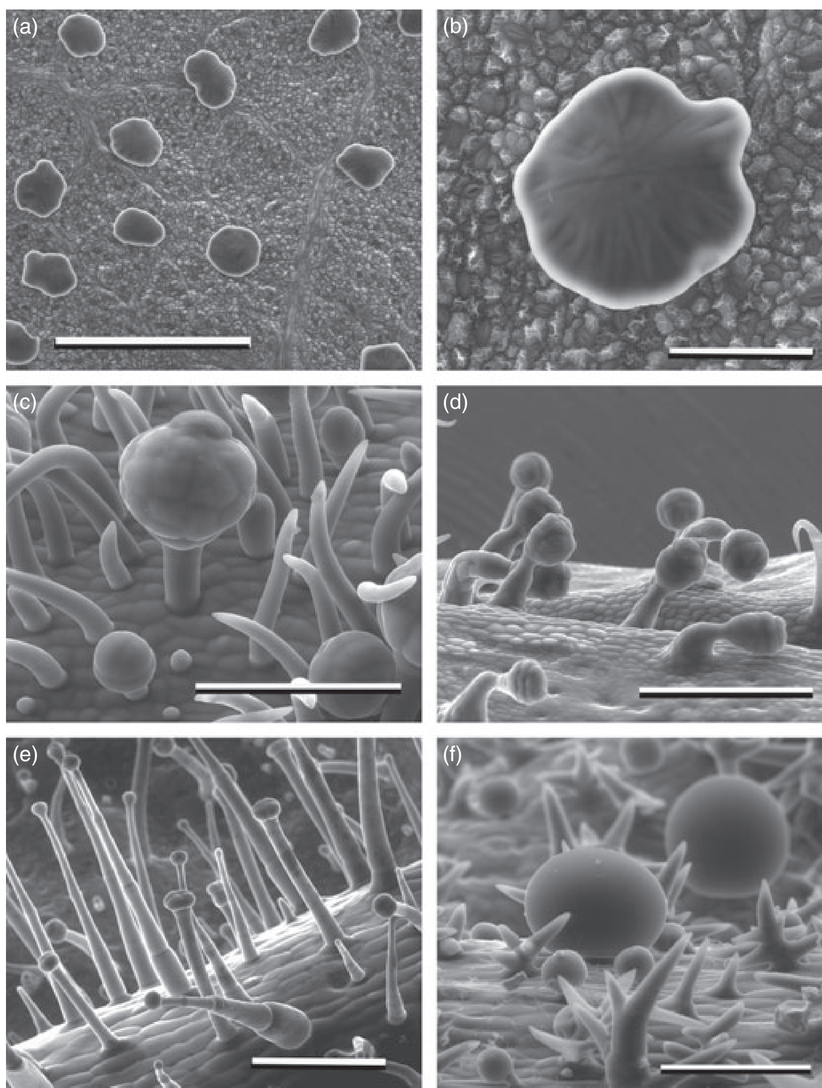

Figure 2 Scanning electron micrographs of diverse eudicot glandular trichomes imaged with a field emission scanning electron microscope using a freezing stage at −10°C and operating under environmental mode. (a) Leaf surface of hops, *Humulus lupulus* L. (Cannabaceae), showing a dozen large peltate glandular trichomes (bar = 500 μm). (b) Higher magnification of a hops peltate glandular trichome showing the upper surface of the large disc of glandular cells (bar = 100 μm). (c) Large club-shaped glandular trichomes among simple hairs on the surface of a developing leaf of *Ailanthus altissima* (Mill.) Swingle (Simaroubaceae) (bar = 100 μm). (d) Large capitate glandular trichomes on the leaf surface of *Ribes alpinum* L. (Grossulariaceae) (bar = 200 μm). (e) Glandular hairs on the leaf surface of *Nicotiana benthamiana* Domin (Solanaceae), consisting of small clusters of glandular cells at the apex of elongated and unbranched stalks (bar = 200 μm). (f) Leaf surface of *Perovskia atriplicifolia* Benth. (Lamiaceae) showing large hemispherical peltate glandular trichomes and smaller capitate glandular trichomes, among dendroid non-glandular trichomes. These peltate glandular trichomes are the typical of mints. They appear hemispherical because of an inflated cuticle that separates from a flat disc of glandular cells below and fills with secreted essential oil (bar = 100 μm).

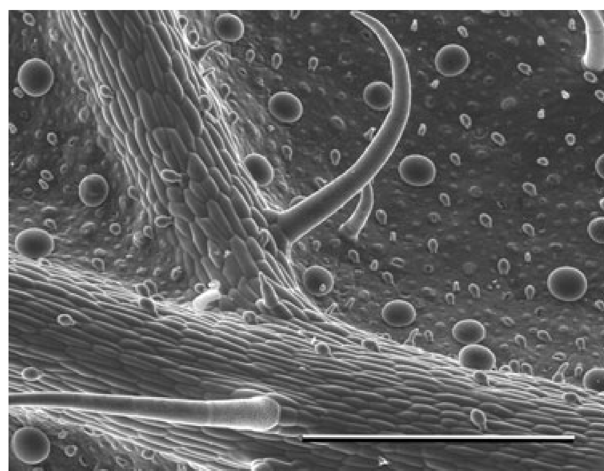

Figure 3 Cryo-scanning electron micrograph of the abaxial leaf surface of peppermint, *Mentha* × *piperita* L. (Lamiaceae), showing the distributions small capitate and large peltate glandular trichomes. The hemispherical, essential oil containing, peltate glands occur more or less evenly spaced over the leaf blade surface, but they are mostly absent from the epidermis above the major veins (bar = 500 μm).

contain a large abundance of Golgi apparatus, while those secreting lipophilic essential oils and resins are often characterized by a large abundance of smooth endoplasmic reticulum (SER) (Schnepf 1974). In retrospect, the high volume of Golgi found in carbohydrate- or protein-secreting glands is unsurprising because of the now well-established roles of the Golgi apparatus in protein and carbohydrate secretion (Hawes, 2005; Hawes and Satiat-Jeunemaitre, 2005; Parsons *et al.*, 2012; Staehelin, 1997). Much less is known about the roles of extensive SER in lipid-secreting glands. Plant glands secreting hydrophobic essential oils and resins share a number of common features. In addition to an extensively developed SER, they often contain unusually shaped amoeboid leucoplasts that are sometimes surrounded by an enclosing layer of periplastic SER. Numerous plastid–SER membrane contacts are apparent (Figure 4). They also share a number of common properties of other metabolically active cells such as a high density of ribosomes, a large nucleus-to-cytoplasm volume ratio and relatively small vacuoles (Schnepf 1974; Schnepf 1993; Schnepf and Klasova, 1972; Dell and McComb, 1978; Durkee *et al.*, 1984; Fahn, 1979, 1988; Werker, 2000).

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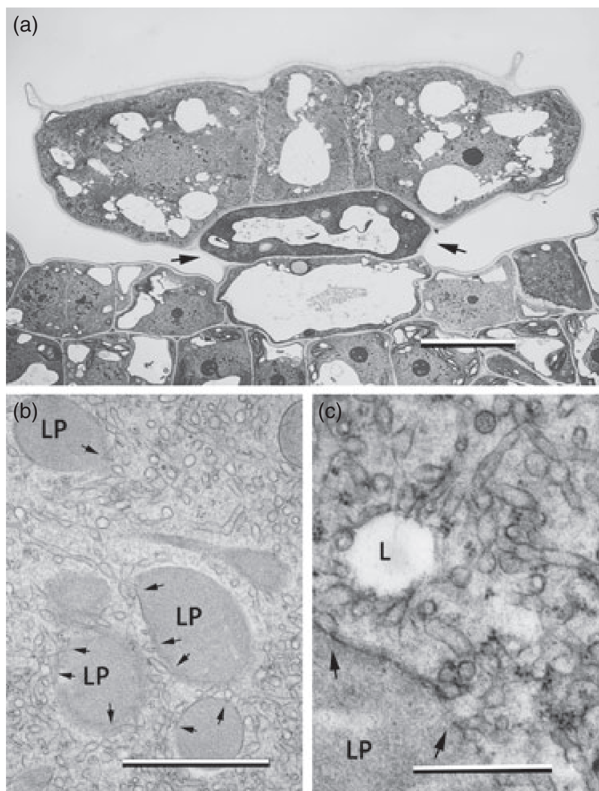

Figure 4 Transmission electron micrographs of peltate glandular trichomes of peppermint, *Mentha* × *piperita* L. (Lamiaceae). (a) Low magnification image of secretory phase peltate gland showing a disc of glandular cells, as stalk cell, and a basal cell. The glandular cells of the disc contain large leucoplasts (LP) and abundant endoplasmic reticulum. Areas of suberization of the lateral walls of the stalk cell are marked with arrows and can be seen as relatively lightly stained region of the walls. The thick cuticle above the glandular disc has separated from the glandular cells, but the subcuticular pocket has collapsed with the extraction of monoterpenes that occurred during tissue processing (bar = 10 μm). (b) High magnification showing a section of a freeze-substituted glandular cell during peak secretory activity. The amoeboid leucoplasts (LPs) have large surface areas with many elongated branches separating bulbous regions. Numerous membrane contacts can be seen between the leucoplasts and the abundant ER (arrows) (bar = 2 μm). (c) Higher magnification of a freeze-substituted cell showing the region of cytoplasm adjacent a leucoplast (LP) with apparent membrane contacts between the ER and the leucoplast (arrows). Remnants of a cytoplasmic lipid body (L), extracted during tissue processing, can be seen in the cytoplasm surrounded by ER membranes (bar = 1 μm).

Plastids of glandular trichome secretory cells

The plastids of the secretory cells of plant glands, which include GTs, idioblasts, secretory cavities, and secretory ducts, are often non-pigmented, unusually shaped, or in more subtle ways different from the chloroplast of neighbouring chlorenchyma tissue. The presence of amoeboid, non-pigmented plastids, which lack thylakoid membranes and have few ribosomes, appears to correlate strongly with the presence of monoterpenes in the secretion product (Cardé, 1984; Cheniclet and Cardé, 1985), but no correlation has been found for the presence of high sesquiterpenoid, alkaloid or phenylpropanoid content in the secretion and the presence of a particular plastid type in the gland cells (Cheniclet and Cardé, 1985). The plastids of glandular cells of some plants contain functional chloroplasts, including the GTs of some members such as the Solanaceae and Asteraceae (Cheniclet and Cardé, 1985). The glandular hairs of *Nicotiana tabacum* L. contain well-developed chloroplasts in their apical glandular cells, as well as abundant transcripts for photosynthesis-related enzymes (Cui *et al.*, 2011; Nielsen *et al.*, 1991), while the GTs of *Artemisia annua* L. contain five layers of cells, three of which contain well-developed chloroplasts and two of which contain leucoplast-like plastids, suggesting potentially different physiological specializations for the different cell layers (Duke and Paul, 1993). Similar GTs with both cells containing chloroplasts and cells containing apparent leucoplasts appear to be common in the Asteraceae and have been described from *Sigesbeckia* and *Helianthus*. (Göpfert *et al.*, 2005; Heinrich *et al.*, 2010).

