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REVIEW

Volatile terpenoids: multiple functions, biosynthesis, modulation and manipulation by genetic engineering

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Abstract

Main conclusion   **Terpenoids play several physiological and ecological functions in plant life through direct and indirect plant defenses and also in human society because of their enormous applications in the pharmaceutical, food and cosmetics industries. Through the aid of genetic engineering its role can by magnified to broad spectrum by improving genetic ability of crop plants, enhancing the aroma quality of fruits and flowers and the production of pharmaceutical terpenoids contents in medicinal plants.**

Terpenoids are structurally diverse and the most abundant plant secondary metabolites, playing an important role in plant life through direct and indirect plant defenses, by attracting pollinators and through different interactions between the plants and their environment. Terpenoids are also significant because of their enormous applications in the pharmaceutical, food and cosmetics industries. Due to their broad distribution and functional versatility, efforts are being made to decode the biosynthetic pathways and comprehend the regulatory mechanisms of terpenoids. This review summarizes the recent advances in biosynthetic pathways, including the spatiotemporal, transcriptional and post-transcriptional regulatory mechanisms. Moreover, we discuss the multiple functions of the terpene synthase genes (TPS), their interaction with the surrounding environment and the use of genetic engineering for terpenoid production in model plants. Here, we also provide an overview of the significance of terpenoid metabolic engineering in crop protection, plant reproduction and plant metabolic engineering approaches for pharmaceutical terpenoids production and future scenarios in agriculture, which call for sustainable production platforms by improving different plant traits.

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Introduction

Terpenoids are structurally diverse and the most abundant group of floral volatiles, encompassing more than 40,000 individual compounds (Buckingham 2004; Muhlemann et al. 2014). A majority of these compounds are of plant origin and have several biological functions in higher plants. Terpenoids are found in most plant species and are essential for plant growth and development. They are the key components of membrane structures (sterols C_{30}), function as photosynthetic pigments (carotenoids C_{40}); abscisic acid (C_{15}) and gibberellins (C_{20}) are phytohormones, and ubiquinones are

involved in mitochondrial electron transport. Many terpenoids (including mono-, sesqui- and diterpenes) are known to be plant secondary metabolites which play fundamental roles in plant–environment and plant-plant interactions (Yu and Utsumi 2009; Dudareva et al. 2013).

Volatile terpenoids (isoprenes, monoterpenes and sesquiterpenes) constitute the largest class of plant volatile compounds. They exhibit several carbon skeletons and extremely variable in chemical structure yet share a common feature of biosynthesis, which occurs in almost all plant organs, including the roots, stems, leaves, fruits and seeds, but their highest amounts are released predominantly from flowers (Dudareva et al. 2013). Floral volatiles are lipophilic liquids in nature, having high vapor pressure and low molecular weight at ambient temperatures. These properties allow them to freely pass through the cellular membranes for release into the adjacent environment (Pichersky et al. 2006). Among all terpenoids, mono- and sesquiterpenes are the most commonly studied classes because of their extensive distribution in the plant kingdom and their essential roles in both human society and plants. Therefore, we will focus more on these two terpene classes in this review article. Terpenoids are synthesized from two inter-convertible C_5 units: isopentenyl diphosphate (IPP) and its allelic isomer dimethylallyl diphosphate (DMAPP). These five-carbon units serve as substrates/precursors for the biosynthesis of terpenoids. Based on their biosynthetic origin, floral volatile organic compounds can be divided into three major classes: terpenoids, benzenoids/phenylpropanoids and derivatives of fatty acids, wherein terpenes constitute 55% of the plant secondary metabolites, alkaloids 27% and phenolics 18% (Muhlemann et al. 2014).

Terpenes play diverse roles in beneficial interactions and in mediating antagonists among organisms (Das et al. 2013). They protect many plant species against pathogens, predators and competitors (Hijaz et al. 2016). Furthermore, herbivore-induced monoterpenoids act as airborne signals to nearby plants in response to insect attack such as (E)-β-ocimene (Arimura et al. 2004). Although terpenes are mostly studied in the above-ground tissues, recently, their novel function in the below-ground environment, as signaling molecules, has been identified (Karban et al. 2014; Delory et al. 2016). For example, β-caryophyllene, released from the roots of maize plants against beetle (*Diabrotica virgifera*) attack, acts as a volatile signal to attract predatory nematodes, which will defend plants indirectly from further damage (Rasmann et al. 2005). Similarly, the *Hedychium coronarium* farnesyl pyrophosphate synthase gene shows a quick response to herbivory and wounding and is involved in floral biosynthesis (Lan et al. 2013). Terpenoids are also beneficial for human beings. For example, limonene is extensively used as a scent compound in cosmetic products (Brokl et al. 2013). The potential anticancer and anti-inflammatory

compound zerumbone from shampoo ginger is a subject of interest in pharmacological studies (Bertea et al. 2005; Yu and Utsumi 2009).

Terpenoids are synthesized from two independent but compartmentally separated pathways: the mevalonic acid (MVA) and methylerythritol phosphate (MEP) pathways. The MEP pathway is mainly responsible for the biosynthesis of mono- and diterpenes, producing approximately 53 and 1% of the total floral terpenoids, respectively. Sesquiterpenes are synthesized from the MVA pathway, contributing approximately 28% of the total floral terpenoids (Muhlemann et al. 2014), as shown in Fig. 1.

The commercial and ecological importance of terpenoids has inspired rapid progress in floral scent engineering for terpenoid production in model plants. The identification of TPS genes, decoding the biosynthetic pathways and enzymes involved in these pathways, has made the manipulation of genetic engineering in plants extremely feasible (Dudareva and Pichersky 2008; Yu and Utsumi 2009). Genetic engineering can improve numerous input and output characteristics in crops, including weed control through allelopathy, pest resistance, increases in the aroma production of fruits and vegetables (by altering the floral scent) and the production of medicinal compounds (Aharoni et al. 2005; Dudareva et al. 2013). Furthermore, model plants (*Arabidopsis*, Tobacco) with altered terpenoid profiles can provide useful information for exploring biosynthesis, regulatory compounds and their ecological importance in plant–environment interactions.

