Appl Microbiol Biotechnol
DOI 10.1007/s00253-015-7128-6

MINI-REVIEW

Sesquiterpene lactone engineering in microbial and plant platforms: parthenolide and artemisinin as case studies

Mohammad Majdi[1,2] · **Morahem Ashengroph**[3] · **Mohammad Reza Abdollahi**[4]

Received: 1 September 2015 / Revised: 24 October 2015 / Accepted: 27 October 2015

Abstract Sesquiterpene lactones (SLs) are one of the most diverse groups of secondary metabolites that mainly have been observed in the Asteraceae. They are composed of a C_{15} skeleton bearing functional groups, e.g., hydroxy, keto, or epoxy. Sesquiterpene lactones have been shown to display several biological activities; hence, their therapeutic effects are indispensable. To overcome low yield of sesquiterpene lactone content in native plants, manipulation of their biosynthetic pathway(s) has become an interesting approach for many researchers. Several genetic engineering strategies have been used in plants or microbial systems for elucidation of the biosynthetic pathway and high-level production of sesquiterpene lactones. Here, we will introduce ongoing research and perspectives about the manipulation of sesquiterpene lactone biosynthesis by various non-traditional metabolic engineering strategies, along with successful examples of high-yield production of sesquiterpene lactones mainly focused on parthenolide and artemisinin in plants and microorganisms.

Keywords Metabolic engineering · Sesquiterpene lactones · Cytochrome P450 · Choloroplast · Terpenes

✉ Mohammad Majdi
m.majdi@uok.ac.ir

[1] Department of Agricultural Biotechnology, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran

[2] Research Center for Medicinal Plant Breeding and Development, University of Kurdistan, Sanandaj, Iran

[3] Department of Biology and Biotechnology, Faculty of Sciences, University of Kurdistan, Sanandaj, Iran

[4] Department of Agronomy and Plant Breeding, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, Iran

Introduction

Terpenes and their biosynthetic pathways

Plant secondary metabolites are commonly considered as important compounds due to their applications as flavors, fragrances, and pharmaceuticals (Berger 2009; Corteau et al. 2000). Terpenes constitute the largest and highly diverse group of secondary metabolites with more than 25,000 compounds (Gershenzon and Dudareva 2007). Terpenes originate from the successive condensation of acetyl-CoA units into C_5 building blocks. They are derived from two distinct pathways, the mevalonic acid (MVA) pathway and a relatively newly discovered one, 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, which are localized in the cytosol and plastids of plant cells, respectively (Back and Chappell 1995; Kirby and Keasling 2009) (Fig. 1). Terpenes are categorized into several groups according to their carbon number as monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), and triterpenes (C_{30}).

Sesquiterpene lactones, structural diversity, and biological activities

Sesquiterpene lactones (SLs) have been considered as the main group of secondary metabolites produced in the Asteraceae (Hristozov et al. 2007). They are generally derived from modifications of sesquiterpene hydrocarbon skeletons by cytochrome P450 monooxygenases (CYP450) and other oxidoreductase enzymes (de Kraker et al. 2002). Cytochrome P450 enzymes catalyze most of the oxidation steps in sesquiterpenoid biosynthesis, including hydroxylation or epoxidation reactions (Kahn and Durst 2000). These enzymes play a key role in adding functional groups to sesquiterpene backbone, as the therapeutic effects of SLs have been

Published online: 14 November 2015

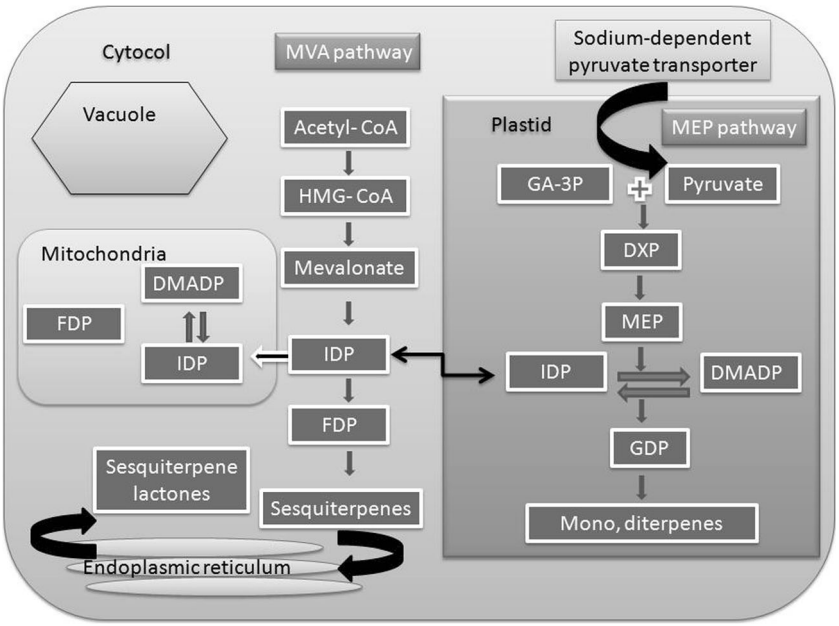


Fig. 1 Pathways related to sesquiterpene lactone biosynthesis and their compartmentalization in different organelles: *DXP* 1-deoxy-D-xylulose 5-phosphate, *GA-3P* glyceraldehyde 3-phosphate, *MEP* methylerythritol phosphate, *DMADP* dimethylallyl diphosphate, *IDP* isopentenyl diphosphate, *GDP* geranyl diphosphate synthase, *MVA* mevalonic acid, *AcetylCoA* acetyl-coenzyme A, *HMG-CoA* 3-hydroxy-3-methylglutaryl CoA, *FDP* farnesyl diphosphate synthase. The role of pyruvate transporter to import pyruvate into plastids and the cross talk of IDP between plastid and cytosol has been shown

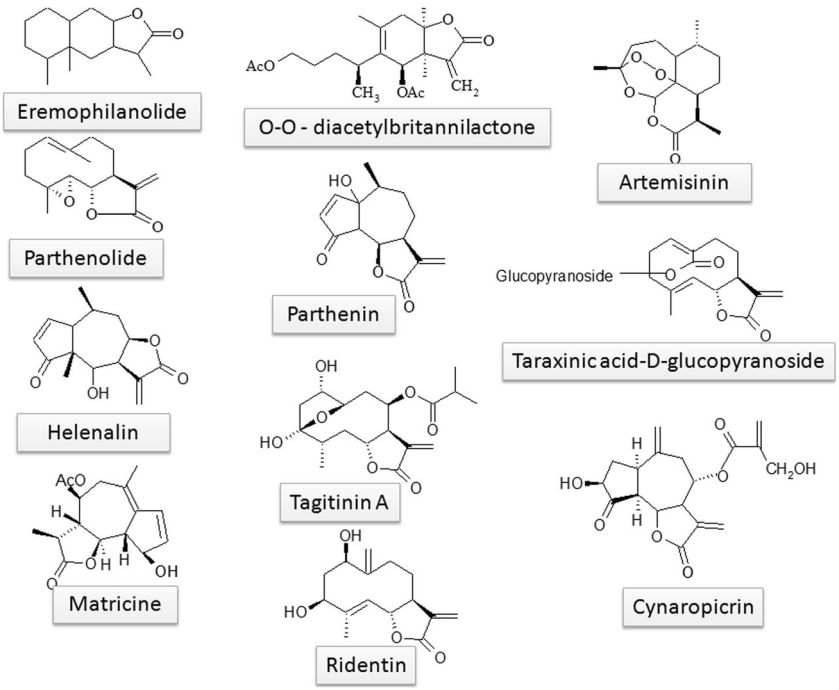
attributed to those functional group(s). The discovery and functional expression of these genes in microbial or plant platforms raised quite a lot of attention recently. SLs are generally colorless, bitter, lipophilic, relatively stable compounds, and containing a α-methylene-γ-lactone ring (Rodriguez et al. 1976). They exhibit a diverse skeletal arrangement comprising several classes, e.g., germacranolides, guaianolides, pseudoguaianolides, and eudesmanolides (de Kraker et al. 1998). SLs have been found in different forms based on the presence of epoxide ring, hydroxy, glycoside, sulfur, halogen, and C_5 acid (tiglic acid or angelic acid) in the modified sesquiterpene (Yuuya et al. 1999) (Fig. 2).

The structural diversity and biological activities of SLs including antibacterial, antifungal, antimalarial, and antiinflammatory properties have been well summarized (Picman 1986; Chaturvedi 2011). Most of pharmaceutically active SLs are produced in low yields in nature, e.g., artemisinin, antimalarial drug from *Artemisia anuua* (Liu et al. 2011a), parthenolide, an anticancer and migraine prophylaxis from *Tanacetum parthenium* (Lesiak et al. 2010; Majdi et al. 2010), *O*,*O*-diacetylbritannilactone, antiapoptotic from *Inula britannica* (Rafi et al. 2005), and helenalin, an anti-inflammatory compound from *Arnica montana* flower heads (Lyss et al. 1997).

Elucidation and metabolic engineering of sesquiterpene lactone biosynthetic pathway

Recent studies mainly have focused on the elucidation of the whole biosynthetic pathway genes of two important sesquiterpene lactones including artemisinin and parthenolide in *A. annua* and *T. parthenium*, respectively. The most widely used sesquiterpene lactone drug is artemisinin (Fig. 3), isolated from *A. annua*, which has been used for malaria therapy in China for more than 1000 years. The antimalarial property of artemisinin has been attributed to its peroxide bridge (Cui and Su 2009). Also, parthenolide raised high attention mainly due to its anticancer and antimigraine properties (Ghantous et al. 2013).