Role of leucoplasts in monoterpene biosynthesis

Leucoplasts have been associated with plant oil- and resin-secreting glands since the 1960s, and their role in monoterpene biosynthesis is now well established. It is important to note that the use of the term leucoplast for amoeboid, non-photosynthetic plastids of oil glands can be confusing because leucoplast is a general term referring to any non-pigmented plastid, including the starch-storing amyloplasts and acyl lipid–storing elaioplasts of seeds, stems and roots. In the 1980s, Cardé and co-workers detailed the development, structure and function of plant oil gland cell leucoplasts from resin ducts, secretory cavities and GTs. While only some of the leucoplasts they studied were from monoterpene-secreting trichomes, the leucoplasts of resin ducts and secretory cavities are very similar to those found in GTs (Cardé, 1984; Fahn, 1979, 1988). Although much of the preliminary work on leucoplasts was carried out on those from secretory cavities and resin ducts, the similarities between these leucoplasts and those of oil-secreting GTs suggest that they are functionally equivalent and relevant to our discussion of GT secretory cells.

Gleizes *et al.* (1983) demonstrated that isolated leucoplasts from secretory cavities of the exocarp of *Citrofortunella mitis* fruits were able to synthesize monoterpene hydrocarbons *in vitro* when provided with the terpenoid precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Cardé (1984) provided descriptions of gland-associated leucoplasts from nineteen divergent species of angiosperms and one pine, including the leucoplasts from GTs of five angiosperm species. These plastids were found to share a number of common features including: a branched amoeboid form; a close association with an enclosing periplastic endoplasmic reticulum; a stroma that lacks thylakoids, but may instead contain tubular membranes; and a stroma that appears to lack 70S ribosomes. Cheniclet and Cardé (1985) carried out a large correlative study involving 45 species and showed that there is a strong correlation between the presence and volume of leucoplasts in gland cells and the quantity of monoterpenes in the secretion produced by the cells. Charon *et al.* (1987) investigated the morphology of leucoplasts from developing *Pinus pinaster* resin ducts. They found that resin duct leucoplasts continued to enlarge forming large complex structures with large central bodies containing numerous deep pockets and small branches, which were all derived from a small number of proplastids present in newly formed gland cells. The authors estimated that the relative

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volume ratio of leucoplasts to cytoplasm within resin duct gland cells was 2.5 times that of the chloroplast-to-cytoplasm volume ratio of pine mesophyll chlorenchyma cells. They also found that the surface area of leucoplasts in a pine resin duct gland cell was three times that of chloroplasts in chlorenchyma cells, and the large leucoplast surface was in close contact with ensheathing periplastic ER (Charon *et al.*, 1987).

Following the discovery of the plastid-resident 2*C*-methyl-D-erythritol 4-phosphate (MEP) pathway for the production of IPP and DMAPP and its role providing these metabolites for most monoterpenes produced in GTs, it was established that the precursors for monoterpene biosynthesis originate within plastids (reviewed in Tissier, 2012a), while immunocytochemical localizations provided direct evidence that the enzymes catalysing the first committed steps of monoterpene biosynthesis reside within the stroma of gland cell leucoplasts (Tholl *et al.*, 2004; Turner and Croteau, 2004; Turner *et al.*, 1999).

Possible roles of the endoplasmic reticulum

Although the role of leucoplasts in monoterpene biosynthesis in GTs has been established, the role of the SER is not well understood but, because it is so abundant, it is likely to be an important part of the secretory machinery. SER is often a prominent feature of secretory phase oil- and resin-secreting glandular cells. For example, it is abundant in glandular cells of *Lonicera periclymenum* L., *Ononis repens* L., *Senecio viscosus* L. (Schnepf and Klasova, 1972), *Passiflora foetida* L. (Durkee *et al.*, 1984), *N. racemosa* L. (Bourett *et al.*, 1994), *A. annua* L. (Duke and Paul, 1993), *Leonotis leonurus* R. Br. (Ascensão *et al.*, 1997), *Mentha* × *piperita* L. (Turner *et al.*, 2000b) and *Zeyheria montana* Mart. (Machado *et al.*, 2006). When the SER is well preserved, its abundance in TEM images of secretory phase gland cells can nearly rival that of sterol-synthesizing glandular cells of animals (Black *et al.*, 2005; Schnepf and Klasova, 1972). At least in part, these similarities may reflect coincidence rather than homologous functions, because nearly all of the sterol biosynthetic enzymes of mammalian steroid and cholesterol-secreting cells are localized to the SER, and the abundance of SER could indirectly reflect the abundance of sterol biosynthetic enzymes, while most of the terpenoid biosynthesis in plant oil gland cells occurs at other subcellular locations. Membrane contact sites (MCSs) of mammalian cell SER are believed to play an important role in the intracellular transfer of steroids and other lipids (Levin, 2004; van Meer *et al.*, 2008; Toulmay and Prinz, 2011). For example, there is evidence that some oxysterol-binding proteins and other lipid transfer proteins that may facilitate non-vesicular transfer of sterols between organelle membranes or function as receptors for lipid signalling are enriched in ER membranes at MCSs (Raychaudhuri and Prinz, 2010; Vihervaara *et al.*, 2011). In general, MCSs between SER and other organelles can play important roles in non-vesicular transport of lipids in eukaryotic cells (Levin, 2004; Levin and Loewen, 2006; van Meer *et al.*, 2008; Toulmay and Prinz, 2011). Exchange of lipids at chloroplast–ER MCSs is important for chloroplast development. For example, fatty acids synthesized within chloroplasts are transported to the ER for further metabolic conversions to lipids, but non-vesicular lipid transfer from the ER back to chloroplasts has been shown to provide diacylglycerol for the biosynthesis of galactoglycerolipids. Both transfers apparently occur at ER–chloroplast MCSs, and recently several ER and chloroplast proteins have been identified and shown by mutational analysis to strongly affect ER-to-chloroplast lipid transfer (Awai *et al.*, 2006; Benning, 2008; Xu *et al.*, 2008; Tan *et al.*, 2011; Wang *et al.*, 2012).

It is tempting to propose a role for ER-leucoplast and ER-plasma membrane MCSs for intracellular transfer of terpenoids in plant oil gland cells. MCSs are common in plant terpenoid-secreting cells. The extensive periplastic ER found surrounding leucoplasts in many species forms numerous evident contacts between the SER and the leucoplasts (Figure 4b,c). Furthermore, an examination of published micrographs suggests that MCSs are also common between the SER and mitochondria and between the cortical SER and the plasma membrane (Bourett *et al.*, 1994; Duke and Paul, 1993; Fahn, 1979; Turner *et al.*, 2000b). The physical properties of monoterpenes indicate that they should partition easily into outer leaflet lipid membranes (Cristani *et al.*, 2007; Turina *et al.*, 2006), and perhaps, intracellular transfer of secreted monoterpenes could be facilitated by the numerous MCSs between the SER and other organelles.

The subcellular distribution of monoterpene biosynthetic enzymes in peppermint peltate gland cells and its implication for metabolite translocation

The Croteau laboratory employed peppermint GTs as a model system for the characterization of plant monoterpene biosynthesis (reviewed in Croteau *et al.*, 2005). One component of this research included descriptions of peppermint gland cell ultrastructure and immunocytochemical localizations of key enzymes using immunogold labelling for TEM. The subcellular distributions of these enzymes suggest that a relatively complicated movement of metabolites occurs within glandular cells during essential oil biosynthesis (Turner and Croteau, 2004; Turner *et al.*, 1999, 2012). The flux of these metabolites was found to be relatively rapid. Turner *et al.* (2000a) used photography and observations of the distribution of GTs of different developmental stages to estimate the rate of filling of the subcuticular oil storage pocket. It was found that each trichome glandular cell secreted a volume approximately equal to its own within about 25 h of secretory activity (Turner and Croteau, 2004; Turner *et al.*, 2000a). Turner and Croteau (2004) estimated the flux of monoterpenes across the secretory cell plasma membranes to be approximately 1.8 fmol/μm^2/h.

In peppermint, IPP and DMAPP for monoterpene biosynthesis are derived exclusively from the plastidial MEP pathway (Eisenreich *et al.*, 1998). Geranyl pyrophosphate (GPP) is produced by the condensation of IPP and DMAPP, and it is not surprising that GPP synthase was localized in plastids (Turner and Croteau, 2004). Label was found only within gland cell leucoplasts, and it became abundant only at secretory phase, indicating that the peltate GTs are likely the sole source of monoterpene biosynthesis within peppermint leaves. The next step in the p-menthane monoterpene pathway involves the cyclization of GPP to (−)-limonene, catalysed by (−)-(4*S*)-limonene synthase. This enzyme was also localized to the leucoplasts of glandular cells within peppermint peltate GTs (Turner *et al.*, 1999). Limonene 6-hydroxylase, which catalyses the oxygenation of (−)-limonene, was localized to the SER of spearmint peltate GTs (Turner and Croteau, 2004). This enzyme catalyses the oxygenation of (−)-limonene at the C6 position, while its close peppermint homolog oxygenates at the C3 position. Limonene 3-hydroxylase of peppermint is presumed to also reside at the SER. The product

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of limonene 3-hydroxylase catalysis, *trans*-(−)-isopiperitenol, is rapidly oxidized to *trans*-(−)-isopiperitenone by isopiperitenol dehydrogenase, which was localized to peltate gland cell mitochondria (Turner and Croteau, 2004). The enzymes catalysing reductions, isopiperitenone reductase (producing (+)-cis-isopulegone from (−)-*trans*-(−)-isopiperitenone), pulegone reductase (producing (−)-menthone and (+)-isomenthone from (+)-pulegone), and (−)-menthone/menthol reductase (producing (−)-menthol from (−)-menthone and (+)-neoisomenthol from (+)-isomenthone), were all localized to the cytoplasm and nuclei of peltate gland cells (Turner and Croteau, 2004; Turner *et al.*, 2012). One of these steps, the conversion of (−)-menthone to (−)-menthol by (−)-menthone/menthol reductase occurs mainly in older leaves, long after new trichomes have stopped forming and after all of the peltate GTs have completed their initial secretion of (−)-menthone-rich essential oil. Over a period of several weeks (−)-menthone is slowly converted to menthol within glandular cells (McConkey *et al.*, 2000; Turner *et al.*, 2012). In summary, these results suggest a rapid movement of metabolites through the glandular cells of the peltate trichomes at secretory phase beginning with the production of (−)-limonene in plastids, followed by the hydroxylation of (−)-limonene at the SER surface, efficient conversion of (−)-isopiperitenol to (−)-*trans*-isopiperitenone within mitochondria, and ultimately by a series of reductions occurring within the cytoplasm. They also imply that (−)-menthone-rich essential oil, initially secreted to the subcuticular space, slowly returns to the gland cells where it is converted to menthol, before it is again secreted into the extracellular oil storage space.