In this review, we emphasize the recent advances in understanding the molecular mechanism of the biosynthetic pathways and its regulation, function and manipulation of genetic engineering of terpenoid production in model plants. Here, we will focus on mono- and sesquiterpenoids.
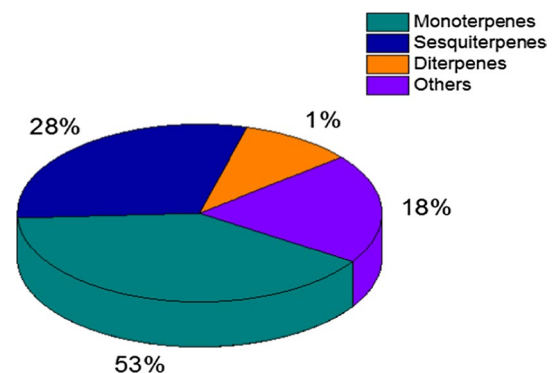

Fig. 1 Pie chart showing the approximate constituents of the total floral terpenoids—mono-, sesqui-, di- and other terpenes (like isoprenoids hemi, tri-, and tetraterpenes). Monoterpenes dominate over half of the pie chart, being operative in plastids and showing wide diversity, while sesquiterpenes compose 1/3 of the pie chart and are distributed in the cytosol, endoplasmic reticulum and peroxisomes

Multifunctionality of volatile terpenoids

Terpenoid metabolites are involved in various physiological and ecological functions based on the differential expression profiles of terpene synthase genes found in response to biotic and abiotic environmental factors throughout plant development. As the largest class of natural plant products, terpenoids possess different functions in mediating beneficial and antagonistic interactions among organisms. They protect many plant species, animals, and microorganisms against pathogens, competitors and predators by transmitting messages to mutualists and conspecifics regarding enemies, the presence of food and mates (Gershenzon and Dudareva 2007). Terpenoids are also involved in the plant–plant interaction, repelling pests and attracting enemies of pests (Unsicker et al. 2009). Volatile terpenoids are involved especially in attracting pollinators, seed dispersal, defense against herbivores from both below- and above-ground (Ali et al. 2012; Delory et al. 2016), plant–plant signaling and protection against pathogens (Huang et al. 2012) (Fig. 2).

Terpenoids as attraction for pollinators

Flowers employ various palettes for mediating the attraction of pollinators to ensure successful reproduction. For pollinators, multisensory inputs (thermal, olfactory, electromagnetic, visual) are necessary for locating breeding sites and food. Many studies showed the role of plant terpenoids in communication between plants and pollinators (Baldwin et al. 2006). Terpenoids are a major cue for attracting pollinators (animals, insects, mammals, birds and bats), serving as vectors in pollen transfer (Abrol 2012; Farré-Armengol et al. 2015) and many other miscellaneous functions in plant biology and ecology (Degenhardt et al. 2009).

Floral scent is an important source of communication between pollinators and flowering plants for their evolution, particularly during long range communication (Dudareva and Pichersky 2000; Farré-Armengol et al. 2013). It has been proven that the information sent by the floral volatiles depends on eliciting a distinct behavioral response and the context and composition of their emission toward the respective pollinators. Long distance floral scent emission mostly contributes to guiding pollinators to flowers, especially for night-emitting plants, for which the production of scent intensity is high to prevail over the low conspicuousness of flowers under low illumination. For example, *Sagittaria latifolia* and *Petunia axillaris*, pollinated by moths, emit larger amounts of volatile terpenoids than day-emitting bee-pollinated plants in the same genus, such as *Silene dioica* and *Petunia integrifolia* (Waelti et al. 2008; Dudareva et al. 2013). Potential pollinators quickly identify and locate the scented flowers, promoting association between pollinators

Fig. 2 A summary of volatile-mediated interactions between plants and their surrounding environment. Plant–animal interactions comprise the attraction of seed disseminators and pollinators by fruit and floral volatiles, the repellence/ attraction of herbivores and the attraction of natural enemies of aggressive herbivores in both the rhizosphere and atmosphere. The above-ground interactions include priming or elicitation of the defense responses of nearby unattacked plants through the leaves of the same plant. Moreover, the below-ground interactions consist of allelopathic activity on the germination, growth and development of competitive nearby plants. Terpenoid volatiles emitted from roots and reproductive organs also possess antimicrobial activity, hence protecting the plants against pathogen attack. Furthermore, leaf volatiles (isoprenes) confer thermotolerance and photoprotection
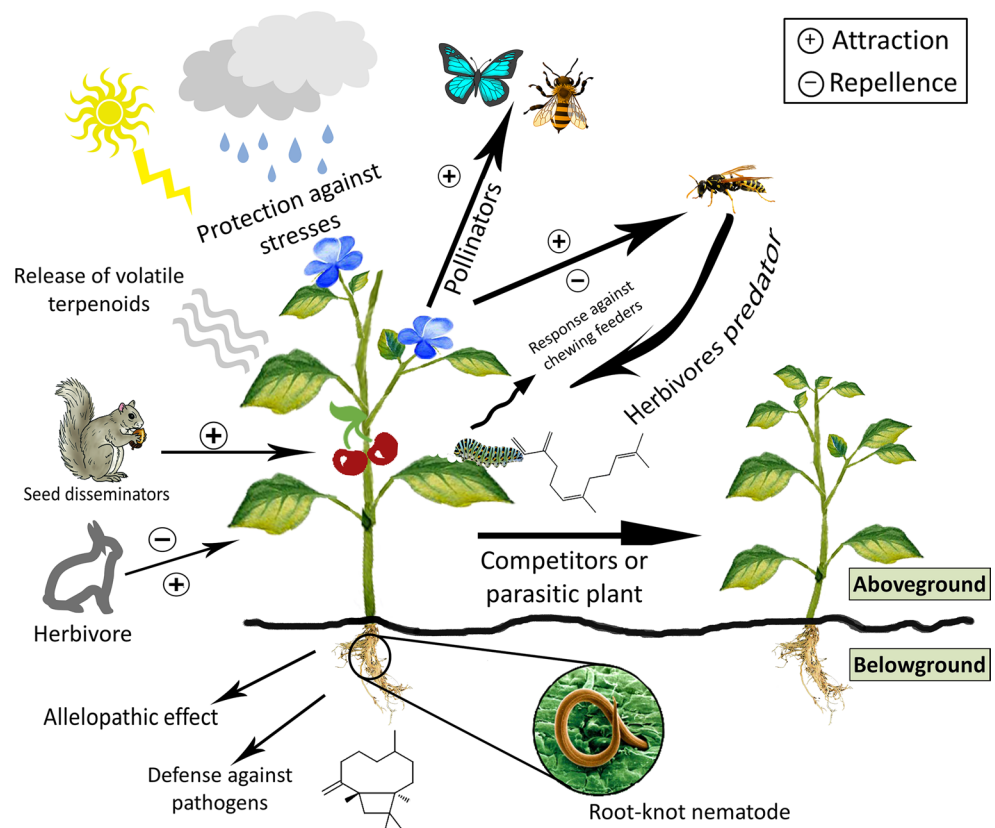
and plants via individual ratios of general compounds or special compounds (Wright and Schiestl 2009). Terpenoid volatiles are emitted not only by petals but also as pollen odors, such as β-ocimene, a universal attractant emitted by plants that attracts long range pollinators (Knudsen et al. 2006), such as bees, moths, butterflies and beetles (Muhlemann et al. 2006; Okamoto et al. 2007), thus benefitting pollinator foraging. They also have been found to attract bumblebees and honey bees (Granero et al. 2005). Volatile terpenoids attract certain pollinators by releasing pheromones; for example, orchids employ floral scents that mimic a blend of pheromones from female pollinators, triggering copulation attempts by male pollinators with flowers (Bohman et al. 2014). Tomato flowers in glasshouses mainly produce four monoterpenes (*p*-cymene, α-pinene, (+)-2-carene, and β-phellandrene); all of these are recognized as herbivore-induced leaf volatiles, possessing toxic properties and functioning in plant defense. The emission of the compounds mentioned above is negatively correlated with bumblebee visitation. Floral scents represent characteristic attributes of plants pollinated via beetles and necrophagous flies, proving a clear link between specific floral scent chemistry and certain pollinator guilds. The complex chemistry between floral volatiles and pollinators is yet to be unraveled. The increase in annual temperature is a potential threat to pollinators that affects their life cycle and flower visitation. The excessive use of pesticides in field crops is a grave threat to potential pollinators, which should be alleviated to improve crop production.