Biosynthesis of SLs in fermentable organisms such as yeast (*Saccharomyces cerevisiae*) and bacteria (*Escherichia coli*) could be a solution to overcome production limitations which exist in this way like the low abundance in native plants and the risks for plant extinction due to overharvesting of the plant species containing these kinds of metabolites. Although total chemical synthesis or semi-synthesis from intermediate precursors both is being used to produce pharmacologically active terpenoids, low yields and high production costs have hindered these strategies as an efficient method. The development of production processes to achieve adequate, sustainable, and reliable amounts of pharmaceutically active SLs is still a major bottleneck for their upscaling and commercial production. Metabolic engineering can be used to introduce or optimize the new pathway(s). During this process, genes from different organisms will be expressed in heterologous system(s) to reconstruct the desired metabolic pathway for production of target compound(s). Pathway reconstitutions for sesquiterpene lactone biosynthesis in microorganism and plants have been successfully carried out; however, additional optimization is still required for achieving the optimal yield. In addition, the generations of new drugs or SL bioconversions in order to improve their effectiveness and reduce their toxicities are feasible through the reconstruction of the biosynthetic pathways in different expression systems (Knaeblein 2004, 2005; Liu et al. 2011b; Liu et al. 2014; Withers and Keasling 2007). However, there are some limitations for plant



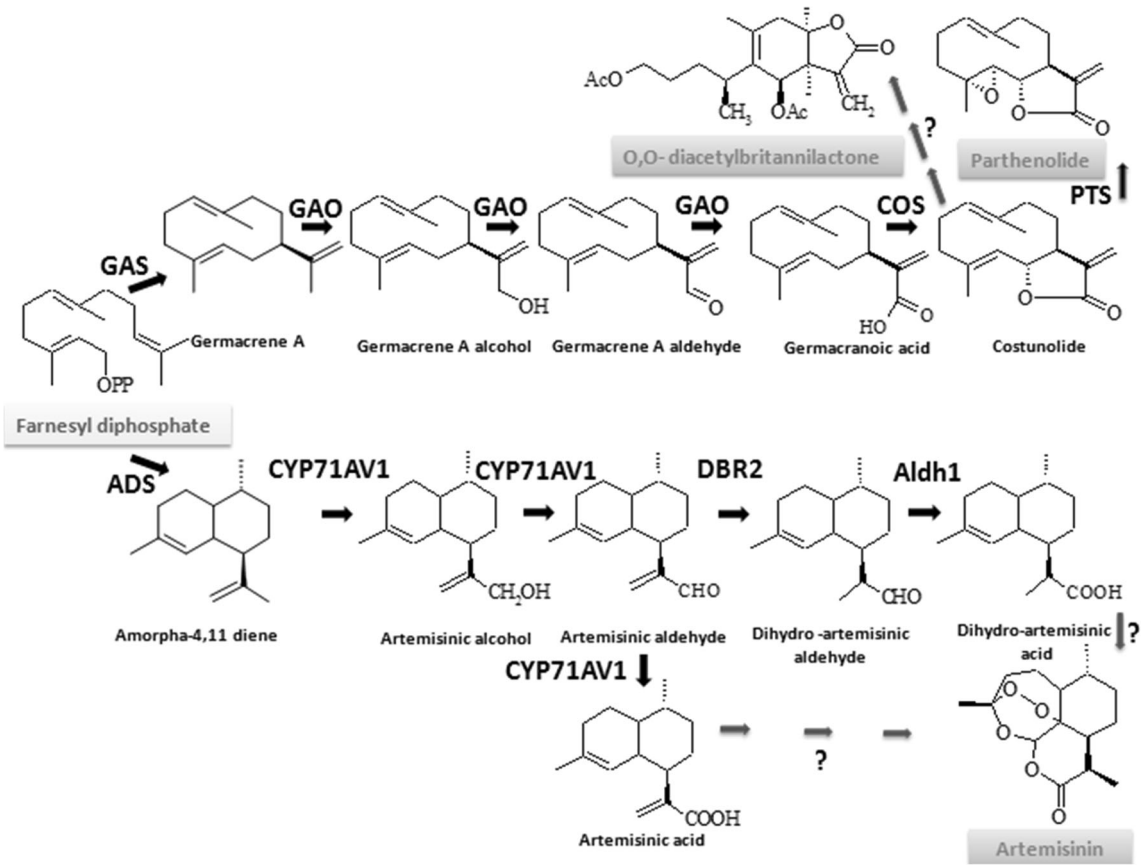


Fig. 2 Structurally diverse sesquiterpene lactones (adapted from Chaturvedi 2011)

Fig. 3 Biosynthetic pathway from farnesyl diphosphate (FDP) toward different sesquiterpene lactones, artemisinin, parthenolide, and *O*,*O*-diacetylbritannilactone. Genes relevant for the biosynthesis of each compound have been indicated: *GAS* germacrene A synthase, *GAO* germacrene A oxidase, *COS* cotunolide synthase, *PTS* parthenolide synthase, *ADS* amorpha 4,11-dine synthase, *CYP71AV1* amorpha-4,11-diene monooxygenase, *Aldh1* aldehyde dehydrogenase, *DBR2* artemisinic aldehyde double-bond reductase. Question symbols shows unidentified genes

metabolic engineering of SLs due to several predicaments as follows: (a) The biosynthetic pathway and its regulation is quite complex, (b) SLs are mostly biosynthesized in non-model plant species with relatively weak genetic information, (c) native plants containing SLs are often recalcitrant to genetic manipulation. Combined genetical, biochemical, and biophysical studies are required to uncover the mechanism by which SLs are accumulated. This includes an understanding of biosynthesis, biodegradation, transport, and localization of SLs. Such studies can be extended to provide novel mechanisms of action of SLs on human health.

Metabolic engineering of SLs: opportunities and bottlenecks

Several review papers have previously described the metabolic engineering of isoprenoids in plants/microorganisms, however they mainly have considered the general metabolic engineering strategies for broad ranges of terpenes including monoterpene, sesquiterpenes, and diterpenes (Kirby and Keasling 2009; Lange and Ahkami 2013; Vickers et al. 2014) or just emphasized on artemisinin as an important antimalarial sesquiterpene lactone in *A. annua* (Farhi et al. 2013; Tang et al. 2014). In most published papers in this issue, the classical metabolic engineering strategies such as overexpression of key genes or downregulation of competing steps have been discussed. It should be considered that in spite of the presence of common metabolic engineering strategies to enhance the universal precursor(s) supply/availability for different terpene backbones, the successful strategy for SL production is more likely different from that already existing for other terpenes due to the several factors that influence SL production, e.g., the nature of target sesquiterpene lactone, plant species, presence of different key genes in the biosynthetic pathway, variability of precursors, different regulatory network mechanisms, transportation, localization, degradation/bioconversion, etc. As mentioned earlier, such clear differences between different terpenes show that the metabolic engineering strategies for SLs need to be separately described in detail. Nevertheless, less discussion still specifically allocated to SLs in the currently available literatures on terpene engineering.

Here, we will review the recent advances in the metabolic engineering and gene discovery of SLs with the main focus on two recently fully elucidated pathways for parthenolide and artemisinin in *T. parthenium* and *A. annua*, respectively. The similarities and differences between these two well-studied SL pathways might pave the way toward more efficient metabolic engineering methods for other SLs as well. Limited examples of SL engineering in other plant species, e.g., chicory (*Cichorium intybus*) and artichoke (*Cynara cardunculus*), are presented as well to improve our understanding of SL engineering. Also, a perspective of the way where SL engineering might pass in the near future will be described based on the new information and advanced technologies. Recent advances in the identification of genes involved in the biosynthesis of SLs with the help of high-throughput sequencing and heterologous expression systems have made metabolic engineering more feasible and promising perspectives that it will be discussed here. Also, quite new points which might have been ignored or not well-intentioned in metabolic engineering strategies so far will be discussed based on the recent successful studies such as the metabolic engineering for increasing the site of biosynthesis (enhancing storage capacity), chloroplast engineering, polycistronic systems, non-native organelle gene expression, transcription factor-based engineering, biotransformation, multifunctional CYP450 expression, the transgene source for metabolice engineering due to the different enzyme activities (from plant species/chemotypes), transient expression approach, improvement of microbial strains, integration of transgene into yeast genome, etc. New aspects will also be explored based on the exclusive bottlenecks that exist for SL production such as further metabolizing precursor/target SL by endogenous enzymes in heterologous hosts or by culture medium, negative effect of elicitors on biomass of hairy roots in tissue culture strategy, or differential plant species responses to the similar metabolic engineering strategy that all has made typical metabolic engineering strategy for SL production challenging and enforced researchers to try out several analytical tools to survey of metabolic changes occurring after genetic manipulation of interested organism(s). In this review, we try to address the metabolic engineering opportunities/bottlenecks that researchers may encounter in SL production as there are still gray areas in this topic that are still needed to be overcome.

Genetically engineered plant cell and organ cultures

Plant cells have been used successfully as "factories" for large-scale production of high-value secondary metabolites. *Agrobacterium rhizogenes* are soil-born bacterial pathogens of plants, and the plasmid DNA from these bacteria can integrate into the genomes of plant cells. The Ri plasmid of *A. rhizogenes* induces rhizogenesis in numerous roots. Such roots can be cultured and stably maintain the biosynthetic characteristics of the original plant. Hairy roots are genetically stable and often show equivalent or better biosynthetic potential for secondary metabolite production compared with parent plants. Genetically modified plant tissue culture, e.g., hairy root cultures, is an interesting approach to overexpress genes encoding rate-limiting enzymes in the biosynthetic pathway of SLs (Khan et al. 2009). The production of SLs can be enhanced by treating the differentiated or undifferentiated cells with elicitors such as methyl jasmonate and salicylic acid (Majdi et al. 2015). For example, treatment of the suspension

culture of *A. annua* with (2,6-di-*O*-methyl)-β-cyclodextrin (DIMEB) and methyl jasmonate showed about 300-fold increase of artemisinin production compared with untreated suspensions (Durante et al. 2011); also, these elicitors both enhanced production of parthenolide in *T. parthenium* (Majdi et al. 2015); hence, elicitation is an effective strategy to elevate SL production.

Hairy roots can trigger SL production in the roots of plant species which do not normally produce significant amounts of SLs in their roots. Some SLs accumulate only in the aerial parts of plants especially in glandular trichomes secretory organs, e.g., artemisinin in *A. annua* and parthenolide in *T. parthenium*, in spite of this, hairy root cultures have been shown to accumulate SLs in some cases as well. For example, artemisinin and parthenolide both normally only accumulate in the aerial parts—where bear glandular trichomes—of *A. annua* and *T. parthenium* respectively, but it has been shown that hairy roots of *A. annua* can be successfully used to produce artemisinin, while this strategy did not respond to parthenolide production in hairy root culture of *T. parthenium* (Liu et al. 1997; Stojakowska and Kisiel 1997; Jaziri et al. 1995). The latter shows that the response might differ from one plant species to another. Thus, for the time being, this strategy could not be used for production of all SLs and should be tested empirically in different plant species. A shackle in this way is that some compounds which promote hairy root growth make negative effects on SL production. For example, the growth of the roots in *A. annua* was enhanced by low concentrations of naphthylacetic acid while inhibited artemisinin production (Liu et al. 1997). SL production can be enhanced in the roots of species which normally produce particular SLs using different elicitors and also by the optimization of growth conditions. The latter strategy has been used successfully for 8-deoxylactucin production in chicory roots (Malarz et al. 2007). The SLs produced by hairy roots are often secreted into the culture medium, so that it can be extracted from medium using appropriate solvents. Secretion of SL into medium will reduce the risk of feedback regulation which is normally caused by their accumulation. Such systems can be scaled up for industrial production of SLs, for example, artemisinin production in transformed hairy roots of *A. annua* (Souret et al. 2003; Cai et al. 1995).

