Mini Review

Title:

"Hidden" Terpenoids in Plants: Their Biosynthesis, Localisation and Ecological Roles

Running title: Biosynthesis and activities of "hidden" terpenoids

* Corresponding author: Dr. K. Yazaki, Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho, Uji 611-0011, Japan. Telephone, +81-774-38-3617; fax, +81-774-38-3623; E-mail, yazaki@rsh.kyoto-u.ac.jp

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"Hidden" Terpenoids in Plants: Their Biosynthesis, Localisation and Ecological Roles

Running title: Biosynthesis and activities of "hidden" terpenoids

Authors:

Kazufumi Yazaki[1], Gen-ichiro Arimura[2], Toshiyuki Ohnishi[3]

Affiliations:

[1] Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho, Uji 611-0011, Japan

[2] Department of Biological Science & Technology, Faculty of Industrial Science & Technology, Tokyo University of Science, Tokyo 125-8585, Japan

[3] College of Agriculture, Academic Institute, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8017, Japan

Abbreviations: FC, furanocoumarin; MEP, methylerythritol phosphate; PT, prenyltransferase; PVOC, plant-derived volatile organic compound; TPS, terpene synthase

Abstract

Terpenoids are the largest group of plant specialized (secondary) metabolites. These naturally occurring chemical compounds are highly diverse in chemical structure. Although there have been many excellent studies of terpenoids, most have focused on compounds built solely of isoprene units. Plants, however, also contain many “atypical” terpenoids, such as glycosylated volatile terpenes and composite-type terpenoids, the latter of which are synthesized by the coupling of isoprene units on aromatic compounds. This mini review describes these “hidden” terpenoids, providing an overview of their biosynthesis, localisation, and biological and ecological activities.

Keywords: glycosylation, mutualism, prenylated coumarin, prenylated flavonoid, shikonin, terpenoid

Introduction

Terpenoids are the largest group of plant specialized (secondary) metabolites, sub-grouped according to the number of five-carbon units in their skeletons; i.e., hemi- (C_5), mono- (C_{10}) sesqui- (C_{15}), di- (C_{20}), sester- (C_{25}), tri- (C_{30}), and tetra- (C_{40}: carotenoids) terpenoids. Their basic backbone structures are synthesized by the enzyme terpene synthase (TPS), following which they are further modified by, for example, hydroxylation, dehydrogenation, acylation or glycosylation (Dudareva et al., 2004, Pichersky et al., 2006), resulting in an array of chemically diverse terpenoid compounds. These include commercially useful products, including terpenoid-based biofuels and high quality natural rubber (Yamashita et al. 2016).

In addition to these typical terpenoids, some contain isoprenoid chains hidden within the carbon skeletons of other metabolic groups. For example, the furan ring of furanocoumarin derivatives is derived from dimethylallyl diphosphate, although the typical C_5 unit does not remain in the structure (Bourgaud et al. 2006). In addition, part of the naphthalene ring of shikonin derivatives is derived from geranyl diphosphate (Yazaki 2001), and some indole alkaloids also contain isoprenoid residues. Isoprene units are also used as prenyl residues for modification of metabolic groups such as polyphenols. These prenylation reactions increase the chemical diversity of phenolic compounds, with these prenylated compounds having various biological activities (Yazaki et al. 2009). These compounds, however, are not categorised as typical terpenoids due to their equivocal chemical structures, despite their being end products of isoprenoid metabolism.

Plant-derived volatile organic compounds (PVOC) in the terpenoid family are predominantly mono- and sesquiterpenoids. Their biosynthesis and accumulation patterns have been well characterised. In some plant species, these compounds may be present in non-volatile form; these include, for example, the monoterepene glycosides, which are hydrolysed to volatile forms upon tissue damage, such as insect attack (Mizutani et al. 2002). This review focuses on these “hidden” terpenoids, and includes an overview on key enzymes involved in their biosynthesis and on their tissue-specific accumulation patterns. This review also summarises their ecological roles in nature and discusses the physiological importance of their “hidden” properties.

Glycosylation contributes to the “hidden” scent of volatile terpenoids

Airborne volatile terpenoids constitute the scent of flowers and fruits of seed plants (Schwab et al., 2008). Their physiological roles include chemical defences against abiotic and biotic stresses (Schilling et al., 2015; Berenbaum et al., 1981), and biologically informative signals with other organisms. The volatility of terpenoids is influenced by their chemical properties, including their hydrophobicity, molecular weight, intermolecular hydrogen bonds, and vapour pressure; and their emission rates depend on abiotic and biotic factors, including temperature, seasonality, irradiance, and interactions with plants and other organisms. For example, the emission rate of *β*-pinene by *Quercus ilex*, an evergreen oak native to the Mediterranean area, is 40 times higher in summer than in winter (Staudt and Bertin, 1998). Further discussion for the biotic factors enhancing the emission is given later in the section of "Ecology of terpenoids".