Terpenoid volatiles: an immediate response in plant defense

The plant kingdom shows two types of responses (direct and indirect defense mechanisms) upon contact with herbivores and pathogen and rodent attacks. The direct mode of defense includes several physical structures such as thorns, trichomes and accumulation of chemicals (phytochemicals) having antibiotic activities. In most plant species, sesquiterpenes and diterpenes act as phytoalexins (Mumm et al. 2008). For example, in *Gossypium* species, gossypol and its associated sesquiterpene aldehydes, derived from the (+)-δ-cadinene precursor, provide inducible and constitutive protection against pests and diseases (Townsend et al. 2005). Fourteen diterpene phytoalexins have been discovered from *Oryza sativa* and divided into four types based on structural distinctions: phytocassanes A–E, oryzalexin S, momilactones A and B, and oryzalexins A–F. Numerous volatile terpenoid compounds possess antifungal and antimicrobial activities in vitro (Bakkali et al. 2008; Johnson and Gilbert 2015), explaining the tissue-specific expression patterns of their biosynthetic genes (Chen et al. 2004). Moreover, only some of them have been characterized due to their

role in defense against pathogens (Rosenkranz and Schnitzler 2016). (*E*)-β-Caryophyllene emitted from the stigma of *Arabidopsis* flowers limited bacterial growth, while plants lacking the secretion of this sesquiterpene showed a dense population of bacteria on the stigma of the flower, resulting in reduced seed weight compared with the wild-type plant. Huang et al. (2012) proved that (*E*)-β-caryophyllene is important for plant fitness and functions in defense against pathogenic bacteria.

The phenomenon "indirect defense response" refers to the plants' characteristics, which protect them against herbivore attacks by promoting the efficacy of herbivore's natural enemies (Dicke and Baldwin 2010). This may include releasing a blend of specific floral volatile scents that attract predators of herbivores after being attacked (Kessler 2010; Peñuelas et al. 2014)). In the model plant *Arabidopsis*, females of parasitoids *Cotesia marginiventris* use TPS10 (a sesquiterpene) to track their lepidopteran host by utilizing the floral scent (Schnee et al. 2006). *Arabidopsis* has been shown to emit two terpenoids, (3,S)-(*E*)-nerolidol and its derivative (*E*)-4,8-dimethyl-1,3,7-nonatriene, when the strawberry nerolidol synthase gene was introduced into the plants, resulting in greater attraction of the predators of predatory mites (Kappers et al. 2005). Caryophyllene, emitted from the roots of Maize plants, is known to be an herbivore-induced below-ground signal, which strongly attracts entomopathogenic nematodes (Rasmann et al. 2005; Delory et al. 2016). Similarly, Ozawa et al. (2000) demonstrated that (*Lotus japonicus*) shoots infested with spider mites released a blend of volatiles that attracted predators of mites (*Phytoseiulus persimilis*). Terpenoids released from *Nicotiana attenuata* affect the expression of several genes of the adjacent conspecifics. Terpenoids released from the air also play a significant role in plant defense against biotic and abiotic stresses (Paschold et al. 2006; Unsicker et al. 2009; Dudareva et al. 2013).

Plant terpenes emission could also be an internal signal for plants to indicate the presence of herbivores and allow the initiation of defense in neighboring tissues (Rosenkranz and Schnitzler 2016). In the case of herbivore damage, significant amounts of volatile terpene blends are emitted from lima bean leaves to stimulate the adjacent leaves to boost nectar secretion to attract herbivore enemies (Heil and Bueno 2007). Developments in chemical ecology increasingly confirm terpenoids' defensive role. The above-mentioned examples strongly support the floral volatile terpenoids' involvement in deterring unwanted floral visitors. Many TPS genes from various crops have been identified that showed defensive roles against herbivore and pathogen attacks, but there is a need to explore them further. Potential TPS genes and their defensive roles can be enhanced with the help of systems biology. Genetic engineering combined with biotechnology and plant breeding is a valuable

approach for enhancing the defensive role of TPS genes in other crops that show less resistance toward pathogen and herbivore attacks.