Metabolic engineering strategies in plants

Elucidation and reconstitution of SL biosynthetic pathway: the key role of CYP450s

Reconstitution of the downstream part of the biosynthetic pathway of target SL in plant tissues with high biomass yield is an attractive approach. This is particularly true if the host plant shares a significant part of the biosynthetic pathway of interest compound. For instance, costunolide is the immediate precursor of parthenolide and the pathway to costunolide is highly active in chicory roots (de Kraker et al. 1998, 2002) (Fig. 3). Identification of costunolide epoxidase (parthenolide synthase) which is responsible for the conversion of costunolide to parthenolide from other plants producing high amount of parthenolide such as feverfew and its overexpression in hairy roots of chicory would be an efficient strategy to produce parthenolide in large scale for commercialization. On the other hand, genetic engineering allows reassembling of a biosynthetic pathway or a part of it into hosts which do not have the relevant specialized pathways. A prerequisite for the engineering of the production of SLs is a deep understanding of their biosynthesis at the molecular level. For most of SLs, the enzymes involved in the early steps have been fully characterized, while the late biosynthetic steps generally need to be elucidated except of parthenolide and aretemisinin in *T. parthenium* and *A. annua*, respectively. There has been some recent progress on the identification of genes responsible for the late steps of some SLs (Teoh et al. 2006; Nguyen et al. 2010; Liu et al. 2011b, Ikezawa et al. 2011; Cankar et al. 2011; Liu et al. 2014) (Fig. 3). Recently, with the help of heterologous expression in yeast and in vitro enzyme assay experiments, multifunctional and sequential oxidation activities of cytochrome P450s have been discovered at the late steps. Such multifunctional activity of cytochrome P450s is not inconsistent with the hypothesis of the presence of independent steps by several distinct enzymes, for example, in *A. annua*, an amorphadiene oxidase (AMO or CYP71AV1) catalyzes three-step oxidations of amorphadiene to artemisinic acid (Fig. 3) (Ro et al. 2006; Teoh et al. 2006). Another cytochrome P450 monooxgenase, germacrene A oxidase catalyzes a three-step oxidation of the hydrocarbon germacrene A to germacra-1(10),4,11(13)-trien-12-oic acid (germacrenoic acid) in *Lactuca sativa* (Nguyen et al. 2010), *C. intybus* (Cankar et al. 2011), and *T. parthenium* (Liu et al. 2014). Also, in the lovastatin biosynthetic pathway in *Aspergillus terreus*, lovA, a cytochrome P450 monoxgenase gene has been found to catalyze a double-bond formation and double hydroxylation reactions to convert dihydromonacolin L acid to monacolin L acid, followed by the regio- and stereo-specific hydroxylation of monacolin L acid to yield monacolin J acid (Barriuso et al. 2011). A valencene oxidase gene (CYP71AV8) from chicory also does germacrene A oxidase activity as well (Cankar et al. 2011). Recently, the germacrene A oxidase from *C. cardunculus* converted the non-natural substrates including amorphadiene, cascarilladiene, and (+)-germacrene D to their oxidized products, but the oxidation activity was less than its native substrate, germacrene A (Eljounaidi et al. 2014). Results obtained from

heterologous expression of several germacrene A oxidases (cytochrome P450 monoxgenases) from different species (belong to the Asteraceae) in yeast showed their high conserved activity and sequence similarity (Nguyen et al. 2010). The gene responsible for conversion of germacrenoic acid to costunolide named costunolide synthase has been isolated and characterized from chicory (CYP71BL3) (Liu et al. 2011b; Ikezawa et al. 2011), lettuce (CYP71BL2), and feverfew (Liu et al. 2014). Also, recently, several CYP450s have been characterized in *T. parthenium*, which have parthenolide synthase (costunolide epoxidase) (CYP71CA1), parthenolide hydroxylase, and costunolide hydroxylase activities (CYP71CB1) (Liu et al. 2014). Bi-functional activity of CYP71CB1 in the latter case is interesting which can hydroxylate both costunolide and parthenolide. All these genes belong to the CYP71 group of cytochrome P450s which are mostly available in David Nelson's cytochrome P450 homepage (http://drnelson.uthsc.edu/cytochromeP450.html) (Fig. 3).

Boosting the precursor's supply: overexpression of key genes and suppression of competing step(s)

To improve SL production, most of the studies have been focused on the elevation of upstream precursors of SLs, usually to boost the FDP availability as penultimate precursor of sesquiterpenes. The latter has achieved by overexpression of genes affecting the rate-limiting steps of the MVA pathway which consequently increased plant SLs. For a specific pathway which competes with another pathway for a common precursor, an accelerated conversion of a common precursor to desire SL can minimize the conversion of this precursor to competing compound(s). This strategy could be considered as a classic genetic engineering strategy. Overexpression of a rate-limiting gene which boosts the first committed step in SL pathway can be considered for efficient increase of SL production in native plant. A number of studies have been conducted in a variety of plants where key genes of the MVA pathway have been manipulated, e.g., 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGR*), farnesyl diphosphate synthase (*FDS*), and sesquiterpene synthase, such as amorpha-4, 11-diene synthase (*ADS*) (Alam and Abdin 2011; Liu et al. 2011a). Along with this, downregulation or antisense suppression of competing intermediates in the branch point of target SLs could increase their production as well. For instance, the transformation of *A. annua* with a cotton farnesyl diphosphate synthase (FDP) via *A. tumefaciens* or *A. rhizogenes* resulted in a 2- to 3-fold increase in artemisinin production (Chen et al. 1999; Chen et al. 2000). It has been documented that amorpha-4,11-diene, dihydroartemisinic acid, dihydroartemisinic aldehyde, and artemisinic acid are upstream precursors of artemisinin in *A. annua* (Bouwmeester et al. 1999; Teoh et al. 2006; Wallaart et al. 1999a, b) (Fig. 3). The conversion of dihydroartemisinic acid to artemisinin in *A. annua* is probably non-enzymatic, which requires light and molecular oxygen (Covello 2008). Heterologous expression of genes involved in artemisinin biosynthesis in *A. annua* has been carried out in tobacco; co-expression of amorphadiene synthase (ADS), amorpha-4,11-diene oxidase (CYP71AV1), and artemisinic aldehyde Δ11(13) double-bond reductase (DBR2) led to the accumulation of artemisinic alcohol and dihydroartemisinic alcohol but not artemisinic acid, so it has been stated that the cellular environment of the transgenic tobacco may favor reduction of intermediates to alcohols rather than oxidation into acids (Zhang et al. 2011).

Also, introduction of DNA construct for antisense suppression of squalene synthase (an enzyme which competes with ADS for FDP) via *Agrobacterium*-mediated transformation led to significant increase in artemisinin content in some transgenic lines of *A. annua* (Wang et al. 2012). In another study, suppression of β-caryophyllene synthase (a sesquiterpene synthase competing for FDP) using antisense technology led to about 55 % elevation in artemisinin production in transgenic lines compared to non-transgenic lines of *A. annua*, showing that the inhibition of competing step for SL biosynthesis could be considered as an alternative way to increase SL content along with overexpression of key genes (Chen et al. 2011). However, it should be borne in mind that silencing of competing steps might cause unwanted effects on plant physiology and growth. It should be considered as well that rate-limiting enzymes from different sources may considerably vary in their catalytic activity and effectiveness; this part has not been well considered in plant metabolic engineering strategies

In another work, reconstruction of artemisinin biosynthetic pathway in tobacco with some technical improvement led to successful production of artemisinin; this was achieved by generation of large recombinant plasmid carrying gene encoding cytochrome P450 reductase (CPR), ADS, CYP71AV1, and truncated HMGR (tHMGR) under the control of CaMV 35S or Rubisco promoters. To elevate artemisinin production, ADS was targeted to mitochondria (mtADS) using a COX4 transit peptide sequence. The success of this approach might be due to several factors: first co-expression of CPR with CYP71AV1, second the expression of tHMGR for precursor supply, third using single vector, and fourth the application of intense light during tobacco growth to promote the conversion of dihydroartemisinic acid to artemisinin (Farhi et al. 2011). The above improvements are not still enough for developing transgenic plants for large-scale production of artemsinin.

Inhibition of further conversion of target sesquiterpene lactone(s) as a new approach: advantages and disadvantages

Inhibition of further conversion of target SLs (the desire end product) to downstream compound(s) might be a new approach which is further depends on the potential of target SL for additional modifications. Most of studies focused on the elevation of target SL but still less intentioned to their additional conversions that probably happen enzymatically or non-enzymatically. For instance, further conversion of parthenolide to epoxy-parthenolide and hydroxyl-parthenolide in *Anvillea radiata* has been reported (Hassany et al. 2004) (Fig. 4), and this would probably occur in other plant species that accumulate parthenolide. In consistent with this hypothesis, enzymatic hydroxylation of parthenolide to hydroxyl-parthenolide and costunolide to hydroxyl-costunolide by CYP450 (CYP71CB1) has been reported recently which further support the latter hypothesis (Liu et al. 2014). Metabolic strategies that aimed to cease the further modification of desired SL to another compound would be promising. For example, it has been reported that expression of parthenolide hydroxylase (parthenolide metabolizing enzyme) is negatively associated with parthenolide accumulation in *T. parthenium*; hence, metabolic engineering that aimed to downregulate parthenolide hydroxylase might increase parthenolide accumulation (Majdi et al. 2015). Also, inhibition of possible degradation or biodegradation mechanisms of target SLs can lead to less lost and enhance their accumulation. In this case, degradation of lactucin, a major SL from chicory roots (*C. intybus* L.) has been reported by UV irradiation (Frey et al. 2002). Probably, subcellular localization plays key role in SL modifications due to the differential enzyme activities/availabilities or the compartment environment circumstances that might favor specialized changes.

It is noticeable that SL modifications can confer advantages and disadvantages based on clinical aims; at first, it should be elucidate that does such modifications affect biological activity of target SL. If such conjugations occur at functional groups of SL (which confer their pharmaceutical activity), then they might miss their biological activity and obviously this is negative evidence. In another aspect, such modifications might be positive as well to develop new drugs with better water solubility; for example, poor water solubility of SLs limits their potential for oral use and conjugation to other compounds could improve their water solubility. A nice example of this aspect is the use of dimethylamino-parthenolide (DMAPT) instead of parthenolide that exhibit oral bioavailability and retain the biological activity of parthenolide as well (Guzman et al. 2007). Also, relatively polar conjugates of parthenolide including parthenolide cysteine and parthenolide glutathione heterologously produced in tobacco were highly active against colon cancer cells (Liu et al. 2014). Improving the water solubility of parthenolide enhances its potential as a drug in near future.