Land plant species evolved mechanisms to sequester highly volatile compounds, including terpenoids, within plant tissues. For example, limonene, a dominant monoterpene in *citrus* spices, accumulates in oil glands located in oil-filled pits on the peel (exocarp) (Fig. 1). Volatile monoterpenes and sesquiterpenes, such as *γ*-terpinene, limonene, *β*-caryophyllene, *α*-humulene and *γ*-elemene, were identified in extracts of type VI glandular trichomes on tomato leaves (Schilmiller et al., 2009). Trichomes are typically classified into eight different groups (I-VIII), among which glandular capitate trichomes are type I, IV, VI and VII. Type VI glandular trichomes represent the most abundant trichome type on leaves and stems of tomato plants and accumulates an array of secondary metabolites (Glas et al., 2012). Specialised types of storage tissues, such as oil glands and glandular trichomes, may have evolved as specific compartments to sequester large quantities of hydrophobic and/or highly reactive compounds, which may show self-toxicity (Fig. 1).

Glycosylation and oxidation contribute to the stable storage of terpenoids in plant cells by reducing their volatility. These reactions may result in increases in hydrophilicity and molecular weight, as well as the formation of additional intermolecular hydrogen bond networks. For example, linalool, a dominant monoterpene alcohol in Arabidopsis, is sequentially metabolised by cytochromes P450 to 8-hydroxy, 8-oxo- and 8-carboxylinalool, with oxidised forms of linalool transformed to glycosylated derivatives with reduced volatility (Boachon et al., 2015). Moreover, the ectopic expression of a strawberry terpene synthase (FaNES1) in Arabidopsis plants produced a set of nerolidol, (*S*)-linalool and glycosylated linalool derivatives (Aharoni

et al., 2003). These findings indicated that the production of volatile terpenoids would result in increased glycosylation activity, allowing the accumulation of these terpenoids in non-volatile forms. Furthermore, the glycosylation of terpenoids has been conserved in many plant species (Fig. 1).

Volatile monoterpenes, such as citronellol, nerol, linalool, and geraniol, accumulate as their water-soluble glycosylated forms in many land plant species, including tea, grape, kiwifruit, rose, and sweet potato plants. The chemical structures of terpene glycosides are primarily *β*-diglycosides, including *β*-primeveroside (6-*O*-*β*-D-xylopyranosyl-*β*-D-glucopyranoside) in young tea leaves (Guo et al., 1993), and *β*-accuminoside (6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranosides) (Gunata et al., 1985). PVOCs can be released from these diglycosides by acid hydrolysis or by enzymatic treatment with glycosidases. For example, in tea plants, *β*-primeveroside is hydrolysed by an endogenous diglycoside-specific *β*-glycosidase, resulting in the release of volatile terpenes (Ma et al. 2001, Mizutani et al. 2002). In grape, water-soluble terpene glycosides can be converted into volatile compounds through acid and/or enzymatic hydrolysis during the wine-making and aging processes (Maicas and Mateo, 2005).

Terpene glycosides are biosynthesised by members of the UDP glycosyltransferase (UGT) superfamily, which transfer sugar moieties to volatile terpenoids. UGTs belonging to the UGT85 family were recently cloned from tea, grape, kiwifruit, rose, and sweet potato plants, and shown to catalyse the glucosylation of not only monoterpenes but of other volatile compounds, including (*Z*)-3-hexenol, 2-phenylethanol, benzyl alcohol and 2,5-dimethyl-4-hydroxy-3(*2H*)-furanone (furaneol), producing their corresponding volatile monoglucosides (Bönisch et al., 2014; Yauk et al., 2014, Ohgami et.al. 2015, Sasaki et al., 2015). These monoglucosides can be further enzymatically modified by malonylation or a second glycosylation to malonyl monoglucosides or diglycosides, respectively (Ginglinger et al., 2013, Ohgami et al., 2015). CsGT2 (UGT94P1) specifically catalyses a second glycosylation of the 6'-hydroxy group of terpene monoglucosides, producing geranyl *β*-primeveroside. These sequential glycosylation and malonylation reactions result in the solubility in water of various airborne terpene compounds. cLogP is a measure of water solubility, with a lower cLogP value indicating higher water solubility. The conversion of geraniol (cLogP 2.97) to geranyl *β*-primeveroside (cLogP 0.46) or geranyl malonyl glucoside

(cLogP -1.07) results in higher water solubility, indicating that glycosylation and malonylation are key to the stable storage of volatile compounds in soluble compartments like vacuoles in seed plants.