Volatile terpenoids: impact on the agroecosystem

Volatile terpenoids have several important applications in human society; the food and pharmaceutical industries exploit their effectiveness and potential as flavor enhancers and medicines (Rosenkranz and Schnitzler 2016). The most extensively used terpene by human beings is rubber, a polyterpene composed of repeated isoprene subunits. Similarly, terpenes fine important use as methanol, cleaners, solvents, camphor, antiallergenic agents, pyrethrins (insecticides), limonene, rosin, nepetalactone (in catnip), digitoxigenin, carvone and hecogenin (a detergent) (Croteau et al. 2000; Thimmappa et al. 2014). The antimicrobial activity of terpenoids is the most significant because of the drastic increase in bacterial resistance to antibiotics, which is a growing cause of concern globally (Islam et al. 2003; Singh and Sharma 2015). In livestock, the addition of terpenes can replace conventional antibiotics, as they can slow down the resistance of bacteria against antibiotics. Plant oils that included terpenes in their composition showed promising in vivo bactericidal activity. Terpenoids also play key roles in the clinical industry (Singh and Sharma 2015). Prabuseenivasan et al. (2006) proved that cinnamon oil showed a broad range of activity against *Pseudomonas aeruginosa.* Moreover, the terpene composition can vary markedly among different species. *Neolitsea foliosa* (Nees) plant oil contains caryophyllene (sesquiterpene), which is responsible for its antibacterial properties (John et al. 2007). Terpenoids have also been used in antibacterial soaps, household products and cosmetics due to their lipid organizational properties (Caputi and Aprea 2011).

The impact of terpenes on both nature and human applications is complicated; however, these compounds have a significant impact on the agroecosystem and maintenance of bio-diversity. For example, blueberry, apple, cucurbits and canola are valuable to varying degrees from the insect pollinator point of view (Losey and Vaughan 2006; Tholl 2015) and for self-incompatible dioecious crops. Floral scents have also been unintentionally used to retain a few pollinator attracting traits as a baseline, without which the crop could not bear fruits. Jabalpurwala et al. (2009) observed that self-incompatible *Citrus grandis* (pummelo) blossoms emit larger quantities of volatile organic compounds than other species such as lemon, orange and grapefruit flowers, which comprise both female and male organs. Few experimental studies have been conducted to evaluate the effects of floral scents on crop pollination. For this purpose, bee visitation was compared between *Bacillus thuringiensis* (Bt) and wild-type (Bt)-expressing eggplant (Dudareva et al.

2013). Commercial bumblebees were strongly attracted by Bt-eggplant instead of the wild-type in a glasshouse environment (Arpaia et al. 2011). The relationship between failure in crop pollination and the composition of floral volatile organic compounds is best demonstrated by alfalfa seed production, which depends on pollination through honeybees that consistently showed low fidelity to alfalfa flowers compared with other weedy plants and crops. To show the link between crops and volatile terpenoid chemistry, Morse et al. (2012) studied tomato production in a glasshouse, where imported bumblebees were regularly used as pollinators, which showed a preference for other forage and flowering plants outside of the glasshouse. The behavior of bees toward tomato plants was attributed to the emission of four monoterpenes (α-pinene, β-phellandrene, (+)-2-carene, and *p*-cymene), which possessed toxic properties and were useful in plant defense. Terpenoids are of utmost importance in crop protection, plant reproduction, and future scenarios in agriculture involving sustainable production platforms. Terpenoids may have other mysterious functions in plants that remain to be revealed.

Biosynthesis and subcellular compartmentation of terpenoids in plants

Terpenoids are the largest class of plant floral volatiles, encompassing 556 scent compounds. For the biosynthesis of most terpenoids in plants, the simple five carbon unit IPP and its allylic isomer DMAPP serve as the initial substrate. IPP is the most frequent precursor of all terpenoids, synthesized by two compartmentally separated and independent pathways, the MVA and MEP pathways (Chen et al. 2011). The MVA pathway comprises six enzymatic reactions, carried out through step-by-step condensation of three molecules of acetyl CoA, which then undergo reduction to the MVA pathway followed by two subsequent steps of phosphorylation and decarboxylation, forming IPP as the ultimate product (Lange et al. 2000; Tholl 2015). Similarly, the MEP pathway involves seven enzymatic steps and begins with the condensation of pyruvate and glyceraldehydes-3-phosphate (G3P) (Nagegowda 2010; Muhlemann et al. 2014). IPP isomerizes to form DMAPP, which serves as a substrate for hemiterpene biosynthesis or combines with one IPP unit to produce geranyl diphosphate (GPP), catalyzed by the GPP synthases (GPS). The condensation of one IPP with one GPP molecule produces farnesyl pyrophosphate (FPP), which is catalyzed in the presence of FPP synthases (FPS). Similarly, GPP and FPP are precursors for the biosynthesis of monoterpenes and sesquiterpenes, respectively (Vranová et al. 2012), whereas prenyl diphosphates in the trans-configuration are assumed to be the ubiquitous naturally occurring substrates for terpene synthases. In wild-type tomato

trichomes, Z, Z-FPP and two prenyl diphosphates, in the cis-configuration, are also naturally occurring substrates for the synthesis of sesquiterpenes catalyzed by FPP synthase (zFPS). Similarly, iso-, mono- and sesquiterpenes synthases convert DMAPP, GPP and FPP (or Z, Z-FPP) to isoprene, monoterpenes and sesquiterpenes, respectively. Based on their biosynthetic origin, all volatile organic compounds can be divided into numerous classes, while several floral scent volatiles are constituents of the terpenoid, phenylpropanoid/benzenoid or fatty acid derivative groups of compounds, and each volatile group is recognized by a few compounds that produce a typical scent (Muhlemann et al. 2014).