Spatial organization of pathways and targeting transgene expression to subcellular compartments

Study of the sesquiterpene biosynthesis potential in different subcellular compartments in plants can give insight into their biosynthesis/regulation in plants and could provide useful tool for the elevation of SL production via metabolic engineering. A relatively successful strategy for the engineering of sesquiterpene production in plants has been developed which takes advantage of the compartmentalization. Generally, sesquiterpenes are biosynthesized in the cytosol via the MVA pathway (Fig. 1). Redirection of the MVA pathway into other compartments is becoming a new successful approach for efficient production of sesquiterpenes. This fascinating approach has been accomplished profitably by production of transgenic plants in which their pathway components have been transmitted into the non-cytosolic compartments, e.g., chloroplasts (Wu et al. 2006) or mitochondria (van Herpen et al. 2010; Liu et al. 2011b; Liu et al. 2014). For example, redirection of sesquiterpene biosynthetic pathways from the cytosol to the chloroplast led to 100–10,000-fold higher terpene accumulation (Wu et al. 2006). Targeting of ADS and FDP synthase into the chloroplasts of tobacco resulted in 25,000-fold increase in amorphadiene production compared with expression of ADS in the cytosol, the normal place of sesquiterpenoid biosynthesis (Wu et al. 2006). In *Arabidopsis,* heterologous expression of a strawberry nerolidol synthase (*Fa*NES1), fused to a mitochondrial targeting sequence led to nerolidol biosynthesis, which was not observed for cytosol-targeted nerolidol synthase (Kappers et al. 2005). Mitochondrial targeting of germacrene A synthase from feverfew (Majdi et al. 2011), using a CoxIV mitochondrial targeting sequence in *Nicotiana benthamiana* led to a significant increase in the production of germacrene A compared with the native cytosolic targeting (Liu et al. 2011b). Targeting the patchoulol synthase (a sesquiterpene synthase) together with FDP synthase into the plastids of tobacco dramatically increased the biosynthesized patchoulol up to 100 times compared with its native cytosolic production (Wu et al. 2006). The increase in sesquiterpene production by targeting the biosynthetic pathway or a part of it to non-native compartments such as chloroplasts (Wu et al. 2006) and mitochondria (Kappers et al. 2005; Liu et al. 2011b) is a promising methodology for elevation of sesquiterpenes and consequently SLs. Several hypotheses have been proposed for the success of the organellar targeting strategy for sesquiterpene engineering including precursor's availability (Kappers et al. 2005), the absence of regulatory mechanism(s) controlling carbon flux toward any

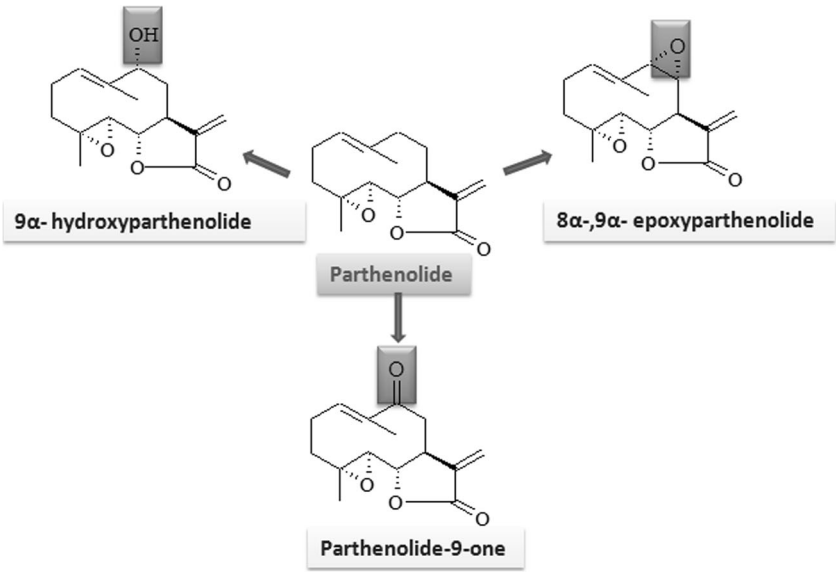


Fig. 4 An example of further modifications of target sesquiterpene lactone by hydroxylation, oxidation, and epoxidation. In this case, parthenolide can convert to its derivatives including hydroxyparthenolide, epoxyparthenolide, and parthenolide-9-one (Hassany et al. 2004)

biosynthetic pathway, and the lack of a competing pathway for precursor(s) expenditure (Wu and Chappell 2008). Some technical improvements have become available for better expression of sesquiterpene synthases in model plants such as *A. thaliana* and *N. benthamiana*, e.g., targeting the sesquiterpene synthases or upstream enzymes to mitochondria or chloroplasts (Kappers et al. 2005; Wu et al. 2006; van Herpen et al. 2010; Liu et al. 2011b; Liu et al. 2014).

Recently, two P450 genes have been isolated and functionally characterized from artichoke (*C. cardunculus*) including CYP71AV9 and CYP71BL5. Co-expression of CYP71AV9 together with GAS led to germacra-1(10),4, 11(13)-trien-12-oic acid in yeast which shows its germacrene A oxidase activity. The co-expression of CYP71BL5 and CYP71AV9 with GAS led to biosynthesis of the free costunolide in yeast and costunolide conjugates in *N. benthamiana*, showing costunolide synthase activity. The substrate specificity of CYP71AV9 was investigated by testing its ability to convert amorpha-4,11-diene,(+)-germacrene D and cascarilladiene to their oxidized products when co-expressed in yeast with the corresponding terpene synthases. In *N. benthamiana* leaves infiltrated with *A. tumefaciens* carrying mitochondrial GAS (mGAS) and cytosolic GAS (cGAS) and chloroplast GAS (chGAS) from *C. cardunculus*, it was observed that cGAS produced 3-fold higher germacrene A compared with mGAS or chGAS. The results of targeting in this study was not consistent with other reports (van Herpen et al. 2010; Liu et al. 2011b), so it has been suggested that the contrary result might be due to the CcGAS properties which may not be compatible with subcellular localization and adapted mostly with cytosolic conditions rather than plastids or mitochondria. It has been reported that CYP450s in some cases can have broad substrate specificity and reacts with a wide range of sesquiterpene backbones. The germacrene A oxidase from *C. cardunculus* was able to convert non-natural substrates including amorphadiene, cascarilladiene, and (+)-germacrene D to their oxidized products, but its oxidation activity was less than its native substrate, germacrene A (Eljounaidi et al. 2014).

It has been proposed that introduction of accumulation mechanisms by engineering of transport systems can be used for secondary metabolite elevation in plants (Yazaki 2005). The different localizations of the target compound and its biosynthetic enzymes indicate that biosynthetic intermediates probably move among organelles or even through cells. In consistent with this gene expression analysis in glandular trichomes of *A. annua* including apical, subapical, and mesophyl cells showed that the expression of three enzymes specific to the artemisinin biosynthetic pathway are active in both apical and subapical cells; hence, these cells are most likely the active site of artemisinin biosynthesis, while artemisinin and/or its precursors are stored in subcuticular cavity (Olsson et al. 2009; Majdi et al. 2011; Olofsson et al. 2012).

Transient expression and the advantages of polycistronic systems

Heterologous expression of genes involved in SL biosynthesis in plants with high biomass and rapid growth characteristics is a good approach for elucidation of biosynthetic pathway or high-yield production. For example, *Nicotiana* spp. are being used as a proper host for heterologous production of SLs and discovery of genes involved in their biosynthetic pathway using nuclear, chloroplast, and transient transformation (Kappers et al. 2005; Wu et al. 2006; van Herpen et al. 2010; Liu et al. 2011b; Liu et al. 2014). A recent advance in

metabolic engineering of sesquiterpenes is the use of transient expression in *N. benthamiana* by *A. tumefaciens*. This method is quite rapid and allows to simultaneously express several genes without the need to produce stable transgenic plants via tissue culture and regeneration. Transient expression is easy to carry out, and the required time for post-agroinfiltration assessment is very short compared with stable transformation (van Herpen et al. 2010). Also, recently, it has been reported that the relative expression level of the transgene could be manipulated by changing the relative amount of *A. tumefaciens* strains carrying genes in transient expression, while achieving this aim is problematic in stable transformation (Ting et al. 2013). The latter strategy has been successfully used for heterologous expression of a germacrene A synthase from feverfew (Majdi et al. 2011), a germacrene A oxidase from chicory (Cankar et al. 2011), and costunolide synthase from chicory (Liu et al. 2011b) in *N. benthamiana* leaves and led to the production of two costunolide conjugates including costunolide-glutathione and costunolide-cysteine (Fig. 5). In vivo enzyme assays indicated that costunolide-glutathione and costunolide-conjugate are formed spontaneously. In this experiment, in co-expressing of germacrene A synthase and germacrene A oxidase, we were expecting the production of germacrenoic acid or its derivative compounds, but these compounds were not detected (Liu et al. 2011b). This suggests that probably germacrenoic acid forms various conjugates which were not detectable in our analysis. On the other hand, in the presence of an additional enzyme, the germacrenoic acid is readily converted to costunolide. Such interactions between endogenous metabolites and the final products of ectopically expressed biosynthetic genes is an important aspect of the metabolic engineering of plant metabolites. For example, the conversion of artemisinic acid to its diglucoside form or costunolide to gluthatione-costunolide or cysteine-costunolide forms might be a mechanism by which they can be stored in vacuole to avoid toxicity through a glucoside-specific H+-coupled transporters (Bartholomew et al. 2002) or ATP-binding cassette (ABC) transporters (Rea 1999; Rea 2007). Recently, parthenolide biosynthesis was reconstituted by transient co-expression of all the biosynthetic genes in *N. benthamiana* (Liu et al. 2014). The produced parthenolide was mainly present as cysteine and glutathione conjugates. In *N. benthamiana*, feverfew germacrene A synthase (*TpGAS*), germacrene A oxidase (*TpGAO*), costunolide synthase (*TpCOS*), and four parthenolide synthase candidates that belong to CYP450s (*Tp8878*, *Tp2116*, *Tp4149*, and *Tp9025*) were transiently expressed under the control of the Rubisco (RBC) promoter. TpGAS was targeted to mitochondria using CoxIV mitochondrial targeting sequence based on the fact that mitochondrial targeting of sesquiterpene synthases have improved sesquiterpene production. *N. benthamiana* leaves were co-infiltrated with different combinations of the transformed *A. tumefaciens* strains to reconstitute the parthenolide biosynthetic pathway step by step. After the infiltration with the *A. tumefaciens* strain carrying the RBC/TpGAS, the *N. benthamiana* leaves emitted the germacrene A into headspace. But when leaves were co-infiltrated with two *A. tumefaciens* carrying the *TpGAS* and *TpGAO*, germacrene A levels in the headspace were dramatically reduced by 90 % compared to *TpGAS* alone, proposing that *Tp*GAO can drastically convert germacrene A. An interesting point was that there was no any new product peak(s) in the headspace or in the dichlorom extracts of the infiltrated leaves, showing that the expected product(s) of the TpGAO enzyme (germacra-1(10),4,11(13)-trien-12-ol, germacra-1(10),4,11(13)-trien-12-al, and germacra-1(10),4,11(13)-trien-12-oic acid) might not be stable in planta or further convert to other compound(s) as such response has been reported before for heterologous expressed *GAO* gene from chicory (Liu et al. 2011a). But co-infiltration of *A. tumefaciens* strains carrying the *TpGAS*, *TpGAO*, and *TpCOS* expression produced costunolide together with cysteine and glutathione (GSH) conjugates of costunolide. No parthenolide was detected in leaf extracts transiently expressing *TpGAS*, *TpGAO*, *TpCOS*, and *TpPTS* (*Tp2116*); consequently, it was hypothesized that the availability of parthenolide precursors should be increased. To this aim, AtHMGR was used to boost the flux. About 4-fold increases in costunolide production were observed when *AtHMGR* co-expressed together with *TpGAS*, *TpGAO*, and *TpCOS* compared to that was observed without *AtHMGR* expression (Liu et al. 2014). When *AtHMGR* co-expressed with *TpGAS*, *TpGAO*, *TpCOS*, and *TpPTS* (Tp2116), free parthenolide was detected, showing that boosting of the precursor supply in the pathway plays a key role in SL biosynthesis. These results show that upstream flux barriers should be released to overcome low-yield production of SLs. In order to functionally characterize *Tp8878* (CYP71CB1) in planta, *A. tumefaciens* strains with the parthenolide pathway constructs plus *AtHMGR* were co-infiltrated into *N. benthamiana* along with *Tp8878* expression construct. Results showed that two new compounds were produced including hydroxycostunolide-GSH and hydroxycostunolide-cysteine (Liu et al. 2014). Also, for monoterpene engineering when geraniol ectopically produced in the heterologous host *N. benthamiana*, the volatile geraniol is mostly converted into non-volatile geraniol glycosides/oxidized geraniol glycosides to avoid toxic effects of free geraniol (Dong et al. 2015). This is a good learned lesson to do not just look for expected compound(s) in metabolic engineering experiments; hence, using broad ranges of detection technologies to uncover any other biosynthesized and unexpected compounds is mandatory. In such experiments, the use of untargeted metabolomics is highly recommended instead of targeted metabolomics because plants usually have non-specific modifying enzymes that are able to convert the precursors of SLs/desired SLs to other compounds, e.g.,