We have attempted to provide a brief overview of the structure and biology of essential oil-producing GTs. These remarkable structures contain, in a few glandular cells, the biosynthetic machinery that can quickly and efficiently turn imported sugar into essential oil. The subcellular organization of these cells is a vital component that allows for efficient transfer of metabolites during oil biosynthesis. In the following paragraphs, we will outline the current knowledge regarding the essential oil biosynthetic machinery in GTs of various angiosperms.

Utility of transcriptome sequencing of glandular trichomes for terpenoid pathway gene discovery

Isolation of secretory glandular trichomes

The ultrastructural features and the limited structural diversity of essential oil or resin constituents contained in GTs were highly suggestive of a substantial metabolic specialization. Beginning in the mid-1980s, several methods for the isolation of gland cells from GTs and subsequent biochemical analysis were developed (Table 1). These protocols involved percoll density gradient centrifugation (Slone and Kelsey, 1985), surface scraping with razor blades (Spring *et al.*, 1987) or powdered dry ice (Yerger *et al.*, 1992), chafing with quartz sand in buffer (Hashidoko and Urashima, 1994), or abrasion with glass beads in a highly viscous buffer (Gershenzon *et al.*, 1987, 1992), with subsequent filtering or other means of size selection. These isolated gland cells were shown to be a rich, if not exclusive, source of enzyme activities related to terpenoid essential oil biosynthesis (Gershenzon *et al.*, 1987, 1989, 1992; McCaskill and Croteau, 1995; McCaskill *et al.*, 1992; Yerger *et al.*, 1992). Yerger *et al.* (1992) were the first to obtain RNA from isolated gland cells, but it was not until the mid- to late 1990s that such RNA was successfully captured

Table 1 Development of techniques for the isolation of secretory glandular trichomes

Glandular trichome isolation technique	Species	References
Mechanical abrasion	*Mentha × piperita*, *Helianthus annuus*	Croteau and Winters (1982); Spring *et al.* (1987)
Percoll density gradient centrifugation	*Artemisia annua*	Slone and Kelsey (1985)
Surface abrasion with glass beads	*Mentha × piperita*, *Mentha spicata*, *Salvia officinalis*, *Tanacetum vulgare*	Gershenzon *et al.* (1987, 1989, 1992)
Adhesive tape	*Thymus vulgaris*	Yamaura *et al.* (1992)
Surface abrasion with powdered dry ice	*Abutilon theophrasti*, *Cucurbita pepo*, *Pelargonium* sp., *Solanum lycopersicum*, *Solanum tuberosum*	Yerger *et al.* (1992)
Abrasion with quartz sand	*Rosa rugosa*	Hashidoko and Urashima (1994)
Laser microdissection	*A. annua*	Olsson *et al.* (2009); Olofsson *et al.* (2012)

for the generation of cDNA libraries (Lange *et al.*, 2000). Olofsson *et al.* (2012) recently introduced a protocol that involves the immersion of hand-sectioned flower buds in an appropriate buffer on glass slides and direct laser microdissection of gland cells. The authors demonstrated that RNA isolated from the cells was suitable for real-time PCR, indicating the potential of this method for the rapid cloning of gland cell-expressed genes (Olofsson *et al.*, 2012).

By modifying previously established methods to collect gland cells (Gershenzon *et al.*, 1987, 1989, 1992), we were able to extract high-quality RNA for the generation of cDNA libraries, and subsequently developed expressed sequence tag (EST) resources (Lange *et al.*, 2000). This effort turned out to be a treasure trove for genes involved in peppermint essential oil biosynthesis and validated the hypothesis that transcript abundance in gland cells should reflect the phenomenal metabolic specialization of peppermint leaf GTs. The general approach of using transcriptome data (or a combination of 'omics' data) from isolated gland cells for gene discovery efforts has now been employed successfully for many GT-bearing plants, and we are highlighting a few examples with relevance for understanding terpenoid biochemistry in the upcoming paragraphs. For large gene families with known conserved motifs or domains, such as terpene synthases, PCR with degenerate primers has been used successfully in many studies, while transcriptome data have been particularly helpful for the identification of genes coding for novel activities with relevance to terpenoid biosynthesis.

Monoterpene and sesquiterpene biosynthesis in the Lamiaceae

The above-mentioned EST project with peppermint gland cells enabled the Croteau laboratory to eventually clone and characterize all genes involved in the fairly complex p-menthane monoterpene pathway (with only one exception) within a few years (Bertea *et al.*, 2001; Burke and Croteau, 1999; Davis *et al.*,

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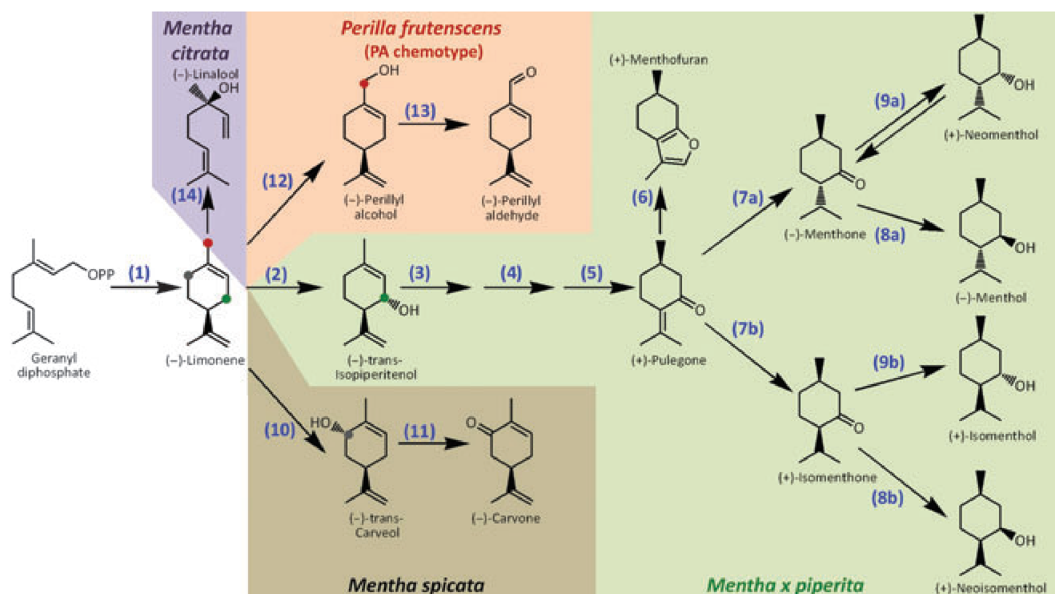

Figure 5 Overview of p-menthane monoterpene biosynthesis in Lamiaceae. The enzymes involved in this pathway are (1), (−)-limonene synthase; (2), (−)-limonene 3-hydroxylase; (3), (−)-*trans*-isopiperitenol dehydrogenase; (4), (−)-*trans*-isopiperitenone reductase; (5), (+)-*cis*-isopulegone isomerase; (6), (+)-menthofuran synthase; (7a), (+)-pulegone reductase [(−)-menthone-forming activity]; (7b), (+)-pulegone reductase [(+)-isomenthone-forming activity]; (8a), (−)-menthone: (−)-menthol reductase [(−)-menthol-forming activity]; (8b), (−)-menthone: (−)-menthol reductase [(+)-neoisomenthol-forming activity]; (9a), (−)-menthone: (+)-neomenthol reductase [(+)-neomenthol-forming activity]; (9b), (−)-menthone: (+)-neomenthol reductase [(+)-isomenthol-forming activity]; (10), (−)-limonene 6-hydroxylase; (11), (−)-*trans*-carveol dehydrogenase; (12), (−)-limonene 7-hydroxylase; and (13), (−)-perillyl alcohol dehydrogenase.

2005; Ringer *et al.*, 2003, 2005) (Figure 5) (Table 2). In addition, the peppermint EST effort was the source of novel gene candidates for the plastidial MEP pathway of terpenoid precursor biosynthesis (Lange and Croteau, 1999a; Lange and Croteau, 1999b; Lange *et al.*, 1998). This pathway had been suggested to exist based on labelling patterns obtained after feeding plants and microbes with carbon sources that were labelled with stable isotopes in specific positions (Eisenreich *et al.*, 1998), but the individual steps had not yet been elucidated at the time. The peppermint EST data set was also used to clone two sesquiterpene synthases, *E,E-trans*-beta-farnesene synthase (Crock *et al.*, 1997) and *cis*-muurola-4(14),5-diene synthase (Prosser *et al.*, 2006). The gene encoding (−)-limonene synthase, which catalyses the committed step in the p-menthane pathway in spearmint and peppermint, had been cloned using 'traditional' approaches by purification of the native protein, internal amino acid sequencing, probe generation with degenerate primers, screening of a leaf cDNA library, and subsequent characterization of recombinantly expressed candidate cDNAs (Colby *et al.*, 1993) (Figure 5). In bergamot mint (*Mentha citrata*), the principal monoterpenes are (−)-linalool and its acetate ester, and a (−)-linalool synthase was cloned and characterized based on the homology to other monoterpene synthases of the Lamiaceae (Crowell *et al.*, 2002). Degenerate PCR with primers designed to amplify conserved domains in cytochrome P450 monooxygenases was also used in the cloning of (−)-limonene 3-hydroxylase from peppermint and (−)-limonene 6-hydroxylase from spearmint (Lupien *et al.*, 1999). A second limonene 3-hydroxylase clone was obtained by PCR from curly mint (*Mentha spicata* L. crispa) (Lücker *et al.*, 2004). Subsequently, a (−)-limonene 7-hydroxylase clone was obtained from *Perilla frutescens* by screening a gland cell cDNA library with a probe representing both the peppermint and spearmint sequences (Mau *et al.*, 2010) (Figure 5). Interestingly, although there is only moderate sequence identity among these three functionally distinct monooxygenases (~60%–70%), it was shown that a single amino acid exchange, using site-directed mutagenesis, was sufficient to change the regiospecificity from a 6-hydroxylase to a 3-hydroxylase (Schalk and Croteau, 2000). Within the genus *Perilla,* various chemotypes accumulate essential oils with different mixtures of monoterpenoids, and several genes involved in the formation of these constituents have been cloned, including terpene synthases catalysing the synthesis of (−)-limonene, myrcene, geraniol and (−)-linalool (Hosoi *et al.*, 2004; Ito and Honda, 2007; Masumoto *et al.*, 2010; Yuba *et al.*, 1996) (Table 2).