Two different and independent pathways are involved in the formation of five carbon isoprene building units, the plastidial MEP and cytosolic MVA pathways; however, the MEP pathway is also disseminated in the endoplasmic reticulum and peroxisomes (Mahmoud and Croteau 2002; Dudareva and Pichersky 2008; Lange and Ahkami 2013), whereas these compartmentally separated isoprenoid biosynthetic pathways are interconnected by a metabolic "crosstalk" (Flügge and Gao 2005; Orlova et al. 2009). Mevalonate-5-diphosphate is produced in *Arabidopsis thaliana* by the MVA pathway through phosphorylation, and the entire reaction is catalyzed by mevalonate kinase and phosphomevalonate kinase (Lluch et al. 2000; Yu and Utsumi 2009). The MVA pathway in the peppermint glandular trichomes is blocked at 3-hydroxy-3-methylglutaryl-CoA reductase, which is an enzyme that controls the rate of the MVA pathway as well as both mono- and sesquiterpenes biosynthesis entirely based on the plastidial IPP. In the tobacco suspension cell line BY-2, the crosstalk between the MEP and MVA pathways has been verified (Hemmerlin et al. 2003; Dudareva et al. 2013), although the specific intermediate has not yet been identified. Studies on snapdragon flowers also indicate that the MEP pathway supplies IPP for the biosynthesis of monoterpenes and sesquiterpenes. Furthermore, the trafficking of IPP occurs unidirectionally from the plastid to the cytosol (Dudareva et al. 2005; Nagegowda 2010). Moreover, both pathways co-operate with each other for a while and provide the IPP precursor for the biosynthesis of terpenoids as mentioned above. The chamomile sesquiterpenes are an adequate example showing clear metabolic crosstalk between the two pathways in which both provide IPP precursors for the production of certain terpenoids that consist of 2 five-carbon isoprene units, which are derivatives of the MEP pathway, while the third unit is formed by both pathways (Towler and Weathers 2007). In the plastids, the MEP pathway (Hsieh et al. 2008) results in the formation of IPP and DMAPP from G3P and pyruvate. However, the classical independent MVA pathway (Vranová et al. 2013) is disseminated in the cytosol, peroxisomes and endoplasmic reticulum (Pulido et al. 2012). The MVA pathway forms IPP starting with acetyl-CoA condensation and is responsible for

the formation of most sesquiterpenes (C15), which account for approximately 28% of all floral terpenoids. In snapdragon flowers, the MEP pathway alone supports the biosynthesis of sesquiterpenes (Dudareva et al. 2005), whereas the MVA and MEP pathways are involved in the formation of sesquiterpenes in the roots and leaves of carrots (Hampel et al. 2005). Terpenoid biosynthetic pathways and their intracellular compartmentation in plants are shown below in Fig. 3.

The formation of the GPP precursor has been confirmed to occur in plastids and is responsible for the biosynthesis of monoterpenes as the final product, while sesquiterpenes are produced in the endoplasmic reticulum, peroxisomes or cytosolic compartments, which are also a site for FPP formation. Nevertheless, two unlikely monoterpene synthases of strawberry that generate several olefinic monoterpenes and linalool lacked plastid targeting signals and were shown to be targeted in the cytosolic compartment (Aharoni et al. 2004; Guirimand et al. 2012). Likewise, the overexpression of the lemon basil α-zingiberene synthase gene in tomato plants resulted in the production of a monoterpene in the cytosol (Davidovich-Rikanati et al. 2008). Santalene and bergamotene synthase (SBS) from tomato contain putative N-terminal plastid targeting peptides (Schilmiller et al. 2009). Plant genomes have evolved to encode diverse FPP synthase isoforms, which are present in the cytosol, mitochondria, plastids or peroxisomes (Chen et al. 2011; Thabet et al. 2012). Terpene biosynthesis occurs at specific stages during plant development and within specific tissues (Vranová et al. 2012).

Numerous TPS enzyme products, including isoprenes, monoterpenes, sesquiterpenes and a few diterpenes in specialized metabolism, are volatile under the normal growing conditions (environmental and temperature) of plants. To date, numerous flower-specific terpene synthases have been characterized and isolated, as they are accountable for the development of monoterpene linalool (*A. thaliana*, *Anthirrhinum majus*, *H. coronarium* and *Clarkia breweri*) (Nagegowda et al. 2008; Ginglinger et al. 2013; Yue et al. 2014), 1,8-cineole (*Citrus unshiu*, *H. coronarium* and *Nicotiana suaveolens*) (Roeder et al. 2007; Li and Fan 2007), myrcene (*Alstroemeria peruviana* and *A. majus*) (Aros et al. 2012), *E*-(β)-ocimene (*A. majus* and *H. coronarium*) (Shimada et al. 2005; Fan et al. 2003, 2007) and the sesquiterpenes α-farnesene (*Actinidia deliciosa* and *H. coronarium*) (Nieuwenhuizen et al. 2009; Yue et al. 2015), nerolidol (*A. chinensis* and *A. majus*) (Green et al. 2011), valencene (*Vitis vinifera*) (Lücker et al. 2004), germacrene D (*V. vinifera*, *Rosa hybrid* and *A. deliciosa*) (Lücker et al. 2004; Nieuwenhuizen et al. 2009) β-ylangene, β-copaene, β-cubebene, α-bergamotene (*Cananga odorata* var. *fruticosa*) (Jin et al. 2015) and β-caryophyllene (*Ocimum kilimandscharicum*, *Daucus carota*) (Yahyaa et al. 2015; Jayaramaiah et al. 2016).