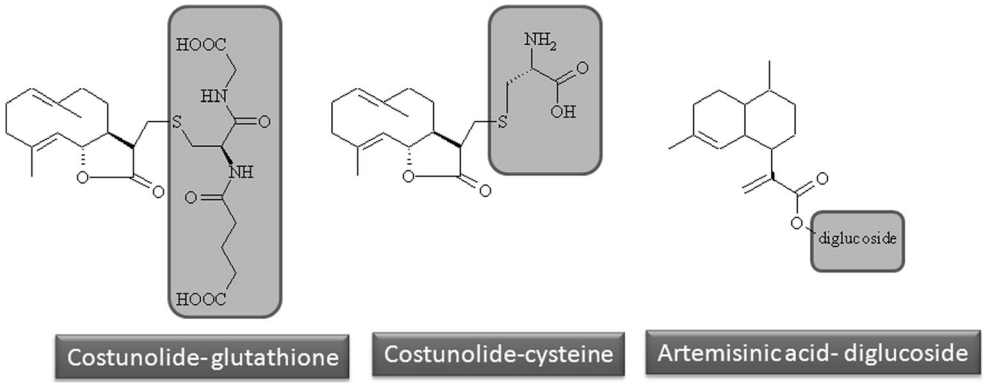


Fig. 5 Spontaneous conjugation of some compounds to target sesquiterpene lactones when their biosynthetic pathways have been expressed in *Nicotiana benthamiana*. Conjugation of glutathione and cysteine to costunolide (Liu et al. 2011b) and diglucoside to artemisinic acid (van Herpen et al. 2010) in transformed *Nicotiana benthamiana.* The *boxes* represent conjugates

dehydrogenases, hydroxylases, glycosyl transferases, and glutathione transferases. To avoid such modifications, directing the gene expression in appropriate spatial and temporal landscapes might be a good solution to overcome this problem, e.g., using glandular trichome-specific promoters for SL engineering (which mainly biosynthesized in such secretory tissues) or expression in specific subcellular compartment (Majdi et al. 2014).

A good example in sesquiterpene lactone biosynthesis in *A. annua* is that two chemotypes exist based on the quantity of artemisinin and its precursors, the low-artemisinin production (LAP) chemotype, and a high-artemisinin production (HAP) chemotype (Wallaart et al. 2000). They both contain artemisinin and arteannuin B, however the HAP chemotype has a relatively high content of artemisinin and dihydroartemisinic acid (DHAA), while the LAP contains high content of arteannuin B and artemisinic acid (AA). Systematic comparison of probable artemisinin biosynthesis genes involved in determining these chemotype (CYP71AV1, DBR2, and aldehyde dehydrogenase (ALDH1)) using transient expression in *N. benthamiana* showed that the enzyme activity of DBR2 and ALDH1 from the two chemotypes did not show a significant difference, while structural differences in CYP71AV1 from LAP (AMOLAP) and HAP (AMOHAP) chemotypes showed a real different enzyme activity. AMOLAP had an extra seven amino acids in N-terminal region compared with AMOHAP. Transient expression of AMOLAP and AMOHAP in *N. benthamiana* revealed that the enzyme activity of AMOLAP was higher than AMOHAP and their co-expression in combination with the other pathway genes lead to qualitatively different product profile (chemotype) (Ting et al. 2013). Agro-infiltrated leaves of *N. benthamiana* with DBR2, ALDH1, and CYP71AV1 genes showed that the chemotype is determined by the function of the CYP71AV1 type and relative dosage of DBR2 and ALDH1 (Ting et al. 2013).

A new approach has been successfully applied to express several consecutive genes using a single promoter based on polycistronic approach. This is achieved by translation of a single open-reading frame with protein-coding sequences separated by viral peptide signal (2A) sequences (Ralley et al. 2004). The 2A peptide leads to ribosomal skipping and consequently production of separate proteins. This strategy has been successfully used to express up to four genes of the glucosinolate pathway (Geu-Flores et al. 2009) or three mitochondrial targeted genes in the biosynthetic pathway of artemisinin (ADS, FDS, tHMGR) from a single promoter in tobacco (van Herpen et al. 2010). In the latter case, amorphadiene synthase fused to FDS and HMGR improved amorphadiene biosynthesis in *N. benthamiana*-infiltrated leaves. In latter case, co-infiltration with CYP71AV1 (a sesquiterpene oxidizing P450 led to almost full conversion of amorphadiene to artemisinic acid-12-β-diglucoside rather than the free artemisinic acid (van Herpen et al. 2010) (Fig. 5). It is also possible to co-express different genes in separate constructs transiently as this strategy successfully has been applied for production of costunolide and parthenolide in *N. banthamiana* (Liu et al. 2011b; Liu et al. 2014).

Increasing the active sites of SL biosynthesis/accumulation

Another new strategy that would be promising in near future is to increase the specific cells where the biosynthesis takes place. In plants, several different specialized biosynthesis/accumulation sites for SLs have been reported, e.g., cavities in *Solidago canadensis* (Curtis and Lersten 1990), ducts in *Ambrosia trifida* (Curtis 1988), laticifers in *L. sativa* (Esau 1965), and glandular trichomes in *A. annua* and *T. parthenium* (Lommen et al. 2006; Majdi et al. 2011). Enhancement of plant potential for SL production could be achieved by increasing the numbers/potential of the active site of biosynthesis/accumulation of SLs, for example, in *A. annua* and *T. parthenium*, glandular trichomes have been shown to

be the main place of biosynthesis/accumulation of artemisinin and parthenolide, respectively (Duke and Paul 1993; Lommen et al. 2006, 2007; Majdi et al. 2011). It is quite logical to assume that metabolic engineering to promote the glandular tichome densities in those plants would elevate corresponding SLs. In this aspect, ectopic formation of glandular trichomes in *Nicotiana tabacum* or *Antirrhinum majus* expressing MYB genes has been reported before (Payne et al. 1999; Perez-Rodriguez et al. 2005), so it should not be far from mind for it to be feasible in other plant species for SL production. In consistent with this hypothesis, more recently up to 5-fold increase in artemisinin content has been observed by metabolic engineering to increase glandular trichomes in *A. annua*. In latter pioneer work in this area, trichome density was increased up to 20 % in leaves and 66 % in flowers of *A. annua* with the expression of β-glucosidase (bgl1) gene, and this strategy resulted in an increase in artemisinin content up to 1.4 % in leaves and 2.56 % in flowers (g DW-1) (Singh et al. 2015). Such elevation in artemisinin content is comparable with the highest yields achieved yet through metabolic engineering. Hence, it is possible to increase artemisinin content or other SLs by manipulating trichomes' density or other biosynthetically active sites as stated above. Of course, this strategy is directly related to the plant species and its main SLs that should be empirically tested in future for favorably.

Chloroplast engineering as a new tool with a bright future for SL production

The expression of foreign genes into plant plastids/chloroplasts offers several advantages compared with their expression through nucleus transformation. Chloroplast engineering is a fascinating approach in plant metabolic engineering which offers more benefits over nuclear genome engineering, e.g., high-level expression of integrated genes—as a consequence of being highly polyploid of plastid genome with more than 500–10,000 genome copies per cell—(Bendich 1987), no need for a signal peptide, and no gene silencing problem and polycistronic expression ability. Chloroplast engineering has been successfully used as a remarkable tool for other isoprenoids, e.g., isoprenoid content of tobacco leaves was changed using this system. Ectopic expression of bacterial genes encoding β-carotene ketolase (CrtW) and β-carotene hydroxylase (CrtZ) in plastid genome of tobacco leaves leads to high amount production of a highly value carotenoid, astaxanthin (Hasunuma et al. 2008a). Cyanobacterial genes encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) in tobacco leads to 350-fold higher DXR activity which increased the levels of special isoprenoids such as chlorophyll a, β-carotene, lutein, solanesol, and β-sitosterol (Hasunuma et al. 2008b). Introduction of lycopene β-cyclase gene from daffodil (*Narcissus pseudonarcissus*) into plastid genome of tomato (*Solanum lycopersicum*) efficiently converts lycopene into provitamin A (β-carotene), in parallel massive elevation in total fruit carotenoid content (Apel and Bock 2009). Recently, a cytoplasmic mevalonate pathway from yeast has been expressed in tobacco chloroplasts (Kumar et al. 2012). In this pioneer work, a multigene strategy has been used to integrate the whole cytosolic MVA pathway (with six genes) into the tobacco chloroplast genome, which leads to higher levels of mevalonate, carotenoids, squalene, sterols, and triacyglycerols than control plants (Kumar et al. 2012). Multigene engineering is possible by translation of polycistrons generated from a single promoter (Quesada-Vargas et al. 2005). Based on these successful examples, it is not far from mind to assume that this methodology can also be applied to produce high levels of SLs. It has been reported that CYP450s P450 are found in the endoplasmic reticulum, where they face the cytosol. There is still no report for sesqiterpene backbone transportation from chloroplast to cytosol (for further conversion by cytochrome P450s); hence, this part remains as a major dilemma for its possibility for SL production. On the other hand, chloroplast engineering is now applicable to only a few plants, e.g., tobacco and lettuce (Verma and Daniell 2007) that has made the strategy development difficult. Consequently, compartmentalization of plant cells exhibits a major congestion for SL production through plant metabolic engineering; hence, this key issue should be genuinely taking into account when defining plans to achieve SLs.