Composite-type terpenoids

Isoprenoid residues are often hidden in quinone compounds, such as the polyprenylated benzoquinone ubiquinone and the naphthoquinone derivative shikonin (Fig. 1). Owing to their various biological activities and their unique lipophilic features as red pigments used to stain cloths, shikonin derivatives have been intensively investigated as characteristic secondary metabolites in Boraginaceae (Tabata 1988). The industrial production with cell cultures of *Lithospermum erythrorhizon* by Mitsui Chemicals resulted in shikonin derivatives constituting more than 10% of the dry weight of these cells (Fujita et al. 1987). Naphthalene rings can be biosynthesised by several independent pathways, for example via *o*-succinylbenzoic acid or geranylbenzoic acid as the key intermediate. Shikonin is biosynthesised via the latter route, but its production is strongly inhibited by light irradiation, via suppression of expression of the gene encoding *p*-hydroxybenzoate geranyltransferase (PGT) (Yazaki et al. 2002, Ohara et al. 2013). Thus, this membrane-bound enzyme likely plays a key role in the reversible regulation of shikonin production (Yazaki et al. 1999). Moreover, shikonin derivatives, being lipophilic metabolites, are secreted by these cells and highly accumulate on cell surfaces. The mechanism involved in the secretion of these lipophilic metabolites has been recently investigated (Tatsumi, 2016). Further comprehensive information on shikonin and the culture of *L. erythrorhizon* cells and the hairy roots of this plant is provided elsewhere (Yazaki, 1999).

Among the composite-type terpenoids, the prenylated phenolics with about 1,000 compounds have been identified to date in plants (Yazaki et al. 2009). The largest group consists of the prenylated flavonoids (ca. 700), which represent about 10% of all flavonoids (7,000) (Fossen et al. 2006). These compounds, which constitute the active components of various medicinal plants, have been actively investigated as pharmaceuticals because they show various biological activities in humans (Yazaki et al. 2009). The biological activities of phenolic compounds are increased by the attachment of prenyl chains (isoprene units), reactions catalysed by prenyltransferases (PTs), with the first crude enzymes characterised in the 1970s (Schröder et al. 1979). The first PT

gene for flavonoids was isolated from cultured *Sophora flavescense* cells, as has the gene encoding the enzyme that catalyzes the prenylation of naringenin at the 8-position (Sasaki, 2008). In intact *S. flavescense*, the prenylated flavonoids with antimicrobial properties accumulate solely in the root bark. Subsequently, many flavonoid-specific PTs, including those involved in glyceoline synthesis in soybeans, were isolated from other plant species (Dai et al. 2014, Li et al. 2014, Yoneyama et al 2016, Shen et al. 2012). Their common features include 1) the presence of a D-rich motif involved in Mg-coupled prenyl diphosphate recognition, 2) their localisation to plastids by the N-terminal transit peptide, and 3) their containing nine transmembrane α-helices (Sasaki et al. 2011).

Coumarin derivatives are a group of over 1500 lactonised phenylpropanoids. Furanocoumarins (FCs), a subgroup with a furan ring attached to a coumarin core, have not been commonly recognised as terpenoid derivatives, because isoprenoid units are not observed in the basic structure of FCs. However, their furan rings are derived from prenyl chains, followed by the cleavage of a C_3 unit to yield the atypical terpenoid derivatives (Bourgaud et al. 2006). Because FCs exhibit characteristic phototoxic activity, their biosynthesis has been extensively investigated (Kitamura et al. 2005). FCs are grouped into two types, linear (e.g., psoralen) and angular (e.g., angelicin) types according to the organisation of their rings. The regiospecificity of prenylation on the coumarin precursor determines their type, i.e., prenylation at the 8- and 6-positions of umbelliferone results in the formation of linear and angular FCs, respectively. The PT gene for coumarin substrate, however, remained unknown until recent years. The first PT gene encoding a membrane-bound protein that catalyses the 8-prenylation reaction of coumarin was identified in parsley (Karamat et al. 2014). Subsequently, another PT with strong preference for 6-prenylation was identified in parsnip (Munakata et al. 2016), and a geranyl diphosphate (GPP)-specific PT for umbelliferone was characterized in lemon (Munakata et al. 2014). All these PT proteins have the three features of flavonoid-specific PTs from legume plants, i.e., a D-rich motif, a transit peptide and multiple membrane-spanning domains. FCs have also been investigated in the context of an arms race in chemical ecology (Berenbaum et al. 2002).