Fig. 3 An outline showing the formation of iso-, mono- and sesquiterpene biosynthetic pathways in plants catalyzed by different terpene synthases and their intra-cellular compartmentation. The plastidial MEP pathway starts with the condensation of pyruvate and GA-3P, which undergo different reactions, producing iso-, mono- and sesquiterpenes as their final product. However, the MVA pathway spans the cytosol, peroxisomes and endoplasmic reticulum and starts with the condensation of acetyl-CoA, which undergoes different reactions, giving rise to mono- and sesquiterpenes as the final products. The cross-talk between the two pathways includes the exchange of IPP between the two compartments, the cytosolic MVA and plastidial MEP pathways, producing different terpenes as end products (iso-, mono- and sesquiterpenes)
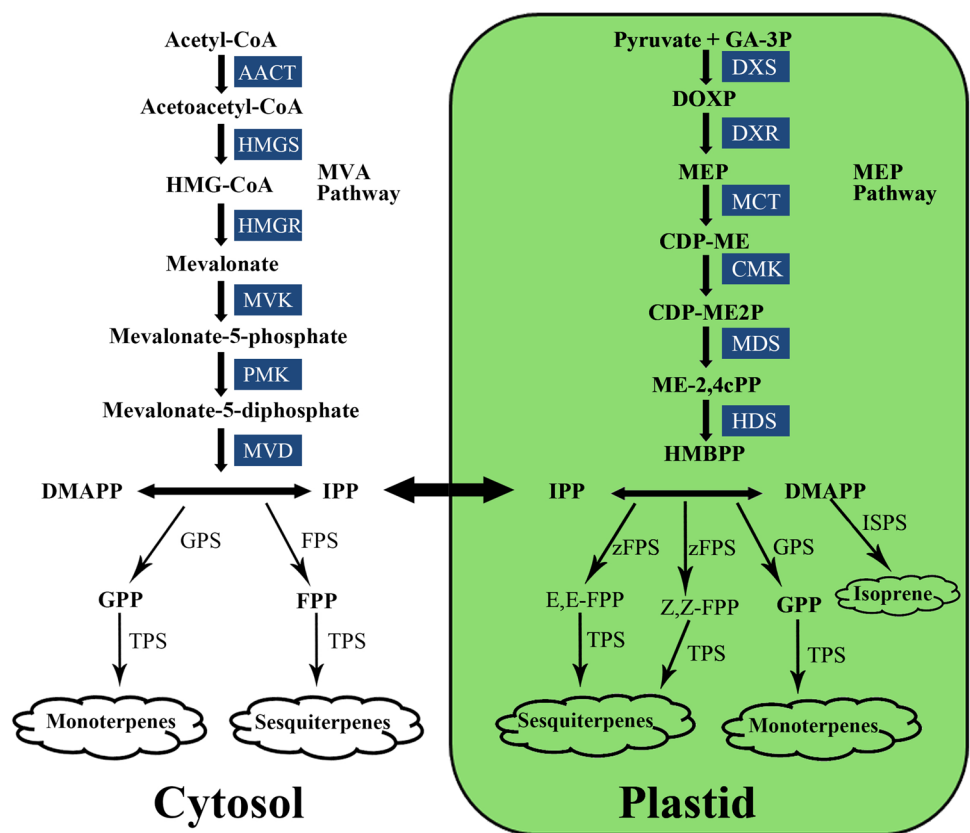
Modulation of plant terpenoid biosynthesis

Terpenoids are frequently synthesized and released at particular times from specific plant tissues. Ample evidence explains the tempo-spatial TPS expression correlating with the volatile terpenoid biosynthesis and emission, suggesting that the volatile terpenoid biosynthesis is mostly regulated at the transcriptional and post-transcriptional levels (Tholl 2015). The regulation of plant terpenoid biosynthesis is complicated and usually divided into two categories during development, the temporal and spatial regulation, in response to biotic and abiotic factors such as light intensity, insect/pathogen damage, nutrients, humidity and temperature conditions (Van Poecke et al. 2001; Hakola et al. 2006).

Developmental, rhythmic and spatio-temporal regulation

The emission of particular volatile compounds into the atmosphere depends on their rates of biosynthesis and emission. Numerous studies showed that terpenoid volatiles are mostly synthesized de novo in a few special physical structures, such as oil glands (Gershenzon et al. 2000) and resin ducts (Miller et al. 2005), which accumulate large quantities of terpenes. Biosynthesis occurs in the epidermal cells of plant tissues, through which they can easily be released

into the atmosphere/rhizosphere (Kolosova et al. 2001; Chen et al. 2004; Tholl and Lee 2011). Several conifers accumulate significant amounts of scent oleoresins in their specialized needles, bark blisters or resin ducts and sapwoods (Zulak and Bohlmann 2010). The biosynthesis and regulation of emission of plant terpenoids are mostly carried out via petals, although other tissues, such as sepals, stamens, pistils and nectaries, also contribute to the floral bouquet of plant species (Farré-Armengol et al. 2013). Sometimes, the regulation of terpenoids is induced via abiotic stresses, pathogens attack or feeding of herbivores (Vranová et al. 2012). In *Arabidopsis*, mono- and sesquiterpene synthases are not expressed in flower petals, and their expression is restricted to nectaries, sepals, anthers and stigma (Tholl et al. 2005). In the snapdragon flower, key volatile benzoid compounds, the monoterpene myrcene, (E)-β-ocimene biosynthesis and developmental regulation occur in the epidermal layer of the upper and lower lobes of petals, controlled by the circadian clock following the diurnal rhythm (Kolosova et al. 2001; Muhlemann et al. 2014). Similarly, *H. coronarium* promoters (*PrHcTPS1* and *PrHcTPS2*) are involved in the temporal and spatial regulation of *HcTPS* genes associated with the biosynthesis of terpenoids (Li and Fan 2011; Li et al. 2014). Likewise, cell-specific expression of the aroma biosynthetic genes was also reported in *C. breweri* and roses (Bergougnoux et al. 2007). In *N. suaveolens*, several

monoterpene nocturnal emissions from the stigma and petals are an outcome of the transcriptional regulation of 1,8-cineole synthase through the circadian clock (Roeder et al. 2007). Pre-revealed changes in the emission of terpenoids that follow nocturnal, diurnal and circadian rhythms over the lifespan of flowers may be associated with the pollination of insects. In *Artemisia annua*, β-pinene emission fluctuates with the day-night rhythm, which is usually higher in the day compared with night (Lu et al. 2002; Tholl and Lee 2011). The majority of terpene synthase genes belong to the TPSa and TPSb sub-families, reaching peak expression according to the bundle of accumulation of individual compounds, whereas in the TPSg sub-family, only the linalool synthase transcripts were observed during the ripening of berries.

Transcriptional and post-transcriptional regulation

During the floral lifespan, the gene expression is transcriptionally regulated by more than one biochemical pathway. The enzymes proficient in using numerous similar substrates, such as acyltransferase and salicylic acid methyltransferase (SAMT), supplied the precursors that controlled the product type (Boatright et al. 2004). A relative investigation on the regulation of monoterpenes and benzenoids emission in snapdragon flowers showed that the orchestrated emission of isoprenoids and phenylpropanoid compounds were regulated upstream by individual metabolic pathways (Muhlemann et al. 2012).