The potential of transcription factor-based strategy

Transcription factors are the key regulators of metabolic networks that can coordinately upregulate multiple steps in certain biosynthetic pathway. Transcription factors (TFs) can be used to modulate plant metabolism without need to fully elucidate the biosynthetic pathway and its rate-limiting steps in a complex metabolic pathway. In addition to pathway flux, TFs can alter the transportation and deposition of metabolites. The use of TFs to upregulate core isoprenoid pathway has not been reported in plants (Lange and Ahkami 2013), but successful examples exist for other secondary metabolites. Elucidation of rate-limiting steps in plants containing relevant SL compounds (which mostly belong to non-model plant species) is actually hard and time-consuming; on the other hand, the optimal level of desired SL can be limited by several consecutive enzymes, in such cases, an alternative methodology might be engineering of the target TF of the pathway instead of the manipulation of structural genes directly involved in the pathway. The potential of transcription factors to enhance desired metabolites have been demonstrated in several experiments, e.g., heterologous expression of the maize transcription factor genes LC (leaf color) and C1 in tomato resulting in high-level flavonol (Bovy et al. 2002), introduction of *Sn* (a maize myc-class gene) results in enhanced phenolic and anthocyanin

accumulation in *Lotus corniculatus* (Robbins et al. 2003), and ectopic expression of specific MYB transcription factors lead to accumulation of anthocyanins in tomato (Butelli et al. 2008). Based on these successful results, overexpression of transcription factors can be developed to elevate the level of biologically active SLs. Two TFs have been characterized for sesquiterpene biosynthesis both belonging to WRKY transcription factors: first *Ga*WRKY1 from cotton (*Gossypium arboreum*), which upregulates the expression of *Cs*AD1-A, (+)-δ-cadinene synthase, thus participating in the regulatory network of biosynthesis of sesquiterpene aldehydes, including gossypol (Xu et al. 2004), and the second one, *Aa*WRKY1 is a trichome-specific TF from *A. annua* which has been shown to be involved in ADS regulation, the first committed step in the biosynthesis of artemisinin (Ma et al. 2009). When *Aa*WRKY1 was ectopically expressed in tobacco, the transcript level of the 5-epi-aristolochene synthase gene (EAS4: a sesquiterpene synthase) upregulated, while it did not affect squalene (a triterpene) synthase gene (SQS) expression (Ma et al. 2009). Recently, two jasmonate-responsive APETALA2/ethylene response factor (AP2/ERF) transcription factors *Aa*ERF1 and *Aa*ERF2 have been found which positively regulate artemisinin biosynthesis in *A. annua* L. by the induction of promoter activities of ADS or CYP71AV1 (Yu et al. 2012). Recently, it has been reported that overexpression of *AaORA* (in which its expression could directly elevate the expression of biosynthetic genes of artemisinin including *ADS*, *CYP71AV1*, and *DBR2)* in *A. annua* led to a 50 and 35 % increase in artemisinin and DHAA content, respectively (Lu et al. 2013). Also, in *Catharanthus roseus*, overexpression of the AP2/ERF transcription factor ORCA3 positively affected the expression of terpene indole-alkaloid biosynthetic genes and led to isoprenoid indole alkaloid increase (van der Fits and Memelink 2000).

Metabolic engineering of microbial systems

Since the yield of SLs in plant is too low and their purification is difficult for commercial uses, genetic manipulation of prokaryotic (*E. coli*) and eukaryotic (*S. cerevisiae*) expression systems as a microbial host for sesquiterpene production has been considered. Chemical synthesis is not feasible in most cases, also classic breeding or genetic engineering is not easy due to unfeasible technological problems, and hence, SL production in microbial platforms such as *S. cerevisiae* or *E. coli* is most likely a good solution and became fascinating approach (Muntendam et al. 2009). Production of secondary metabolites in tightly controlled bioprocesses using engineered microorganisms is emerging as a suit platform for different natural products including SLs. Here, we will briefly review the biosynthesis and bioengineering of SLs in microbial systems and their implications and show the recent progresses/problems in these areas based on the empirical experiments.

Metabolic engineering strategies in yeast

The yeast (*S. cerevisiae*) system offers advantages for SL production compared with *E. coli* or plants: (1) FDP, a common precursor for sesquiterpenes, is natively available in *S. cervisiae*; (2) *S. cervisiae* is a competent microorganism for genetic manipulation and fermentation, while the latter is difficult in plants; (3) *S. cervisiae* is a suit platform for cytochrome P450 enzyme activities due to the presence of endoplasmic reticulum (ER) membrane, while *E. coli* does not bear ER; (4) decreasing the culture times and increasing productivity in *S. cervisiae* fermentation in comparison with plant cell cultures; (5) no or less modification of heterologously expressed SLs compared with plants; and (6) no feedback of end product due to the secretion of SLs into medium has been reported, while negative feedback has been reported in plants. It has been proposed that production of SLs, e.g., artemisinin from microbial systems would be a better strategy compared with classic method (which is based on the extraction from native plants) in terms of cost, quantity, and amenity (Hale et al. 2007). One of the most important factors to overcome in engineering of SL pathways in microbial hosts is to functionally express the enzymes responsible for downstream modification of sesquiterpene backbone such as cytochrome P450s. Cytochrome P450s are able to catalyze a wide range of chemical reactions, e.g., hydroxylation, epoxidation, ring formation, aryl migration, and carbon-carbon bond cleavage, and their role has been described as a useful tool for pharmacology (Morant et al. 2003). Plant P450s are found in endoplasmic reticulum, where they express in concert with an NADPH-dependent CPR (Hamann and Moller 2007). Co-expression of P450s and NADPH-dependent cytochrome P450 reductases can be accomplished by integration of an NADPH-dependent cytochrome P450 reductase from plant resources into yeast genome. This can be expressed into yeast genome permanently such as WAT11 and WAT21 yeast strains which can express the *Arabidopsis* CPR*s*, ATR1 (GenBank ID X66016), and ATR2 (GenBank ID X66017), respectively (Urban et al. 1997). WAT11 has been successfully used for isolation and characterization of chicory costunolide synthase (Liu et al. 2011b) and valencene oxidase gene (CYP71AV8) (Cankar et al. 2011). In another strategy based on episomal expression, one can clone a CPR into yeast expression vector and transform it into yeast, e.g., expression of *A. annua* CPR (Ro et al. 2006). Developing the engineered yeast strains for production of universal intermediates for SLs can serve as a nice host for large-scale production. Recently, *S. cerevisiae* has become a suit platform for SL engineering such as the precursors of artemisinin (artemisinic acid) (Ro et al. 2006) and parthenolide (costunolide) (Ikezawa et al. 2011; Liu et al.

2011b; Liu et al. 2014). Accumulation of intermediates might inhibit the interested pathway by direct or indirect negative feedback on a preceding step; in such cases, efficient conversion to the next step would prevent the feedback regulation/downregulation of the target pathway. Enzymes from different sources may quite differ in their catalytic activity, for example, several sesquiterpene synthases are responsible for converting FDP into various sesquitepene structures; however, some of them are not that much specific to produce just a single sesquiterpene and are able to simultaneously produce several sesquiterpene lactone structure (Pazouki et al. 2015). The perfect sesquiterpene synthase for metabolic engineering purposes is the one that efficiently produces only one sesquiterpene; for instance, it has been reported that germacrene A synthases from different species showed different activity when they were expressed in *S. cervisiae* (Liu et al. 2011b). Heterologous expression of two germacrene A synthase from chicory (Bouwmeester et al. 2002) and a germacrene A synthase from feverfew (GenBank JF819848.1) (Majdi et al. 2011) in *S. cerevisiae* showed a significant difference in the efficiency of germacrene A production. The germacrene A produced by feverfew germacrene A synthase was 2.5-fold higher than of germacrene A synthases from chicory (GenBank AF498000.1; GenBank AF497999.1) when they were expressed in yeast (Liu et al. 2011b). This increase in germacrene A production is comparable with 2–3-fold increase in sesquiterpene hydrocarbon accumulation by knockout/downregulation of *ERG9*, a gene encoding squalene synthase—which is the first committed step in competing sterol pathway—(Takahashi et al. 2007; Ro et al. 2006). However, it is less than 5-fold increase compared with overexpression of truncated *HMGR*—a rate-limiting step which irreversibly fluxing carbon to the MVA pathway—(Ro et al. 2006). This promises a new metabolic engineering strategy based on structural gene selection from different origins or protein engineering to enhance the enzyme activity which would improve beneficial in terms of yeast strain improvement for production of SLs.

To date, most of the yeast metabolic engineering strategies rely on the overexpression of key genes, e.g., *HMG1*, or downregulation of competing steps, e.g., *ERG9*, to increase the FDP availability for target sesquiterpene production (Jackson et al. 2003; Ro et al. 2006; Takahashi et al. 2007; Paradise et al. 2008; Asadollahi et al. 2010). FDP is a branch point of the MVA pathway where several enzymes use it as a precursor for synthesis of various compounds in cells. It has been recognized that FDP is a feedback regulatory agent for its synthesis (Dimster-Denk et al. 1999); hence, higher rate of FDP conversion to target sesquiterpene using efficient sesquiterpene enzyme (along with other strategies to enhance FDP supply) would be a promising strategy for redirecting FDP to the target sesquiterpene compound(s). The recent strategy would allow activation of carbon flux to FDP synthesis and inhibition of FDP negative feedback on its pathway. In consistent with the latter, it has been reported that FDP is a positive signal for degradation of HMGR, a key enzyme in the early step of MVA pathway (Gardner and Hampton 1999).

Optimization of fermentation process is a key factor both in gene discovery and production of desired SLs which should be seriously taken into account. For example, co-expression of lettuce germacrene A oxidase with germacrene A synthase in yeast produced side products such as costic acids and ilicic acid in the acidic condition, while fermentation in a neutral condition led to only germacrenoic acid production, raising this clue that germacrenoic acid is unstable in the acidic condition (Nguyen et al. 2010). Various buffers can be used for neutralization of yeast culture to avoid side products, e.g., 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 3-(*N*-morpholino)propanesulfonic acid (MOPS). An interesting observation is the effect of buffer type on target SL production; in this aspect, our experiment aimed to produce costunolide in yeast by co-expression of germacrene A synthase, germacrene A oxidase, and costunolide synthase showed that the transformed yeast in MOPS buffer (pH 7.5) produced higher cosunolide (3-fold) compared with that cultured in HEPES buffer (pH 7.5) (Liu et al. 2011b), so probably there is interaction between the buffer and intermediate(s)/final product(s) in the culture medium. Oxygen availability has been mentioned as a key factor in SL production as well, for example, co-expression of amorpha-4,11-diene synthase and CYP71AV1 in yeast under restrictive oxygen conditions leads to dihydroartemisinic acid production instead of artemisinic acid (Rydén 2010). In routine, microbial platforms used for SL engineering as *E. coli* do not contain P450s, while *S. cerevisia* does; so, yeast would be a preferable host for P450 expression and consequently SL production. Codon optimization could be used for expression of plant sesquiterpene genes in microbial host as it has been used for production of Taxol (a diterpene) in yeast or other terpenoids in *E. coli* (Martin et al. 2003; Engels et al. 2008; Yin et al. 2015). It has been reported that codon bias is a key factor which influences plant P450 expression in *E. coli*; this is due to the higher codon bias in *E. coli* compared with yeast (Batard et al. 2000). Relatively, the functional expression of cytochrome P450 enzymes in yeast is a really great advantage over *E. coli* for SL production.