Gang *et al.* (2001) obtained ESTs from gland cells of three sweet basil (*Ocimum basilicum*) lines that accumulate chemically distinct essential oils. While the major constituents of sweet basil oils are phenylpropenes and their derivatives, the Pichersky laboratory was also successful in identifying and characterizing the functions of genes encoding six monoterpene synthases (forming β-myrcene, (−)-endo-fenchol, terpinolene, (+)-linalool or geraniol as major products) and four sesquiterpene synthases (forming α-zingiberene, α/β-selinene, germacrene D or γ-cadinene as major products) (Iijima *et al.*, 2004a,b) (Table 2). The group also characterized a gene coding for an enzyme that oxidizes geraniol to geranial (CAD1) and a second gene encoding an enzyme capable of oxidizing both geraniol and nerol (GEDH1) (Iijima *et al.*, 2006). An analogous effort allowed Crocoll *et al.* (2010) to elucidate the roles of three monoterpene synthases (forming sabinene, γ-terpinene or *trans*-β-ocimene as major products) and three sesquiterpene synthases (forming germacrene D, bicyclogermacrene or *E*-β-caryophyllene as major products) in oregano cultivars with terpenoid compositional

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Table 2 Functionally characterized genes involved in terpenoid biosynthesis in secretory glandular trichomes

Species (Family)	Function (based on main product(s))	References
Asteraceae		
Artemisia annua	Amorpha-4,11-diene synthase (KCS12)	Chang *et al.* (2000)
	Amorpha-4,11-diene synthase (pAC12)	Mercke *et al.* (2000)
	Amorpha-4,11-diene synthase	Wallaart *et al.* (2001)
	Multifunctional sesquiterpene oxidase (CYP71AV1)	Teoh *et al.* (2006)
	Multifunctional sesquiterpene oxidase (CYP71AV1)	Ro *et al.* (2006)
	Artemisinic aldehyde-Δ11(13) reductase (DBR2)	Zhang *et al.* (2008b)
	Aldehyde dehydrogenase 1 (ALDH1)	Teoh *et al.* (2009)
	Alcohol dehydrogenase 2 (ALDH2)	Polichuk *et al.* (2010)
	Dihydroartemisinic aldehyde reductase (Red1)	Rydén *et al.* (2010)
	2-Alkenal reductase (DBR1)	Zhang *et al.* (2008a)
	β-Caryophyllene synthase (QHS1)	Cai *et al.* (2002)
	epi -Cedrol synthase (ECS)	Mercke *et al.* (1999)
	8-*epi* -Cedrol synthase	Hua and Matsuda (1999)
	β-Farnesene synthase (β-FS)	Picaud *et al.* (2005)
	Germacrene A synthase (AaGAS)	Bertea *et al.* (2006)
	(+)-Linalool synthase (two clones; QH1; QH5)	Jia *et al.* (1999)
	(−)-β-Pinene synthase (QH6)	Lu *et al.* (2002)
Helianthus anuus	Germacrene A synthase (two clones; HaGAS1, HaTPS1a; HaGAS2, HaTPS1b)	Göpfert *et al.* (2009)
	Multiproduct sesquiterpene synthase (HaCS, HaTPS2)	Göpfert *et al.* (2009)
	Germacrene A acid 8β-hydroxylase (CYP71BL1)	Ikezawa *et al.* (2011)
Solidago canadensis	(+)-Germacerne A synthase (Sc1)	Prosser *et al.* (2002)
Tanacetum parthenium	E-β-Caryophyllene synthase (TpCarS))	Majdi *et al.* (2011)
	Germacrene A synthase (TpGAS)	Majdi *et al.* (2011)
Cannabinaceae		
Cannabis sativa	Olivetol synthase (OLS)	Taura *et al.* (2009)
	Aromatic prenyltransferase (PT)	Page and Boubakir (2011)
	Δ1-Tetrahydrocannabinolic acid synthase (THCAS)	Sirikantaramas *et al.* (2004)
	Cannabidiolic acid synthase (CBDAS)	Taura *et al.* (2007)
	(−)-Limonene synthase (CsTPS2)	Günnewich *et al.* (2007)
	(+)-α-pinene synthase (CsTPS1)	Günnewich *et al.* (2007)
Humulus lupulus	Valerophenone synthase (VPS)	Okada and Ito (2001)
	Prenyltransferase 1 (HIPT-1)	Tsurumaru *et al.* (2010, 2012)
	Geranyl diphosphate synthase (heterodimer; GGPPS SSU and LSU)	Wang *et al.* (2009)
	Myrcene synthase (HIMTS2)	Wang *et al.* (2008)
	β-Caryophyllene/α-Humulene synthase (HISTS1)	Wang *et al.* (2008)
	Germacrene A synthase (HISTS2)	Wang *et al.* (2008)
Cistaceae		
Cistus creticus	Geranylgeranyl diphosphate synthase (CcGGDPS1, CcGGDPS2)	Pateraki and Kanellis (2008)
	Copal-8-ol diphosphate synthase (CcCLS)	Falara *et al.* (2010)
Lamiaceae		
Agastache rugosa	(+)-Limonene synthase (ArLMS)	Maruyama *et al.* (2002)
Lavandula angustifolia	β-Phellandrene synthase (LaβPHLS)	Demissie *et al.* (2010)
	(+)-Limonene synthase (LaLIMS)	Landmann *et al.* (2007)
	(−)-Linalool synthase (LaLINS)	Landmann *et al.* (2007)
	trans-α-Bergamotene synthase (LaBERS)	Landmann *et al.* (2007)
Mentha citrata	(−)-Linalool synthase	Crowell *et al.* (2002)
Mentha × *piperita*	Geranyl diphosphate synthase (heterodimer; pMp13.18 and pMp23.10)	Burke and Croteau (1999)
	(−)-Limonene 3-hydroxylase (PM17, CYP71D13; PM2, CYP71D13)	Lupien *et al.* (1999)
	(−)-trans-Isopiperitenol dehydrogenase (ISPD)	Ringer *et al.* (2005)
	(−)-trans-Isopiperitenone reductase (IspR)	Ringer *et al.* (2003)
	(+)-Menthofuran synthase (MFS)	Bertea *et al.* (2001)
	(+)-Pulegone reductase (PulR)	Davis *et al.* (2005)
	(−)-Menthone:(−)-menthol reductase (MMR)	Davis *et al.* (2005)
	(−)-Menthone:(+)-neomenthol reductase (MNR)	Davis *et al.* (2005)
	E,E-trans-β-Farnesene synthase (p43)	Crock *et al.* (1997)
	cis-Muuroladiene synthase (MxpSS1)	Prosser *et al.* (2006)

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Table 2 Continued

Species (Family)	Function (based on main product(s))	References
Mentha spicata	(−)-Limonene synthase (pLC5.2)	Colby *et al.* (1993)
	(−)-Limonene 6-hydroxylase (SM12, CYP71D18)	Lupien *et al.* (1999)
	(−)-Carveol dehydrogenase (ISPD)	Ringer *et al.* (2005)
M. spicata L. Crispa	Limonene 3-hydroxylase (Lim3h)	Lücker *et al.* (2004)
Ocimum basilicum	β-Myrcene synthase (MYS)	Iijima *et al.* (2004a)
	(−)-endo-Fenchol synthase (FES)	Iijima *et al.* (2004b)
	Terpinolene synthase (TES)	Iijima *et al.* (2004b)
	(+)-Linalool synthase (LIS)	Iijima *et al.* (2004b)
	Geraniol synthase (GES)	Iijima *et al.* (2004a)
	α-Zingiberene synthase (ZIS)	Iijima *et al.* (2004a)
	α/β-Selinene synthase (SES)	Iijima *et al.* (2004a)
	Germacrene D synthase (GDS)	Iijima *et al.* (2004a)
	γ-Cadinene synthase (CDS)	Iijima *et al.* (2004a)
	Geraniol oxidase (CAD1)	Iijima *et al.* (2006)
	Geraniol/nerol oxidase (GEDH1)	Iijima *et al.* (2006)
Origanum vulgare	Sabinene synthase (OvTPS1)	Crocoll *et al.* (2010)
	γ-Terpinene synthase (OvTPS2)	Crocoll *et al.* (2010)
	trans -β-Ocimene synthase (OvTPS7)	Crocoll *et al.* (2010)
	(−)-Germacrene D synthase (OvTPS3)	Crocoll *et al.* (2010)
	Bicyclogermacrene synthase (OvTPS4)	Crocoll *et al.* (2010)
	E-β-Caryophyllene synthase (OvTPS6)	Crocoll *et al.* (2010)
Perilla citridora	Geraniol synthase (PcTps-C)	Ito and Honda (2007)
Perilla frutescens	(−)-Limonene synthase (PFLC1)	Yuba *et al.* (1996)
	(−)-Limonene 7-hydroxylase	Mau *et al.* (2010)
	Myrcene synthase (PTS-5526)	Hosoi *et al.* (2004)
	Geraniol synthase (PfTPS-PL)	Ito and Honda (2007)
Perilla hirtella/Perilla setoyensis	Geraniol synthase	Masumoto *et al.* (2010)
	(−)-Linalool synthase	Masumoto *et al.* (2010)
Pogostemon cabli	γ-Curcumene synthase (PatTpsA)	Deguerry *et al.* (2006)
	(+)-Germacrene A synthase (PatTpsCF2)	Deguerry *et al.* (2006)
	(−)-Germacrene D synthase (two clones; PatTpsBF2; PaTpsB15)	Deguerry *et al.* (2006)
	(−)-Pathoulolol synthase (PatTps177)	Deguerry *et al.* (2006)
Salvia fruticosa	1,8-Cineole synthase (Sf-CinS1)	Kampranis *et al.* (2007)
Salvia officinalis	(+)-Bornyl diphosphate synthase (SBS)	Wise *et al.* (1998)
	1,8-Cineole synthase (SCS)	Wise *et al.* (1998)
	(+)-Sabinene synthase (SSS)	Wise *et al.* (1998)
Salvia pornifera	Sabinene synthase (Sp-SabS1)	Kampranis *et al.* (2007)
Salvia stenophylla	(+)-3-Carene synthase	Hölscher *et al.* (2003)
Schizonepeta tenuifolia	(+)-Limonene synthase (dLMS)	Maruyama *et al.* (2001)
Solanaceae		
Nicotiana tabacum	Z-abienol synthase (NtABS)	Sallaud *et al.* (2012)
	α-Cembratrienol/β-cembratrienol synthase (CYC-1)	Wang and Wagner (2003)
	Cembratrienol synthase (NSCBTS-2a; NsCBTS-2b; NsCBTS-3; NsCBTS-4)	Ennajdaoui *et al.* (2010)
	α-Cembratrienol/β-cembratrienol monooxygenase (CYP71D16)	Wang and Wagner (2003)
	8-Hydroxy-copalyl diphosphate synthase (NtCPS2)	Sallaud *et al.* (2012)
Solanum habrochaites	α-and β-Bisabolene synthase (ShTPS14)	Bleeker *et al.* (2011)
	β-Elemene synthase (ShTPS15)	Bleeker *et al.* (2011)
	Germacrene B synthase (SSTLH1)	van der Hoeven *et al.* (2000)
	Germacrene D synthase (SSTLH2)	van der Hoeven *et al.* (2000)
	Limonene synthase (ShLMS)	Gonzales-Vigil *et al.* (2012)
	β-Phellandrene synthase (ShPHS1)	Gonzales-Vigil *et al.* (2012)
	α-Pinene synthase (ShPIS)	Gonzales-Vigil *et al.* (2012)
	α-Santalene/α-bergamotene/β-bergamotene synthase (Sst2) (ShSBS)	van der Hoeven *et al.* (2000); Sallaud *et al.* (2009)
	Valencene synthase (ShTPS17)	Bleeker *et al.* (2011)
Solanum lycopersicum (formerly Lycopersicon esculentum)	Neryl diphosphate synthase (NDPS1)	Schilmiller *et al.* (2009)
	Z,Z -farnesyl diphosphate synthase (zFPS)	Sallaud *et al.* (2009)