The formation of volatile flower terpenoids through various independent pathways is not merely dependent on the biochemical properties of the enzymes involved in the biosynthesis but also on the contribution of the transcriptional factors (TFs). Until now, only a few TFs had been identified in the regulation of biosynthetic genes. Recently, R2R3-type MYB TF, ODORANT1 (ODO1), isolated from the petunia flower, was highly expressed in petal tissues and was involved in the regulation of a major portion of the shikimate pathway (Verdonk et al. 2005; Muhlemann et al. 2014). EOBII (EMISSION OF BENZENOIDS II), another R2R3-type MYB TF positively regulates the ODO1 that activates the biosynthetic gene (isoeugenol synthase) promoter (Colquhoun et al. 2010). Similarly, the MYB4 transcriptional factor was a repressor of one of the enzymes in the cinnamate-4-hydroxylase, phenylpropanoid pathway and controlled the flux in petunia flowers (Colquhoun et al. 2011). Similarly, MYC2 activated two sesquiterpene synthase genes (*TPS11*, *TPS21*) through the jasmonic acid (JA) and gibberellic acid signaling pathway in *A. thaliana* during inflorescence (Hong et al. 2012). In roses, the up-regulation of terpenoid pathways occurred via the overexpression of the PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1) transcriptional factor (Zvi et al. 2012). The enhancement in the concentration of terpenes in response to different stresses was usually connected to the increased transcriptional activity of a particular biosynthetic terpene gene (Nagegowda 2010; Nagegowda et al. 2010; Xi et al. 2012). In *Medicago sativa*, pathogen attacks cause metabolic and transcriptional changes in the plant cells (Suzuki et al. 2005).

Genetic engineering for the production of plant terpenoids

Genetic engineering in plants/model plants for terpenoid production is currently a dynamic research area. Metabolic engineering offers a gigantic potential for enhancing plant resistance to pests in agriculture and forestry due to the abundance and contribution of secondary volatile metabolites in altering the floral scent bouquets, improving fruit quality, eradicating undesirable compounds and improving plant defenses (direct and indirect defenses) for exploring, the effect of changes in the volatile emission upon insect behavior (Mahmoud and Croteau 2002; Capell and Christou 2004; Tholl 2015). Recent advances in the identification of genes and enzymes responsible for volatile compound biosynthesis have made metabolic engineering dramatically realistic (Aharoni et al. 2006; Dudareva and Pichersky 2008; Dudareva et al. 2013). In most studies, the overexpression of TPS genes is a promising method for tackling these issues by manipulating terpene formation in transgenic plants. The first reported transgenic plants with modified monoterpene profiles were petunia (*Petunia hybrida*) and mint species (*Mentha* spp.) (Krasnyanski et al. 1999; Aharoni et al. 2005). It has been demonstrated that upon herbivore attack, plants emit a blend of volatiles, which is composed of more than 200 compounds that are directly responsible for deterring, intoxicating or repelling herbivorous insects (Seybold et al. 2006). Alternatively, they can attract the parasitoids and natural predators of offending herbivores, resulting in securing the plant from damage (Mercke et al. 2004; Degen et al. 2004; Dudareva et al. 2013). The transformation of petunia with the (S)-limonene synthase gene from *C. breweri* results in linalool production that repels aphids (Lücker et al. 2001). The repellent/attraction properties of various terpenoid volatiles in petunia flower were observed via silencing the biosynthetic genes individually responsible for the production of scent compounds (Kessler et al. 2013). Another example of floral scent engineering is the introduction of a maize sesquiterpene synthase gene (*TPS10*) into *Arabidopsis*, leading to the production of a significant amount of various sesquiterpenes that are exploited by the female parasitoid wasp *C. marginiventris* to navigate to their potential lepidopteran host (Schnee et al. 2006; Delory et al. 2016). Manipulation of genetic engineering for sesquiterpenes production seems a challenging task and is less successful compared with that for monoterpenes due to lack of suitable precursors.

The manipulation of terpenoid biosynthesis is an effective strategy for improving the quality of floral scent, aroma and flavor of fruits, herbs and vegetables (Pichersky and Dudareva 2007; Dudareva and Pichersky 2008). The first attempt to revolutionize the flavor of fruits was made through genetic engineering of tomato plants by overexpression of the linalool synthase gene from *C. breweri* under the control of the E8 promoter (fruit-specific promoter), which improved the aroma of fruits that were originally discarded by humans (Lewinsohn et al. 2001; Nagegowda 2010).

Plant metabolic engineering approaches for pharmaceutical terpenoids production

A large amount of different terpenoids have been described from which many of them are separated from different medicinal plants, like *Taxus chinensis*, *Salvia miltiorrhiza*, *A. annua*, *Panax ginseng* and *Ginkgo biloba*. Terpenoids isolated from taxol, ginkgolides and artemisinin have great effects on several diseases, such as paclitaxel derived from taxol, a diterpenoid derived from *Taxus brevifolia*, a significant anticancer agent. Structurally unique ginkgolides diterpenoids families are highly particular platelet-activating factor receptor antagonists (Lu et al. 2016). Similarly nowadays, a sesquiterpene (artemisinin) produced from *A. annua* is the best therapeutic towards drug resistant and malaria causing *Plasmodium falciparum* strains (Weathers et al. 2011). Several plants metabolic engineering approaches holds a significant pledge to regulate the pharmaceutical terpenoids biosynthesis based on Agrobacterium-mediated genetic transformation. Effective strategies to enhance the production of pharmaceutical terpenoids include the overexpression of genes involved in the terpenoid biosynthetic pathways and via suppress the competing metabolic pathways. Furthermore, regulating the relative transcription factors, primary metabolism and endogenous phytohormones can also considerably boost up their yield. Combination of all these approaches can help to increase the supply of limited terpenoid drugs, ultimately minimizing the price of expansive drugs and promote standards of people's living.

Gene promoters

Gene promoters can be very useful in the manipulation of genetic engineering, especially those promoters that drive gene expression at a particular time and a specific place; for example, cell types, tissues, and organs are interesting targets for the genetic engineering of the metabolic pathway (Benedito and Modolo 2014). To coordinate gene expression, promoters interact with the RNA polymerase under certain conditions and are common to plant genetic studies (e.g., actin, CaMV35S and ubiquitin). Specific promoters for glandular trichomes are favorable tools for this purpose. For example, the trichome-specific promoter of the cytochrome P450 gene (*CYP71AV1*) from *A. annua* has been studied and is a promising tool for genetic engineering of artemisinin biosynthesis in the whole plant system.