Metabolic engineering strategies in *E. coli*

Beside yeast manipulation, an alternative way for SL production instead of is biosynthetic reconstruction in *E. coli*. This can lead to the production of target SLs or its precursor(s) compound(s); in the latter case, it can be converted to target SL with minor feasible chemical changes. *E. coli* has been employed as a good system for characterization of several terpene synthases, beside this pathway reconstruction in *E. coli* opened an applicable gate

for economical or industrial production of sesquiterpenes and probably SLs in near future. Introduction of the MVA pathway of *S. cerevisiae* into *E. coli* led to terpenoid precursor production (Martin et al. 2003). To increase the *E. coli* strain potential for generation of intermediate compounds and to avoid the toxicity of accumulated precursors (which can inhibit the growth and flux via pathway), some technical improvements have been envisaged, e.g., using a truncated HMGR, codon optimization for functional expression in *E. coli*, and application of stronger promoter (Pitera et al. 2007; Gralla et al. 1980). The downstream branch-point enzymes such as sesquiterpene synthases or cytochrome P450s would subsequently add to generate target SL. A significant progress in *E. coli* manipulation for sesquiterpene production was production of amorphadiene by heterologous expression of the mevalonate or DXP pathway plus ADS (Martin et al. 2003). Recent advances in high-level production of amorpha-4,11-diene in *E. coli* have proved the performance of this system for large-scale production of sesquiterpene/SLs using two-phase partitioning bioreactor (Newman et al. 2006). *E. coli* strain engineering and fermentation process optimization has been successfully used (Tsuruta et al. 2009). Replacing *HMGS*, HMG-CoA synthase (ERG13, GenBank ID 854913) and *tHMGR*, truncated version of HMG-CoA reductase (HMG1, GenBank ID 854900) from yeast with their ortholog genes from *Staphylococcus aureus*, resulted in double amorpha-4,11-diene production, provided an alternative source of artemisinin (Tsuruta et al. 2009). The functional expression of cytochrome P450s in *E. coli* remained a big challenge. Protein engineering based on the construction of fusion proteins might be an alternative strategy for expression of P450s (which associated with the endoplasmic reticulum) in *E. coli*. The engineering of special characteristics in cytochrome P450s such as membrane binding, applying alternative reducing sources or fusion proteins, have been shown to be a proper solution (Miles et al. 2000). For example, two mammalian cytochrome P450s have been successfully expressed in *E. coli*, the active P450 fusion proteins containing the flavoprotein domain of rat NADPH-cytochrome P450 reductase (Fisher et al. 1992). Recently, up to 100 mg l^{-1} of costunolide has been produced in *E. coli* by expression of three genes (*GAS*, *GAO*, *COS*) involved in the biosynthetic pathway of costunolide and eight genes involved in converting acetyl-CoA into FDP in the MVA pathway (Yin et al. 2015), the latter achieved by screening and optimization of cultured medium and inducing temperature. Results of the latest study showed that application of glucose as carbon source and yeast extract elevated both cell growth and costunolide production in *E. coli* which indicates that high-cell density cultivation may be feasible for production of costunolide at an industrial scale (Yin et al. 2015); hence, these items should take in account for SL production via *E. coli* culture.

Addition of precursors and biotransformation

Some cultures are able to transform cheap precursors into costlier chemicals. Biotransformation of secondary metabolites such as terpenoids by microorganisms, mammals, or algae for adding the functional groups have been reported (Furusawa et al. 2005). It is noticeable that inactive compounds might convert to active form by microbial transformation and vice versa (Musharraf et al. 2010). Many microorganisms are able to convert terpenoid region-selectively and stereo-selectively which is most likely due to the action of cytochrome P450s or dioxygenases (Martin et al. 2008; Krings et al. 2009; Spakowicz and Strobel 2015). Beside this, for the time being, some problems still exist which are associated with the structure of SLs, e.g., poor solubility in water and absorption which makes their oral use so difficult. Such problems motivated scientist to find a new SL derivative, for example, using DMAPT instead of parthenolide (Guzman et al. 2007). These kinds of modifications can be expected by using microorganisms for SL transformation to achieve a variety of new useful drugs.

Biotransformation of sesquiterpenoids such as dehydrocostuslactone, costunolide, α-, β-, and γ-cyclocostunolides, α-santonin, and atractylon from crude drugs and (−)-ambrox by microorganisms such as *Aspergillus niger* has been successfully conducted (Hashimoto et al. 2001). Artemisinin converted to four different hydroxylated derivatives by transformation with fungus *Cunninghamella elegans*, among which 7β-hydroxyartemisinin cannot be synthesized chemically (Parshikov et al. 2004, 2012), while *Cunninghamella echinulata* and *A. niger* transformed artemisinin to 6β-hydroxyartemisinin and 4α-hydroxydeoxyartemisinin, respectively (Zhan et al. 2002). Hence, biotransformation could be a proper strategy to produce those SLs which cannot be chemically synthesized. To develop a more potent and less neurotoxic agents for the oral use of artemisinin comparative molecular field analysis (CoMFA) and hologram QSAR (HQSAR) carried out by artemisinin analogues with known in vitro antimalarial activity. Quantitative structure activity relationships (QSARs) suggested some modifications which seem to promote antimalarial activity (Avery et al. 2002).

Although biotransformation strategy has been poorly exploited on SLs, it can be applied for efficient production of SLs with low cost (even for the biosynthesis of new drugs with less toxicities and side effects to human health). Further region-specific and stereo-specific hydroxylation of SLs might promote their water solubility or generate the new

modification sites for further conversion to new drugs. Reconstitution of parthenolide biosynthetic pathway in *N. benthamiana* led to the production of cysteine and glutathione conjugates of parthenolide in which these conjugates affect water solubility of parthenolide. Biological assay showed that parthenolide conjugates were less effective than free parthenolide but still had considerable anticancer activity, especially against colon cancer cells (Liu et al. 2014). Based on this issue that it is feasible to improve the water solubility of SLs, the production of water-soluble conjugates of SLs through metabolic engineering of heterologous hosts might be a good solution to develop SLs as anticancer drugs.

Challenges

Several studies have showed the potential of genetic engineering for the improvement of SL production. However, there are genetic barriers on growth and development of gentically modified plants, and still some challenges exist to achieve efficient production of the desired sesquiterpene. For example, leaf chlorosis, reduced stature, and vein clearing were observed in transgenic tobacco when patchoulol and FDP synthase ectopically expressed in plastids (Wu et al. 2006). Such abnormal growth of engineered plants might be due to the depletion of terpene precursors, e.g., GDP and FDP which are essential for production of the metabolites' needs for normal plant growth; also, the probable toxic effects of transgenes are indispensable. A key point is that for a successful metabolic engineering in plants, several issues should be considered. It is important to produce sufficient content of the desired SL; however, this will be mainly determined by the biochemical nature of the host plant which for the time being is not well known for most of the plant species. For example, some newly synthesized sesquiterpenes are being converted by endogenous enzymes that normally exist in host plants. These enzymes have broad substrate specificity, such as hydroxylases, dehydrogenases, and glucosyltransferases. Metabolic engineering strategies to increase SL production may be affected by unpredicted metabolic consequences such as further modification of the desired SL or its negative effects on plant growth and development. Based on endeavors toward terpene engineering, it has been denoted that metabolic engineering of sesquiterpenes is more challenging and pretty complicated. Sesquiterpene engineering is not as easy as the engineering of other terpenes, for example, monoterpenes are biosynthesized exclusively through the methylerythritol phosphate (MEP) pathway in the plastids while both the cytosolic MVA and plastidic MEP pathways are most likely involved in sesquiterpene biosynthesis. The precursor trafficking of terpenes between organelles depends on the plant species, tissue, and physiological state of the plant (Steliopoulos et al. 2002; Dudareva et al. 2005). Furthermore, a larger quantity of FDP is usually being used for sterol biosynthesis compared with sesquiterpene biosynthesis; thus, it might be less available for sesquiterpene synthases.

Conclusions

SL compounds are highly valuable and play a key role in human health due to their pharmaceutical properties, e.g., artemisinin as a potent antimalarial drug in *A. annua* and parthenolide as an anticancer compound in *T. parthenium*. However, the concentrations of SLs in native plants are usually quite low, which makes them difficult for economical extraction. In recent years, an increasing number of attempts have been carried out to uncover SL biosynthetic pathways or their regulatory mechanism including biosynthesis, transportation, and accumulation. At the time being, some knowledge is available about the SL biosynthesis in plants/microorganisms and some attempts have been conducted for their heterologous expression in microbial or plants platforms, nevertheless further elucidation of their biosynthetic pathway and regulatory mechanisms is needed to develop the high-yield production platforms. Based on recent improvement in the elucidation of SL biosynthetic pathways and some technical improvements for transformation of related genes, a sustainable plant/microbe-based platforms for SL production would be feasible in near future.

Acknowledgments This work was financially supported by the Iran National Science Foundation [Grant No. 90007936] and the University of Kurdistan.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Alam P, Abdin M (2011) Over-expression of HMG-CoA reductase and amorpha-4,11-diene synthase genes in *Artemisia annua* L. and its influence on artemisinin content. Plant Cell Rep 30:1919–1928

Apel W, Bock R (2009) Enhancement of carotenoid biosynthesis in transplastomic tomatoes by induced lycopene-to-provitamin A conversion. Plant Physiol 151:59–66

Asadollahi MA, Maury J, Schalk M, Clark A, Nielsen J (2010) Enhancement of farnesyl diphosphate pool as direct precursor of sesquiterpenes through metabolic engineering of the mevalonate pathway in *Saccharomyces cerevisiae*. Biotechnol Bioeng 106:86–96

Avery MA, Alvim-Gaston M, Rodrigues CR, Barreiro EJ, Cohen FE, Sabnis YA, Woolfrey JR (2002) Structure-activity relationships of the antimalarial agent artemisinin. 6. The development of predictive

in vitro potency models using CoMFA and HQSAR methodologies. J Med Chem 45:292–303

Back K, Chappell J (1995) Cloning and bacterial expression of a sesquiterpene cyclase from *Hyoscyamus muticus* and its molecular comparison to related terpene cyclases. J Biol Chem 270:7375–7381

Barriuso J, Nguyen DT, Li JW, Roberts JN, Mac Nevin G, Chaytor JL, Marcus SL, Vederas JC, Ro DK (2011) Double oxidation of the cyclic nonaketide dihydromonacolin L to monacolin J by a single cytochrome P450 monooxygenase, LovA. J Am Chem Soc 133: 8078–8081

Bartholomew DM, Van Dyk DE, Lau SMC, O'Keefe DP, Rea PA, Viitanen PV (2002) Alternate energy-dependent pathways for the vacuolar uptake of glucose and glutathione conjugates. Plant Physiol 130:1562–1572