Prenyl residues are attached via a C-C or C-O bond to plant specialised metabolites. *O*-Prenylated compounds are often seen in Laminaceae and Rutaceae. For example, citrus species contain many *O*-prenylated coumarins, such as bergamottin and

8-geranyloxypsoralen, which show high accumulation in pericarp. However, all PT genes identified thus far encode *C*-PTs that form C-C bonds with prenyl residues and aromatic components. Although the enzymatic activities of *O*-prenylases have the same three features common to *C*-PTs (Munakata et al. 2012), no *O*-PT gene has been isolated to date.

Coumarin compounds show high accumulation in the oil glands of citrus peels, i.e., spherical apoplastic spaces in which various oily compounds, including monoterpenes, are also sequestered (Lange 2015). Compounds of both families are biosynthesised in plastids, which contain isoprenoid precursors provided by the MEP pathway. Both families of compounds are subsequently secreted from these source cells and accumulate in the apoplastic space to form oil glands. This pathway appears to be common to lipophilic secondary metabolites such as prenylated phenolic compounds. In contrast, ubiquinones are primary metabolites biosynthesised at the inner membrane of mitochondria (Ohara et al. 2004). The mechanisms by which these hydrophobic terpenoids are secreted from cells after biosynthesis in plastid remain unclear, as there are three membranes, the inner and outer membranes of plastids and the plasma membrane. A recent report suggested mechanisms for the secretion of those lipophilic metabolites (Tatsumi 2016).

Ecology of terpenoids

PVOCs, the majority of which are volatile terpenoids, are released from various plant organs and tissues, including flowers and green tissues. PVOCs are involved in the interaction of immobile plants with mutualists, such as pollinators, with enemies, and with neighbouring plants via inaudible talk. The emission of PVOCs is specific to species, cultivars, genotypes, and organs, as well as environments. Floral scents are typical, due to their diurnal rhythms and dependence on developmental stage and floral part, resulting in specific interaction(s) with their mutualists. For example, three *Lithophragma* species emit species-specific floral scent profiles during the day, probably to attract their major pollinators, which are active during the day (Friberg et al. 2014). In contrast, *Mirabilis jalapa* emits its major floral scent, the monoterpenoid (*E*)-*β*-ocimene, mostly during the evening (Effmert et al. 2005). The peak release time of this monoterpenoid coincides with flower opening and the activity of its crepuscular pollinators, hawk moths (Martinez del Rio and Búrquez 1986). The emission patterns of

floral volatiles have evolved to adapt to the behaviour of insect pollinators, resulting in sophisticated mutualism.

Unlike the constitutive emission of floral terpenoids, plants occasionally emit PVOCs in response to biotic and environmental stresses. The emission of most stress-induced PVOCs is thought to be mediated by the *de novo* expression of the genes encoding the responsible proteins, such as TPS and cytochromes P450, as well as by a burst of volatiles from storage organs, including glandular trichomes, oil ducts, and secretory cavities, where PVOCs are hidden, based on damage to each. One class of volatiles is induced in response to attack by herbivores. These PVOCs benefit their host plants by interacting with herbivores, such as ovipositing butterflies and host-seeking aphids, directly repelling these herbivores (Unsicker et al. 2009), and by interacting with the natural enemies of these herbivores, recruit bodyguards, such as predators and parasitic wasps (Sabelis et al. 2007). Intriguingly, PVOCs recruit bodyguards both above and below ground. For example, maize roots release (*E*)-*β*-caryophyllene into the soil in response to feeding by larvae of the beetle *Diabrotica virgifera*. This volatile sesquiterpenoid, which can freely and speedily diffuse through the soil, functions to attract an entomopathogenic nematode, the natural enemy of the root pests (Rasmann et al. 2005).