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Table 2 Continued

Species (Family)	Function (based on main product(s))	References
	α-Bergamotene synthase (SlTPS38)	Falara *et al.* (2011)
	α-and β-Bisabolene synthase (SlTPS14)	Falara *et al.* (2011)
	Camphene/tricyclene synthase (SlTPS3)	Falara *et al.* (2011)
	β-Caryophyllene/α-humulene synthase (SlTPS12)	van der Hoeven *et al.* (2000)
	1,8-Cineole synthase (SlTPS8)	Falara *et al.* (2011)
	Germacrene C synthase (SlTPS9)	Bleeker *et al.* (2011)
	(+)-Linalool/(E)-nerolidol synthase (LeMTS1, SlTPS5)	van Schie *et al.* (2007)
	β-Myrcene/limonene synthase (SlTPS7)	Falara *et al.* (2011)
	β-Phellandrene/β-myrcene/sabinene synthase (LeMTS2, SlTPS4)	van Schie *et al.* (2007)
	β-Phellandrene (SlPHS1, SlTPS20)	Falara *et al.* (2011)
	Valencene synthase (SlTPS17)	Bleeker *et al.* (2011)
	Viridiflorene synthase (SlTPS31)	Bleeker *et al.* (2011)
Solanum lycopersicum (cherry tomato)	Germacrene C synthase (pLE20.3)	Colby *et al.* (1998)
Solanum pennelii	α-Phellandrene synthase (SpPHS1)	Schilmiller *et al.* (2009)

differences in the essential oil. ESTs were also acquired from gland cells of lavender (*Lavandula angustifolia*), which led to the cloning and characterization of a β-phellandrene synthase (Demissie *et al.*, 2010; Lane *et al.*, 2010). Three other monoterpene synthases, (+)-limonene synthase, (−)-linalool synthase and *trans*-α-bergamotene synthase, had been cloned previously from lavender using a homology-based PCR strategy (Landmann *et al.*, 2007) (Table 2). Several monoterpene synthases were cloned by degenerate PCR from common sage (*Salvia officinalis*) [(+)-bornyl pyrophosphate synthase, 1,8-cineole synthase and (+)-sabinene synthase; Wise *et al.*, 1998;], Blue Mountain sage (*Salvia stenophylla*) [(+)-3-carene synthase; Hölscher *et al.*, 2003;], and Greek sage (*Salvia fruticosa*) (another 1,8-cineole synthase; Kampranis *et al.*, 2007) (Table 2). The specific roles of other terpene synthases contributing to the unique essential oil of Greek sage might now be within reach as EST resources from gland cells have become available (Chatzopoulou *et al.*, 2010). Patchouli (*Pogostemon cablin*) accumulates a complex essential oil consisting of over 20 different sesquiterpenes. A homology-based PCR approach allowed Deguerry *et al.* (2006) to identify candidate cDNAs which were demonstrated to code for five different sesquiterpene synthases (forming γ-curcumene, germacrene A, germacrene D or pathoulol as major products). Interestingly, the sum of the reactions products from the recombinantly expressed sesquiterpene synthases resembled the composition of sesquiterpenes in patchouli oil (Deguerry *et al.*, 2006). Two additional (+)-limonene synthases were cloned from other species belonging to the Lamiaceae, *Schizonepeta tenuifolia* (Maruyama *et al.*, 2001) and *Agastache rugosa* (Maruyama *et al.*, 2002) (Table 2).

Terpenoid biosynthesis in the Solanaceae

Glandular trichomes are fairly common on leaves of several species within the genus *Solanum*. ESTs and 454 next-generation sequencing data were acquired from different types of gland cells isolated from cultivated tomato (*Solanum lycopersicum*), currant tomato (*S. pimpinellifolium*) and two pest-resistant wild tomato species that grow at elevated altitudes (*S. habrochaites* and *S. pennellii*) (Bleeker *et al.*, 2011; Fei *et al.*, 2004; McDowell *et al.*, 2011; Sallaud *et al.*, 2009; Schilmiller *et al.*, 2009). Analogous sequencing data were reported for gland cells of tobacco (*N. tabacum*) (Cui *et al.*, 2011; Harada *et al.*, 2010). These cell type-specific studies in tomato and tobacco were expanded also to include proteomic data (Amme *et al.*, 2005; van Cutsem *et al.*, 2011; Schilmiller *et al.*, 2010) and metabolomic evidence (McDowell *et al.*, 2011).

As part of these efforts, the functions of several sesquiterpene synthase genes were characterized (Bleeker *et al.*, 2011; Sallaud *et al.*, 2009; Schilmiller *et al.*, 2009) (Table 2), but the most unexpected findings relate to genes involved in novel pathways for the biosynthesis of monoterpenes and sesquiterpenes in tomato. Sallaud *et al.* (2009) noticed high transcript expression levels for a putative sesquiterpene synthase and a *cis*-prenyltransferase. This was unusual because *cis*-prenyltransferases were thought to be involved almost exclusively in the biosynthesis of long-chain terpenoids such as dolichols (for glycoproteins) and polymers such as rubber, while it was a generally accepted that mono-, sesqui- di-, tri- and tetraterpenoids are derived from shorter-chain *E,E*-prenyl pyrophosphates (Bouvier *et al.*, 2005). *In vitro* assays with recombinant proteins encoded by the above-mentioned tomato *cis*-prenyltransferase and sesquiterpene synthase genes provided unequivocal evidence for functions as *Z,Z*-farnesyl pyrophosphate synthase (zFPP synthase) and santalene/bergamotene synthase (SBS) (Sallaud *et al.*, 2009) (Figure 6) (Table 2). The majority of characterized *E,E*-FPP synthases is localized to the cytoplasm, but there are also reports of plastidial, mitochondrial and peroxisomal isoforms (Cunillera *et al.*, 1997; Sanmiya *et al.*, 1999; Hemmerlin *et al.*, 2003; Thabet *et al.*, 2011). Interestingly, the 5′-end of both the tomato zFPP synthase and SBS genes had sequence properties indicative of a plastidial targeting sequence. Sallaud *et al.* (2009) demonstrated that, based on the results obtained with fusions with green fluorescent protein in transfected tobacco protoplasts, both zFPP synthase and SBS were indeed present exclusively in chloroplasts, thus indicating that the authors identified a new pathway for sesquiterpene biosynthesis, which appears to co-exist with cytosolic sesquiterpene formation. Schilmiller *et al.* (2009) found an analogous pair of genes, also based on an EST approach with gland cells isolated from tomato GTs, with a putative role in monoterpene biosynthesis. The recombinant enzymes were characterized as neryl pyrophosphate synthase (NPP synthase; a *cis*-prenyltransferase) and a monoterpene synthase that produces

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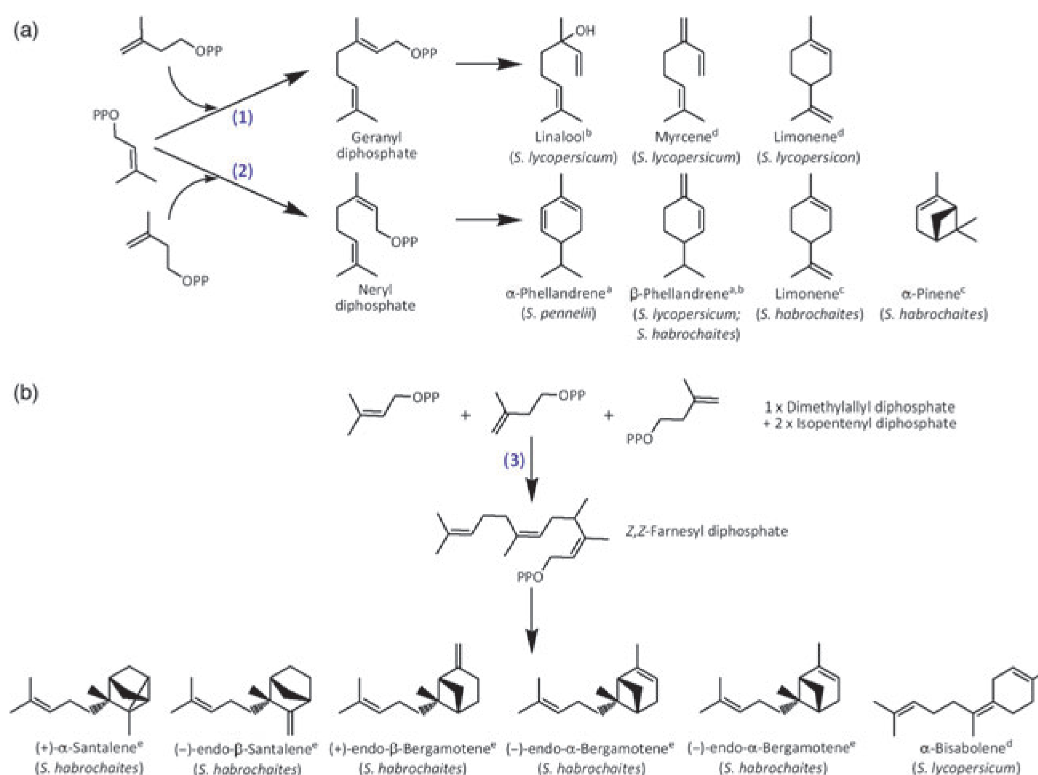

Figure 6 Role of *cis*- and *trans*-prenyltransferases in Solanaceae. (a) The enzymes involved in monoterpene biosynthesis are (1) geranyl pyrophosphate (GPP) synthase and (2) neryl pyrophosphate (NPP) synthase (Schilmiller *et al.*, 2009). (b) The enzyme involved in sesquiterpene biosynthesis is (3) z,z-farnesyl pyrophosphate (zFPP) synthase (Sallaud *et al.*, 2009). Selected end products formed from GPP, NPP or zFPP in *in vitro* assays are shown, while products derived from *E,E*-FPP are omitted for clarity. Relevant references: [a]Schilmiller *et al.*, 2009; [b]van Schie *et al.*, 2007; [c]Gonzales-Vigil *et al.*, 2012; [d]Falara *et al.*, 2011; and [e]Sallaud *et al.*, 2009.