Transcription factors

In organisms, gene expression is regulated via complex regulatory networks, which are synchronized by transcriptional factors (TFs). TFs are proteins that perform as hubs or master regulatory genes through repressing or promoting the RNA polymerase binding to specific promoter regions. TFs can be used as powerful regulators of plant secondary metabolism, as most TFs can initiate the peculiar gene expression coding for enzymes in biochemical pathways. In *A. annua*, the artemisinin biosynthesis is positively regulated via two TFs (AaERF1 and AaERF2) belonging to the AP2/ERF family (Yu et al. 2012). Nevertheless, there is a need to determine whether these TFs indirectly regulate the glandular trichome development, expression of other genes and membrane transport expression or directly regulate them via promoting the expression of biosynthesis genes. Similarly, another TF (*AaWRKY1*) was shown to activate the expression of amorpha-4,11-diene synthase (ADS) and others in the artemisinin pathway (Ma et al. 2017). These findings also highlight the fact that TFs are dynamic tools for manipulating the complete transcriptional networks related to developmental or specific biosynthetic pathways. This approach will help attain food plants with higher nutritional values for both human and animal consumption. The potential use of TFs in heterologous systems can boost the synthesis of important secondary compounds.

Subcellular localization

Localization of genes (TPS) and appropriate prenyl diphosphate synthase in mitochondria or plastids is preferable to cytosolic localization due to the tightening regulation of cytosolic prenyl diphosphate pools. By introduction of potent antimalarial drug (artemisinin) in tobacco via two mega biosynthetic pathways into the nuclear genomes and chloroplasts, resulted in enhanced level of artemisinin without interrupting plant health. A comprehensive evaluation was conducted by Wu et al. (2006) to evaluate the effect of targeting different gene products to cytosol or plastids. The most appropriate example is the accumulation of amorpha-4,11-diene (ADS) and FPS in plastids, resulting in approximately a 5000-fold increase in ADS concentration in model tobacco plants over another model plant (unchanged ADS gene) having a cytosolic gene product (Wu et al. 2006). Likewise, a mitochondrial-localized heterologous TPS gene was first studied by Kappers et al. (2005), which revealed that the overexpression of the *FaNES1* gene from strawberry

in the model *Arabidopsis* plant with an engineered 'Met' mitochondrial targeting sequence resulted in two new terpenoids involved in attracting predatory mites that potentially aid in defense against herbivores. The potential use of genetic engineering in the subcellular localization of TPS genes provides new insights for further investigation.

Host organism selection

The selection of an appropriate host organism for genetic transformation is important and should be done carefully. To date, the study of plant metabolic engineering involves transgenic plants expressing the heterologous gene relevant for the accumulation of terpenoids using quick transformation procedures. Nevertheless, the production of engineered terpenoids in model plants was very low, and the highest accumulation levels reported were approximately 0.01% of fresh weight biomass. However, diterpene production in tobacco glandular trichomes is relatively high; the heterologous gene expression has not yet resulted in high production novel diterpenoids. Therefore, high terpenoid accumulation levels have been found for volatiles emitted from engineered model plants. If the purpose of genetic engineering is to adapt to the trophic interaction of plants with respect to insects, then the terpenoid volatiles released from model plants can have constructive effects against disease resistance (Kappers et al. 2005; Yu et al. 2012). On the other hand, volatilization is undesirable if terpenoid collection is desired, which poses a strategic question for scientists. There are limitations in genetic engineering for non-model plants producing terpenoids, the main limitation being the low efficacy of transformation as well as the lengthy period for the regeneration of transgenic plants. Metabolic engineering has been successfully employed in peppermint and lavender plants to magnify the essential oil production (Lange and Ahkami 2013).

Volatile terpenoids are widely used in different industries, as mentioned above, for multiple uses by human beings. Moreover, genetic manipulation for enhanced terpenoid production in the future would be beneficial for human beings. Much more work in this field is needed regarding the manipulation of genetic engineering in flowering plants. Genetic engineering techniques can revolutionize the use of terpenoids in both plants and human beings.

Conclusion and prospects

Genetic engineering has greatly enhanced our understanding of the function, biosynthesis, and regulation of volatile terpenoids in the past two decades. Recent efforts have demonstrated the viability of improving plant defenses and enhancing the aroma quality of fruits and flower via

metabolic engineering. The information attained provides new insights into terpenoid metabolism and will help in the metabolic engineering of plants for attracting pollinators and increasing the amount of the valuable products of volatile terpenoids. Since plant defense mechanisms are beneficial to agriculture, there is an increasing focus on terpenoids and phytoalexins, which contribute to plant defense responses and controlling pests, weeds and pathogens. Recently, plant terpenoids have been exploited for biofuel development and its by-products. Strategies in plant metabolic engineering clutches a promising role to upregulate the pharmaceutical terpenoids contents in medicinal plants. Pharmaceutical terpenoids production can be improved greatly via a series of metabolic regulation in medicinal plants. In future, with the constant development of metabolic engineering in plants, more high value pharmaceutical terpenoids will be upregulated. The spatial, temporal and transcriptional regulation of terpenoid biosynthesis during the development and response to environmental stresses is correlated to the physiological or ecological functions, such as repelling herbivores, pathogens and attracting pollinators as well as natural enemies of herbivores. Transcriptional and post transcriptional factors could also play important roles. Transcriptional factors include a group or groups of significant regulatory genes, which can perform key functions in genes that respond to stress mechanism. The overexpression of TFs can activate a group of loci, which function in a systematic approach for reacting to unfavorable environmental conditions. Hence, genetic engineering of transcriptional factors would be a powerful technique for improving the genetic ability of crop plants to address abiotic stresses. Transcriptional factors also play essential roles in signaling pathways under temperature stress. On the other hand, the identification of new TFs and overexpression cannot be neglected. Genomic, metabolic and proteomic tools and validation of in vitro cell culture systems could be helpful in elucidating the terpenoid biosynthesis regulatory mechanisms. Genetic engineering plays fundamental roles in the improvement of crop plants at every step, from the engineering of new biosynthetic pathways to altering the floral scent in scentless crops. Many aspects of terpenoids in both plants and ecosystem are yet to be elucidated.

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