Composite-type terpenoids also help defend plants against pathogens and herbivores. For example, shikonin derivatives accumulate exclusively in the roots, especially in epidermal cells of young plants, and in the cork layer on the surface of grown roots. Shikonin derivatives have been reported to exhibit anti-bacterial activity against soil-borne microorganisms, suggesting that these compounds act as a chemical barrier to protect root tissues from biological stresses (Brigham, 1999). Although most prenylated phenolic compounds are not volatile, shikonin derivatives, which have the ability to sublimate, may diffuse into soil to form an extended barrier in the rhizosphere.

The mobility of compounds in air, soil and water is important in chemical ecology. PVOCs emitted by herbivore-damaged plants into the atmosphere interact with undamaged neighbouring plants, alerting them to their current or future risk of damage by herbivores and enhancing their fitness (Arimura and Pearse 2017). These plant behaviours have been dubbed plant-plant communication, or “talking trees”. Despite previous scepticism, plant-plant communications have become an accepted component of plant biology. The nature and significance of plant-plant communication have been

evaluated using sagebrush (*Artemisia tridentata*), one of the leading model species of wild plant-plant communications. Sagebrush, which is native to North America, emits a sweet and pungent aroma, consisting of 1,8-cineole, camphor, α- and *β*-thujone, especially in response to physical damage. The profiles of these volatile compounds vary widely among individuals, but most individuals belong to one of two chemotypes, dominated by thujone or camphor (Karban et al. 2014). Sagebrush defences were induced to a greater extent by PVOCs from a closely related than an unrelated plant species (Karban et al. 2013), probably because plant chemotypes (i.e. quantitative and qualitative compositions of their PVOCs) can distinguish relatives from strangers, thereby enhancing fitness among related plants (Karban et al. 2014).

Plants can also negatively communicate with other surrounding plants via volatile and non-volatile terpenoids as invisible signals, a process termed allelopathy (Fischer 1986; Kato-Noguchi et al. 2010). Allelopathy is the direct or indirect negative as well as positive effect of one plant on another, mediated by allelochemicals released into the environment. Host plants may acquire more of the available nutrients, water and/or light owing to a reduction in resource competition. For example, over their entire life cycle, the roots of rice plants secrete phytotoxic levels of momilactone A and B, non-fragrant allelochemicals and defensive diterpenoids, into the soil (Kato-Noguchi and Peters 2013; Toyomasu et al. 2014). Interestingly, momilactones produced by rice and by the moss *Hypnum plumaeforme* (Hypnaceae) have both allelopathic and defensive properties, indicating a gap in the molecular evolution between Hepatophyta and monocots (Okada et al. 2016). The co-occurrence of a structural class of terpenoids in different taxa may be due to convergent evolution of the responsible gene(s) or variable regulation of these genes (Arimura and Maffei 2016). Although the expression or function of these gene products may be absent from some lineages, later members of these lineages may show activation of these genes (Wink 2003).

Linear-type FCs evolved in Lamiaceae to defend against herbivory, including the specialist *Papilio machaon*. *P. machaon* has evolved a P450 enzyme to decompose these toxic compounds, but plants in turn have developed a angular-type FC that is more difficult to decompose than linear FCs, a process called an arms race. The product specificity of PT determines the type of FCs that form (Munakata et al. 2016). Interestingly, the terpenoid pathway is associated with the co-evolution of these metabolic systems in both plants and insects.

Terpenoids in secondary metabolism including hidden terpenoids described above may be regarded as molecules that are dispensable for plant growth and development, but are indispensable for plant survival. Those terpenoids have enormous structural diversity, with high intraspecific variability within plant taxa, even among genotypes. This enables these plants to secure individual habitats that may involve various characteristics, including natural enemies, competitors and friends. As summarized in this mini review, terpenoids may be indispensable for ecological communications and plant evolution. Hidden terpenoids like glycosylated PVOCs are effective tools for plants as they are stored as inactive form, while in emergency situations, only hydroxylation is required to quickly provide free PVOCs for use. Further physiological studies may reveal additional properties of these atypical terpenoids.