β-phellandrene, and various other monoterpenes, from NPP (Figure 6). It had previously been shown that many monoterpene synthases are capable of using NPP as a substrate, but generally with a much lower activity compared to GPP (reviewed in Davis and Croteau, 2000). It will now be important to evaluate whether the efficient synthesis of monoterpenes from NPP and sesquiterpenes from zFPP is a metabolic peculiarity of tomato gland cells or if this is a more common process that deserves the attention of the broader plant biochemistry community.

Mite-infested *S. lycopersicum* was used as the source for cDNAs encoding a linalool/nerolidol synthase and a multi-product monoterpene synthase (van Schie *et al.*, 2007). A PCR-based approach led to the discovery of a germacrene C synthase gene in cherry tomato leaves (Colby *et al.*, 1998). A segregation analysis of a population generated by a cross between *S. lycopersicum* and *S. habrochaites* allowed van der Hoeven *et al.* (2000), to identify two families of sesquiterpene synthases with activities for the synthesis of β-caryophyllene/α-humulene (*S. lycopersicum*), germacrene B/germacrene D (*S. habrochaites*) and α-santalene/α-bergamotene/β-bergamotene (*S. habrochaites*). An antisense silencing strategy was employed to unravel the functions of α-cembratrienol/β-cembratrienol synthase and α-cembratrienol/β-cembratrienol monooxygenase, which are involved in the biosynthesis of cembradiols, the principal diterpene components of tobacco (*N. tabacum*) GTs (Wang and Wagner, 2003). The genes involved in the biosynthesis of *Z*-abienol, the major labdane diterpene of certain oriential tobacco cultivars, were recently cloned from *N. tabacum* (Sallaud *et al.*, 2012). The authors characterized the functions of the encoded enzymes as 8-hydroxy-copalyl diphosphate synthase (NtCPS2) and *Z*-abienol synthase (NtABS) (Table 2). Interestingly, it was demonstrated that the NtCPS2 gene, which is expressed preferentially in GTs, is present in over 100 tobacco cultivars, but contains polymorphisms that lead to truncated (presumably inactive) gene products in cultivars that lack *Z*-abienol.

Sesquiterpene lactone biosynthesis in the Asteraceae

Sesquiterpene lactones (STLs) are characteristic terpenoid natural products of the Asteraceae, and the distribution of different STLs has been used for the chemotaxonomic classification of this diverse plant family (Hristozov *et al.*, 2007). The unravelling of the pathway for the biosynthesis of artemisinin, an STL that has become a critical component in contemporary antimalarial formulations (Sinclair *et al.*, 2009), was aided tremendously by EST and next-generation sequencing data for transcripts expressed specifically in gland cells (Wang *et al.*, 2009; Weathers *et al.*, 2011) (Figure 7). The gene coding for the enzyme that catalyses the first committed step in artemisinin biosynthesis, amorpha-4,11-diene synthase, was cloned more than 10 years ago using degenerate primers for the amplification of terpene synthases (Mercke *et al.*, 2000; Chang *et al.*, 2000; Wallaart *et al.*, 2001), but all other known genes with relevance for this pathway owe their discovery to the use of gland cell-specific cDNA libraries (Ro *et al.*, 2006; Teoh *et al.*, 2006, 2009; Zhang *et al.*, 2008b) (Table 2). Two GT-specific genes involved in off-

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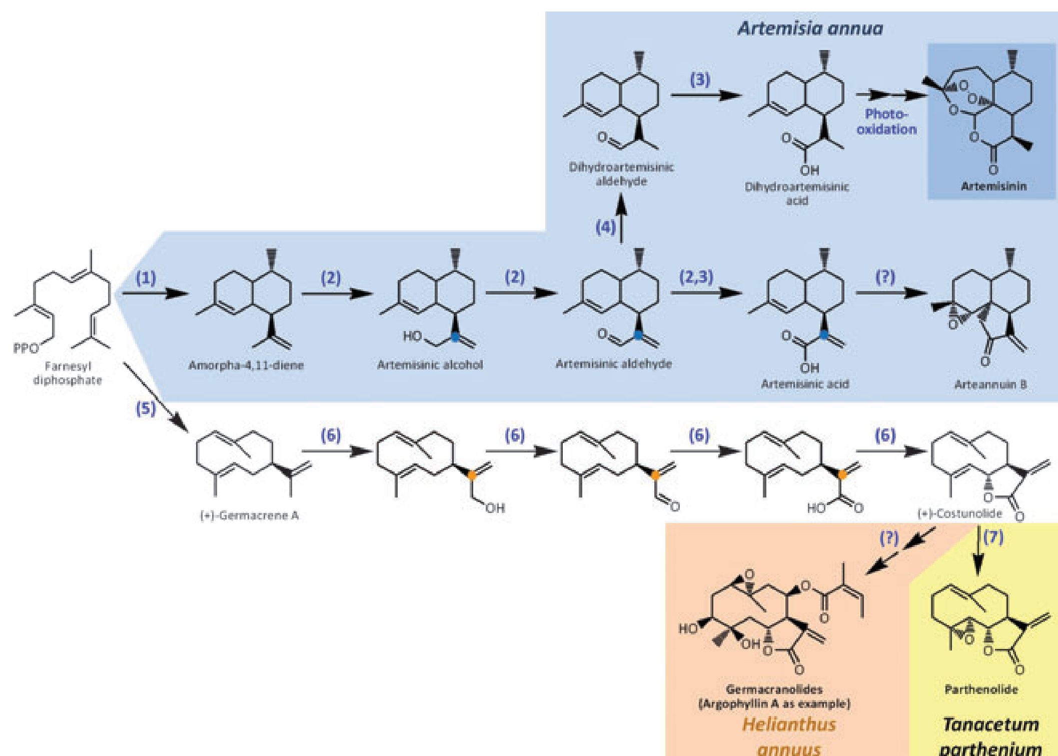

Figure 7 Overview of sesquiterpene lactone biosynthesis in Asteraceae. The enzymes involved in this pathway are (1), amorpha-4,11-diene synthase; (2), multifunctional amorpha-4,11-diene monooxygenase (CYP71AV1); (3), aldehyde dehydrogenase (ALDH1); (4), artemisinic aldehyde Δ11(13) double bond reductase (DBR2); (5), germacrene A synthase; (6), germacrene A oxidase (GAO); (7), costunolide synthase; and (8), costunolide epoxidase/parthenolide synthase.

pathway steps, encoding 2-alkenal reductase and dihydroartemisinic aldehyde reductase, were also discovered (Rydén *et al.*, 2010; Zhang *et al.*, 2008a). Other GT-localized terpene synthase genes cloned from *A. annua* using PCR-based approaches include (+)-linalool synthase (Jia *et al.*, 1999), β-pinene synthase (Lu *et al.*, 2002), *epi*-cedrol synthase (Hua and Matsuda, 1999; Mercke *et al.*, 1999), β-caryophyllene synthase (Cai *et al.*, 2002), germacrene A synthase (Bertea *et al.*, 2006) and β-farnesene synthase (Picaud *et al.*, 2005) (Table 2).

Members of the Asteraceae often contain multiple types of GTs, which frequently produce different natural products. STLs of sunflower (*Helianthus annuus*) and feverfew (*Tanacetum parthenium*) are mostly accumulated in capitate GTs that occur on anther appendages of disc florets, but are also found on leaves, while leaf uniseriate GTs usually contain sesquiterpene hydrocarbons (Göpfert *et al.*, 2005; Majdi *et al.*, 2011). Gland cell-specific cDNA libraries have been used to clone genes encoding germacrene A synthases (sunflower and feverfew), an (E)-β-caryophyllene synthase (feverfew) and a germacrene A acid 8β-hydroxylase (sunflower) (Göpfert *et al.*, 2009; Ikezawa *et al.*, 2011; Majdi *et al.*, 2011) (Figure 7). Other genes involved in sesquiterpene biosynthesis, which are not necessarily associated with GTs, were cloned from leaf RNA based on extensive EST data sets (germacrene A synthase from globe artichoke (*Cynara cardunculus*) (Menin *et al.*, 2012), germacrene A oxidase from lettuce (*Lactuca sativa*) (Nguyen *et al.*, 2010), and germacrene A acid 6α-hydroxylase from lettuce (Ikezawa *et al.*, 2011)). Degenerate PCR was employed to clone germacrene A synthases from chicory (*Cichorium intybus*) (Bouwmeester *et al.*, 2002), lettuce (Bennett *et al.*, 2002), goldenrod (*Solidago canadensis*) (Prosser *et al.*, 2002) and sunflower (Göpfert *et al.*, 2010), and germacrene A oxidases were cloned from selected genera across the Asteraceae (sunflower, chicory, snow lotus (*Saussurea lappa*), and *Barnadesia spinosa* (Nguyen *et al.*, 2010). Although it is currently unknown whether these genes play roles in STL biosynthesis in GTs, there is emerging evidence for differential roles of genes that code for different isoforms of the same enzyme. For example, in sunflower, two germacrene A synthase genes are expressed in organs that bear capitate GTs, while a third germacrene A synthase gene is expressed in organs that lack these specialized anatomical structures (Göpfert *et al.*, 2009, 2010). It will now be important to establish the implications of gene redundancy for STL formation in different plant organs and the specific roles of GTs in the defence against herbivores.

Diterpene biosynthesis in the Cistaceae

The genus *Cistus* consists of perennial shrubs that grow naturally throughout the Mediterranean region. The leaves are covered with trichomes, some of which are GTs that accumulate an aromatic resin enriched in labdane-type diterpenes. Falara *et al.* (2008) obtained ESTs from *Cistus creticus* gland cells and subsequently cloned and characterized a germacrene C synthase. Follow-up work with terpene synthase candidate genes from this effort resulted in the identification of a copal-8-ol pyrophosphate synthase with likely involvement in the biosynthesis of oxygen-heterocyclic labdanes (Falara *et al.*, 2010). These studies have laid the foundation for the identification of

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the remaining genes and enzymes in the pathways towards labdane diterpenes, which have various intriguing biological activities (Carson and Hammer, 2011).

Biosynthesis of terpenophenolics in the Cannabinaceae

Terpenophenolics are natural products of mixed biosynthetic origin, where the terpenoid pathway contributes at least one moiety. The bracts of female cones of hop (*Humulus lupulus*), contain an abundance of GTs, also called lupulin glands, which contain volatile terpenoids as well as non-volatile prenylated phloroglucinols (bitter acids) and prenylflavonoids (Stevens and Page, 2004). Several groups have used ESTs from lupulin glands to identify candidate genes involved in terpenoid and terpenophenolic biosynthesis, which ultimately resulted in the cloning and characterization of geranyl pyrophosphate synthase (Wang *et al.*, 2009), sesquiterpene synthases responsible for the synthesis of caryophyllene, humulene and germacrene A (Wang *et al.*, 2008), an O-methyltransferase involved in the biosynthesis of the prenylflavonoid xanthohumol (Nagel *et al.*, 2008), and a prenyltransferase of the bitter acid biosynthetic pathway (Tsurumaru *et al.*, 2010, 2012) (Figure 8) (Table 2). The gene encoding the enzyme responsible for catalysing the first committed step in the biosynthesis of terpenophenolics, valerophenone synthase, was also cloned using mRNA extracted from lupulin glands (Okada and Ito, 2001). It now appears possible to develop strategies to modulate, either by molecular breeding or by biotechnological means, the amounts of xanthohumol for beer brewing and prenylflavonoids for treating conditions that are responsive to these phytoestrogens.

Some subspecies of *Cannabis sativa* accumulate large amounts of cannabinoid terpenophenolics, most prominently the psychoactive Δ^9-tetrahydrocannabinolic acid (THCA), in GTs of flowers, leaves and stalks of female plants. The enzymes that determine the chemotypic differences between *Cannabis* subspecies, THCA synthase and cannabidiolic acid synthase, were originally purified in native form, peptide sequences were obtained, and the corresponding genes were cloned using degenerate PCR (Sirikantaramas *et al.*, 2004; Taura *et al.*, 2007) (Figure 8) (Table 2). A degenerate PCR approach, based on homology to known polyketide synthases, was also used to clone the gene for the enzyme that putatively catalyses the first committed step of the cannabinoid pathway, olivetol synthase (Taura *et al.*, 2009). The same candidate gene was also identified by an independent research team based on its high abundance in an EST data set obtained with GTs from *Cannabis* (Marks *et al.*, 2009). The authors reported that the expression of this gene in *Escherichia coli* yielded a recombinant protein capable of catalysing conversions of malonyl-CoA and hexanoyl-CoA, but the reaction products were not identified. It was, however, confirmed that neither olivetol nor olivetolic acid was formed (Marks *et al.*, 2009). These discrepancies with regard to the function of candidate genes for THCA biosynthesis have as yet not been resolved satisfactorily. The gene encoding an aromatic prenyltransferase, which is responsible for the condensation of olivetolic acid with geranyl pyrophosphate, was recently identified (Page and Boubakir, 2011). ESTs from *Cannabis* gland cells were also used to clone and characterize genes encoding (−)-limonene and (+)-α-pinene synthases (Günnewich *et al.*, 2007). Taken together, the available genomic resources for *Cannabis* and the fairly well-established biochemistry of the THCA biosynthetic pathway enable breeding approaches aimed at generating marijuana and hemp strains with desirable chemical profiles.

In this paragraph, we attempted to highlight the importance of EST projects for the identification of candidate genes involved in the biosynthesis of various terpenoids in GTs. Another review on this general topic was published recently (Tissier, 2012a), but that article is more focused on the roles of diverse gene products in the context of GT-localized pathways. We are aware of the fact that many more genes that are expressed preferentially or exclusively in gland cells have been characterized, but this review

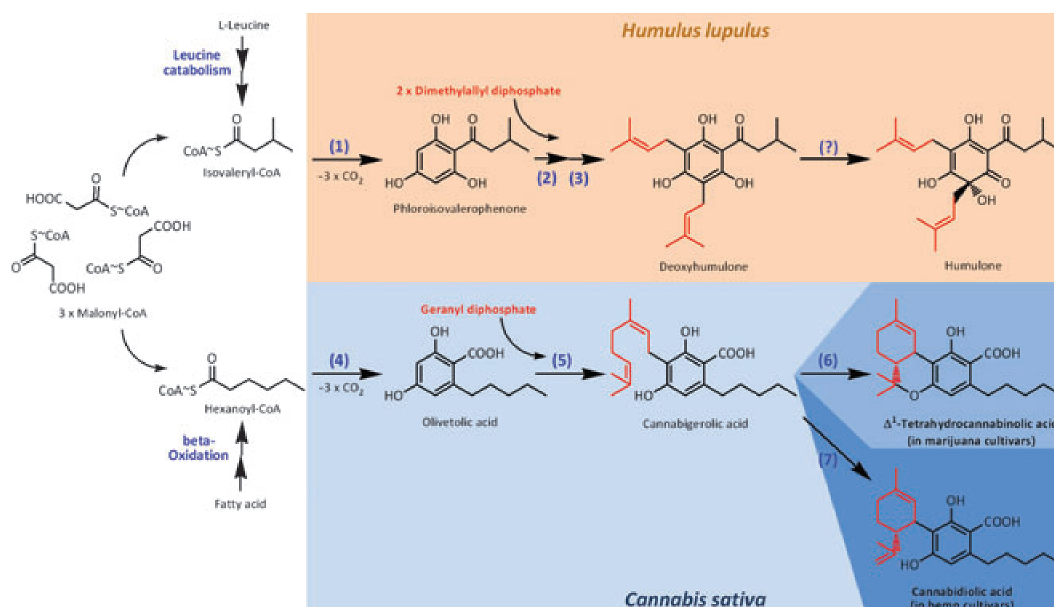

Figure 8 Overview of the biosynthesis of terpenophenolic metabolites in Cannabinaceae. The enzymes involved in this pathway are (1), valerophenone synthase; (2), aromatic prenyltransferase 1 (HlPT-1); (3), aromatic prenyltransferase 2; (4), olievetolic acid synthase (or possibly olievetol synthase in combination with an oxidase); (5), olievetolate geranyltransferase; (6), tetrahydrocannabinolic acid synthase; and (7), cannabidiolic acid synthase.

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is centred on terpenoids and terpenoid-derived metabolites that accumulate in GTs, and we thus do not provide examples pertaining to other GT-localized pathways. Because of space limitations, we were also not able to cover the biosynthesis of terpenoids that are not produced in GTs (e.g. volatiles in flowers or sesquiterpene lactones in roots). We apologize to all authors whose work we have not been able to cite because of these constraints. For a broader listing of plant monoterpene and sesquiterpene synthases the reader is referred to an excellent review by Degenhardt *et al.* (2009). Transcriptome and proteome data sets can be accessed through various dedicated plant trichome databases (http://www.planttrichome.org/trichomedb/index.jsp; http://www.trichome.msu.edu).

Screening terpenoid diversity in genetic variants is becoming a powerful approach in secretory glandular trichome functional genomics

The genomes of several GT-bearing, terpenoid-accumulating plant species have now been sequenced (or are in advanced stages of sequence assembly), including those belonging to the Solanaceae (Potato Genome Sequencing Consortium, 2011; Tomato Genome Consortium, 2012; http://solgenomics.net/). This has enabled genome-wide surveys of genes putatively involved in terpenoid biosynthesis. Falara *et al.* (2011) investigated the 44-member terpene synthase (TPS) family in cultivated tomato (*S. lycopersicum*). On the basis of the *in silico* analyses, 29 of these TPSs were potentially functional and transcript was detected for 26 putative TPS genes in at least one tissue. Previous studies had established the *in vitro* activities of eight of the encoded TPSs, and Falara *et al.* (2011) reported activities for another 10 TPSs. It appears that, in tomato, there is limited structural diversity in the diterpene pathway (derived from a C20 precursor), as the two recognizable diterpene synthase genes were demonstrated to be involved in the biosynthesis of gibberellin hormones (Falara *et al.*, 2011; Rebers *et al.*, 1999). In contrast, a wide variety of monoterpenes and sesquiterpenes (derived from C10 and C15 precursors, respectively) were accumulated in or emitted by tissues rich in GTs, including as major components tricyclene, α-and β-pinene, camphene, sabinene, β-myrcene, limonene, β-caryophyllene, and α-humulene (Falara *et al.*, 2011) (Table 2). The detected monoterpene and sesquiterpene mixtures could be accounted for based on the properties of recombinantly expressed TPSs and their *in vivo* spatial distribution profiles. Recent advances in functional genomics, which take advantage of natural variation, have further facilitated the identification of genes underlying terpenoid diversity. In this context, the use of introgression mapping populations has shown impressive potential. One population was generated by replacing a part of the genome of a cultivated tomato variety, *S. lycopersicum* cv. M82, with a single analogous segment from a wild species, *S. pennellii* LA0716 (Eshed and Zamir, 1994, 1995). This was done for 76 large segments which, as a whole, cover essentially the entire genome. Assessing the phenotypic variation among all of these nearly isogenic lines (NILs) can then be used to map quantitative trait loci (QTLs). Schilmiller *et al.* (2010) used a mass spectrometry-based high-throughput screening method to profile monoterpenes and sesquiterpenes of leaf GTs in the M82 parent cultivar and 65 NILs, which allowed the authors to identify regions with relevance for controlling the amounts and composition of terpenoids. A second NIL population was generated by introgressing segments from the wild accession *S. habrochaites* f. *typicum* LA1777 into the *S. lycopersicum* cv. M82 genome (Monforte and Tanksley, 2000). This population was used as a resource for studying the divergence of terpenoid profiles and differences in the utilization of precursors via the plastidial MEP and the cytosolic mevalonate (MVA) pathways (Besser *et al.*, 2009). An approach based on screening natural variation was also used to clone further terpene synthase genes involved in the formation of α-pinene and limonene from NPP and 7-epizingiberene from zFPP in *S. habrochaites* (Gonzales-Vigil *et al.*, 2012).