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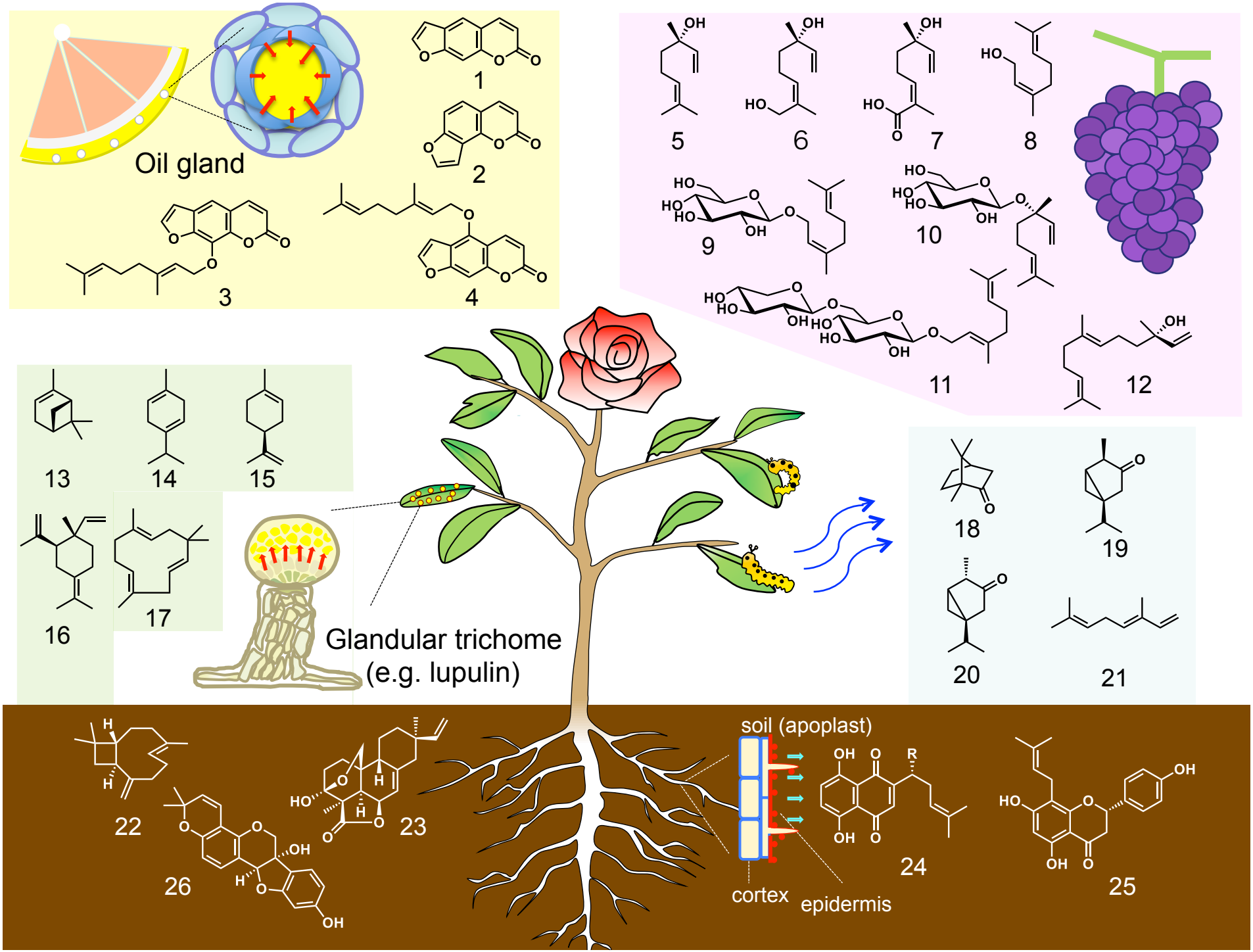
Figure legend

Fig. 1. “Hidden terpenoids”, defined as atypical terpenoids derived via isoprenoid pathways but without the typical appearance of terpenoids, and non-volatile forms of PVOC. Their specific tissues and organs for their localisation/accumulation are also revealed. Red arrows represent the secretion of indicated terpenoids from tissues or cells. Structures drawn in this Figure are: psoralen (1), angelicin (2), 8-geranyloxypsoralen (3), bergamottin (4), (*S*)-linalool (5), 8-hydroxylinalool (6), 8-carboxylinalool (7), nerol (8), neryl β-D-glycopyranoside (9), linalyl β-D-glycopyranoside (10), geranyl β-primeveroside (11), (*E*)-nerolidol (12), (-)-α-pinene (13), γ-terpinene (14), *l*-limonene (15), γ-elemene (16), α-humulene (17), camphor (18), α-thujone (19), β-thujone (20), (*E*)-β-ocimene (21), (*E*)-β-caryophyllene

(22), momilactone B (23), shikonin derivative (24), 8-prenylnaringenin (25), glyceollin I (26).


Oil gland
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20
21
Glandular trichome
(e.g. lupulin)
soil (apoplast)
cortex
epidermis
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23
24
25
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