Very recently, the draft genome sequences of two *Cannabis* cultivars and vast transcriptome data from various tissues were reported (van Bakel *et al.*, 2011). The authors cite a patent application announcing the cloning and characterization of an olivetolate geranyltransferase, a missing link in the cannabinoid biosynthetic pathway (Page and Boubakir, 2011) (Table 2). These data sets allow comparisons between a high-potency marijuana cultivar (*C. sativa* cv. Purple Kush), which accumulates THCA, and hemp varieties ('Finola' and 'USO-31'), which are used for fibre and seed production, thus enabling future molecular breeding and metabolic engineering approaches for optimizing desirable traits.

An F1 hybrid population of *A. annua* was subjected to next-generation sequencing of ESTs from various tissues by Graham *et al.* (2010). This allowed the authors to identify, based on *in silico* comparisons, single-nucleotide polymorphisms, short sequence repeats and insertions/deletions with potential utility as molecular markers in breeding programs. Comparisons of variation in four traits (artemisinin content, leaf area, GT density and fresh weight) were used to select lines with the highest overall artemisinin yield, while maintaining agronomic performance (Graham *et al.*, 2010). The *Artemisia* population was also used for the generation of genetic linkage maps and the subsequent identification of QTLs for traits related to artemisinin production. Interestingly, one of the yield QTLs was the gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase, which is involved primarily in the biosynthesis of precursors for plastidial terpenoids, but in *Artemisia* both the MVA and MEP pathways appear to contribute to the synthesis of artemisinin in the cytosol (Towler and Weathers, 2007). The genomic resources now available for *A. annua* should enable a more expedient selection of high artemisinin producers in molecular breeding programs.

Understanding the regulation of terpenoid metabolism in secretory GTs

While the cloning and functional characterization of components of terpenoid biosynthesis has been highly successful, the regulation of most gland cell-localized pathways is only understood at a very rudimentary level. The expression levels of genes involved in the terpenoid pathway have been determined based on transcript counts in diverse GT-bearing plants (Besser *et al.*, 2009; Bleeker *et al.*, 2011; Chatzopoulou *et al.*, 2010; Cui *et al.*, 2011; Falara *et al.*, 2008, 2011; Gang *et al.*, 2001; Harada *et al.*, 2010; Lane *et al.*, 2010; Lange *et al.*, 2000; Marks *et al.*, 2009; McDowell *et al.*, 2011; Wang *et al.*, 2009). However, only a few studies have attempted to assess the correlation of transcript and/or protein abundance patterns with the accumulation of the terpenoid end products in GTs. The genes encoding two gland cell-specific monoterpene synthases correlated well with the

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amounts of the monoterpene γ-terpinene in various oregano cultivars (Crocoll *et al.*, 2010). Majdi *et al.* (2011) found two sesquiterpene synthase genes to be expressed in various tissues of feverfew, but with the by far highest transcript levels in leaf GTs, the distribution of which also correlated with the accumulation levels of the sesquiterpene lactone parthenolide. Olsson *et al.* (2009) localized the expression of three genes involved in the biosynthesis of artemisinin to the apical cells of *Artemisia* GTs, which is the hypothesized site of synthesis of this sesquiterpene lactone. The expression of farnesyl pyrophosphate (FPP) synthase and two sesquiterpene synthases was followed over eight developmental stages of GTs on sunflower florets. While FPP synthase transcript was detectable at all developmental stages, the expression of the sesquiterpene synthases correlated with the secretory stage when sesquiterpenes are formed (Göpfert *et al.*, 2009). van Bakel *et al.* (2011) compared the expression patterns of all known genes involved in the biosynthesis of terpenophenolics in a marijuana and a hemp strain of *Cannabis*. The most striking difference was the enrichment of transcript for tetrahydrocannabinolic acid synthase in marijuana (synthesizes a psychoactive cannabinoid) and cannabidiolic acid synthase in hemp (synthesizes a non-psychoactive cannabinoid). An interesting outcome of this study was that several genes involved in precursor biosynthesis via the MEP pathway were differentially expressed between the cultivars, with generally higher transcript levels of genes encoding early enzymes but lower expression levels of the last two genes in the hemp strain (van Bakel *et al.*, 2011). Xie *et al.* (2008) reported a generally tight correlation between transcript counts (from an EST project), protein levels (based on semi-quantitative proteomics data) and terpenoid metabolites in GTs of basil lines with chemically diverse essential oil composition. For example, genes and enzymes of the MEP and downstream terpenoid pathways were highly expressed in the SD line, which accumulates high amounts of the monoterpenes, neral and geranial, while only very low levels of expression of these genes and enzymes were detected in the EMX-1 line, which accumulates primarily the phenylpropene methylchavicol. The authors also presented preliminary evidence that post-translational modifications (in particular phosphorylation) could play a role in the fine regulation of the MEP pathway (Xie *et al.*, 2008).

While the above-mentioned studies have laid the foundation for a better understanding of terpenoid biosynthesis in GTs, the only well-characterized model system is the monoterpene pathway in peppermint. The Croteau laboratory succeeded in cloning the genes involved in the p-menthane monoterpene pathway and characterized the kinetic properties of all encoded enzymes (reviewed in Croteau *et al.*, 2005). Gene expression patterns, enzyme activities and the dynamics of monoterpenoid essential oil accumulation in GTs were followed over an entire growth period (from leaf emergence to harvest) (Gershenzon *et al.*, 2000; Lange *et al.*, 1998; McConkey *et al.*, 2000). The developmental patterns of GT formation and oil secretion were also studied, and the cellular and subcellular localization of the relevant enzymes was established (Turner and Croteau, 2004; Turner *et al.*, 1999, 2000a, 2012). These studies demonstrated a very close correlation between transcript abundance and enzyme activity for all gene/enzyme pairs, indicating that, under favourable growth conditions in greenhouses, essential oil biosynthesis in peppermint gland cells is primarily controlled at the transcriptional level. This wealth of physiological and biochemical information allowed our laboratory to develop kinetic mathematical models of monoterpene biosynthesis in peppermint GTs (Rios-Estepa *et al.*, 2008, 2010), which can be used to accurately simulate the time course of accumulation of all major essential oil components. However, we observed discrepancies between measured and simulated monoterpenoid essential oil profiles under certain stress conditions, in particular the accumulation of the pathway intermediate (+)-pulegone and a dead-end side product, (+)-menthofuran. As the high levels of these metabolites in the oil are undesirable from a commercial point of view, we evaluated the biochemical basis of this phenomenon. The mathematical expressions reflecting Michaelis–Menten kinetics of pathway enzymes were modified to simulate the predicted experimental outcome if one assumed an inhibitory effect of an intermediate or end product. This was done for all realistic mechanisms of inhibition and all metabolites, by modifying the Michaelis–Menten expression for one enzyme at a time (Rios-Estepa *et al.*, 2008). The best agreement between experimental and simulated essential oil profiles was observed with an expression reflecting a situation where (+)-menthofuran acts as a competitive inhibitor of (+)-pulegone reductase. Our modelling thus generated a testable hypothesis. Follow-up experiments demonstrated that this inhibitory mechanism was indeed occurring (Rios-Estepa *et al.*, 2008), indicating the utility of mathematical modelling for hypothesis generation and guiding experimental design. We also used modelling to further evaluate how essential oil composition and yield in peppermint GTs is affected by various environmental stresses and in transgenic plants with modulated expression levels of genes involved in the monoterpene pathway. One of the most important conclusions from this effort was that the density and developmental distribution of GTs, among various potential factors, had the highest impact on oil yield, while specific pathway genes exerted a fairly high level of control over monoterpenoid oil composition (Rios-Estepa *et al.*, 2010).

Because of the exceptional depth of knowledge about the regulation of essential oil biosynthesis in peppermint gland cells, the monoterpene pathway has been the target of extensive and highly successful metabolic engineering efforts aimed at enhancing oil yield and composition, which cannot be covered in any detail in the present review article. Current achievements include the adjustment of the accumulation of undesirable side products (e.g. (+)-pulegone and (+)-menthofuran) (Figure 5) and significant yield increases (up to 80% more oil in transgenic plants compared to the Black Mitcham cultivar wild-type control) in multi-year field trials (Lange *et al.*, 2011; Mahmoud and Croteau, 2001; Mahmoud *et al.*, 2004). The greatest challenge remains the control of *Verticillium* wilt disease, which causes dramatic yield reductions in mints, particularly in peppermint (Dung *et al.*, 2010).

Future directions

As outlined in the preceding chapters, impressive progress has been made with regard to characterizing the genes and enzymes involved directly in terpenoid biosynthesis in GTs. A recent review summarizes the current status regarding the identification of promoters that are responsible for the GT-specific expression of transcripts (Tissier, 2012b). Such promoters are potentially of great utility for metabolic engineering approaches aimed at modulating transcript abundance of specific terpenoid biosynthetic or regulatory genes in transgenic plants. Systematic studies are now needed to learn more about the regulatory elements within promoters that enable an expression of the associated genes specifically in GTs. There have also been reports of

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transcription factors that regulate the expression of genes involved in terpenoid biosynthesis in GTs, particularly in *Artemisia* (Liu *et al.*, 2011). Further research is essential to establish the potential of using these regulatory proteins for metabolic engineering efforts. While our understanding of non-GT patterning in model plants has improved substantially over the last 20 years, we are currently lacking even a basic appreciation of the regulation of the patterning of GTs. Lastly, another area of need for research relates to the mechanisms controlling the transport of terpenoids within GTs. Various genetic and genomic community resources for GT research have become available over the last 10 years, and these developments have paved the path for further elucidating the functions of these fascinating anatomical structures. The primary goals of metabolic engineering of terpenoid metabolism in GTs pertain to yield increases and enhancements of the natural composition. A more recent development is the use of GTs as 'biochemical factories' for the production of commercially desirable terpenoids (Schilmiller *et al.*, 2008). Targets include the antimalarial sesquiterpene artemisinin, the flavour sesquiterpene nootkatone and, long term, chemically more complex metabolites such as the anticancer diterpene paclitaxel (taxol®). Preliminary data suggest that novel terpenoids can be accumulated in mint GTs (Lange *et al.*, 2010), but it remains to be established if commercially relevant yields of the target metabolites are achievable. GT-specific promoters and terpenoid pathway regulators as metabolic engineering tools have the potential to enable a step change in the sustainable production of high-value terpenoids.

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