Batard Y, Hehn A, Nedelkina S, Schalk M, Pallett K, Schallera H, Werck-Reichharta D (2000) Increasing expression of P450 and P450-reductase proteins from monocots in heterologous systems. Arch Biochem Biophys 379:161–169

Bendich AJ (1987) Why do chloroplasts and mitochondria contain so many copies of their genome? Bioessays 6:279–282

Berger RG (2009) Biotechnology of flavours—the next generation. Biotechnol Lett 31:1651–1659

Bouwmeester HJ, Wallaart TE, Janssen MH, Posthumus MA, Schmidt CO, De Kraker JW, König WA, Franssen MC (1999) Amorpha-4, 11-diene synthase catalyses the first probable step in artemisinin biosynthesis. Phytochemistry 52:843–854

Bouwmeester HJ, Kodde J, Verstappen FWA, Altug IG, de Kraker JW, Wallaart TE (2002) Isolation and characterization of two germacrene A synthase cDNA clones from chicory. Plant Physiol 129:134–144

Bovy A, de Vos R, Kemper M, Schijlen E, Almenar Pertejo M, Muir S, Collins G, Robinson S, Verhoeyen M, Hughes S, Santos-Buelga C, van Tunen A (2002) High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes LC and C1. Plant Cell 14:2509–2526

Butelli E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen EGWM, Hall RD, Bovy AG, Luo J, Martin C (2008) Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat Biotechnol 26:1301–1308

Cai G, Li G, Ye H, Li G (1995) Hairy root culture of *Artemisia annua* L. by Ri plasmid transformation and biosynthesis of artemisinin. Chin J Biotechnol 11:227–235

Cankar K, Houwelingen A, Bosch D, Sonke T, Bouwmeester H, Beekwilder J (2011) A chicory cytochrome P450 mono-oxygenase CYP71AV8 for the oxidation of (+)-valencene. Febs Lett 585:178–182

Chaturvedi D (2011) Sesquiterpene lactones: structural diversity and their biological activities. In: Tiwari V, Mishra B, editors. Opportunity, Challenge and Scope of Natural Products in Medicinal Chemistry. Kerala. India: Research Signpost; 2011:313–334

Chen DH, Liu CJ, Ye HC, Li GF, Liu BY, Meng YL, Chen XY (1999) Ri-mediated transformation of *Artemisia annua* with a recombinant farnesyl diphosphate synthase gene for artemisinin production. Plant Cell Tissue Organ Cult 57:157–162

Chen D, Ye H, Li G (2000) Expression of a chimeric farnesyl diphosphate synthase gene in *Artemisia annua* L. transgenic plants via *Agrobacterium tumefaciens*-mediated transformation. Plant Sci 155:179–185

Chen JL, Fang HM, Ji YP, Pu GB, Guo YW, Huang LL, Du ZG, Liu BY, Ye HC, Li GF, Wang H (2011) Artemisinin biosynthesis enhancement in transgenic *Artemisia annua* plants by downregulation of the β-caryophyllene synthase gene. Planta Med 77:1759–1765

Corteau R, Kutchan TM, Lewis NG (2000) Natural products (secondary metabolites. In: Buchanan B, Gruissem W, Jones R (eds) Biochemistry and Molecular Biology of Plants, American Society of Plant Physiologists

Covello PS (2008) Making artemisinin. Phytochemistry 69:2881–2885

Cui LW, Su XZ (2009) Discovery, mechanisms of action and combination therapy of artemisinin. Expert Rev Anti-Infe 7:999–1013

Curtis JD (1988) Secretory reservoirs (ducts) of two kinds in giant ragweed, *Ambrosia trifida* (Asteraceae). Am J Bot 75:1313–1323

Curtis JD, Lersten NR (1990) Oil-reservoirs in stem, rhizome, and root of *Solidago-canadensis* (Asteraceae, tribe Astereae). Nord J Bot 10: 443–449

de Kraker JW, Franssen MCR, de Groot A, Konig WA, Bouwmeester HJ (1998) (+)-Germacrene A biosynthesis—the committed step in the biosynthesis of bitter sesquiterpene lactones in chicory. Plant Physiol 117:1381–1392

de Kraker JW, Franssen MCR, Joerink M, de Groot A, Bouwmeester HJ (2002) Biosynthesis of costunolide, dihydrocostunolide, and leucodin. Demonstration of cytochrome P450-catalyzed formation of the lactone ring present in sesquiterpene lactones of chicory. Plant Physiol 129:257–268

Dimster-Denk D, Rine J, Phillips J, Scherer S, Cundiff P, DeBord K, Gilliland D, Hickman S, Jarvis A, Tong L, Ashby M (1999) Comprehensive evaluation of isoprenoid biosynthesis regulation in Saccharomyces cerevisiae utilizing the Genome Reporter Matrix. J Lipid Res 40:850–860

Dong L, Jongedijk E, Bouwmeester H, van Der Krol A (2015) Monoterpene biosynthesis potential of plant subcellular compartments. New Phytol. doi: 10.1111/nph.13629

Dudareva N, Andersson S, Orlova I, Gatto N, Reichelt M, Rhodes D, Boland W, Gershenzon J (2005) The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. Proc Natl Acad Sci USA 102:933–938

Duke SO, Paul RN (1993) Development and fine structure of the glandular trichomes of *Artemisia annua* L. Int J Plant Sci 154:107–118

Durante M, Caretto S, Quarta A, De Paolis A, Nisi R, Mita G (2011) β Cyclodextrins enhance artemisinin production in *Artemisia annua* suspension cell cultures. Appl Microbiol Biotechnol 90:1905–1913

Eljounaidi K, Cankar K, Comino C, Moglia A, Hehn A, Bourgaud F, Bouwmeester H, Menin B, Lanteri S, Beekwilder J (2014) Cytochrome P450s from *Cynara cardunculus* L. CYP71AV9 and CYP71BL5, catalyze distinct hydroxylations in the sesquiterpene lactone biosynthetic pathway. Plant Sci 223:59–68

Engels B, Dahm P, Jennewein S (2008) Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metab Eng 10:201–216

Esau K (1965) Plant Anatomy, 2nd edn. John Wiley & Sons, New York

Farhi M, Marhevka E, Ben-Ari J, Algamas-Dimantov A, Liang Z, Zeevi V, Edelbaum O, Spitzer-Rimon B, Abeliovich H, Schwartz B, Tzfira T, Vainstein A (2011) Generation of the potent anti-malarial drug artemisinin in tobacco. Nat Biotechnol 29:1072–1074

Farhi M, Kozin M, Duchin S, Vainstein A (2013) Metabolic engineering of plants for artemisinin synthesis. Biotechnol Genet Eng Rev 29: 135–148

Fisher CW, Shet MS, Caudle DL, Martin-Wixtrom CA, Estabrook RW (1992) High-level expression in *Escherichia coli* of enzymatically active fusion proteins containing the domains of mammalian cytochromes P450 and NADPH-P450 reductase flavoprotein. Proc Natl Acad Sci USA 89:10817

Frey U, Claude J, Crelier S, Juillert M (2002) UV degradation of sesquiterpene lactones in Chicory extract kinetics and identification of reaction products by HPLC-MS. Chmia 56:292–293

Furusawa M, Hashimoto T, Noma Y, Asakawa Y (2005) Biotransformation of citrus aromatics nootkatone and valencene by microorganisms. Chem Pharm Bull 53:1423–1429

Gardner RG, Hampton RY (1999) A highly conserved signal controls degradation of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in eukaryotes. J Biol Chem 274:31671–31678

Gershenzon J, Dudareva N (2007) The function of terpene natural products in the natural world. Nat Chem Biol 3:408–414

Geu-Flores F, Olsen CE, Halkier BA (2009) Towards engineering glucosinolates into non-cruciferous plants. Planta 229:261–270

Ghantous A, Sinjab A, Herceg Z, Darwiche N (2013) Parthenolide: from plant shoots to cancer roots. Drug Discov Today 18:894–905

Gralla JD, Carpousis AJ, Stefano JE (1980) Productive and abortive initiation of transcription in vitro at the lac UV5 promoter. Biochemistry 19:5864–5869

Guzman ML, Rossi RM, Neelakantan S, Li X, Corbett CA, Hassane DC, Becker MW, Bennett JM, Sullivan E, Lachowicz JL, Vaughan A, Sweeney CJ, Matthews W, Carroll M, Liesveld JL, Crooks PA, Jordan CT (2007) An orally bioavailable parthenolide analog selectively eradicates acute myelogenous leukemia stem and progenitor cells. Blood 110:4427–4435

Hale V, Keasling JD, Renninger N, Diagana TT (2007) Microbially derived artemisinin: a biotechnology solution to the global problem of access to affordable antimalarial drugs. Am J Trop Med Hyg 77: 198–202

Hamann T, Moller BL (2007) Improved cloning and expression of cytochrome P450s and cytochrome P450 reductase in yeast. Protein Expr Purif 56:121–127

Hashimoto T, Noma Y, Asakawa Y (2001) Biotransformation of terpenoids from the crude drugs and animal origin by microorganisms. Heterocycles 54:529–559

Hassany BE, Hanbali FE, Akssira M, Mellouki F, Haidour A, Barrero AF (2004) Germacranolides from *Anvillea radiate*. Fitoterapia 75:573–576

Hasunuma T, Miyazawa S, Yoshimura S, Shinzaki Y, Tomizawa K, Shindo K, Choi S, Misawa N, Miyake C (2008a) Biosynthesis of astaxanthin in tobacco leaves by transplastomic engineering. Plant J 55:857–868

Hasunuma T, Takeno S, Hayashi S, Sendai M, Bamba T, Yoshimura S, Tomizawa K, Fukusaki E, Miyake C (2008b) Overexpression of 1-deoxy-Dxylulose-5-phosphate reductoisomerase gene in chloroplast contributes to increment of isoprenoid production. J Biosci Bioeng 105:518–526

Hristozov D, DaCosta FB, Gasteiger J (2007) Sesquiterpene lactones-based classification of the family Asteraceae using neural networks and k-nearest neighbors. J Chem Inf Model 47:9–19

Ikezawa N, Goepfert JC, Nguyen DT, KimS-U O'MPE, Spring O, Ro DK (2011) Lettuce costunolide synthase (CYP71BL2) and its homolog (CYP71BL1) from sunflower catalyze distinct regio-and stereo-selective hydroxylations in sesquiterpene lactone metabolism. J Biol Chem 286:21601–21611

Jackson BE, Hart-Wells EA, Matsuda SP (2003) Metabolic engineering to produce sesquiterpenes in yeast. Org Lett 15:1629–1632

Jaziri M, Shimomura K, Yoshimatsu K, Fauconnier ML, Marlier M, Homes J (1995) Establishment of normal and transformed root cultures of *Artemisia annua* L. for artemisinin production. J Plant Physiol 145:175–177

Kahn R, Durst F (2000) Function and evolution of plant cytochrome P450. Recent Adv Phytochem 34:151–189

Kappers IF, Aharoni A, van Herpen TWJM, Luckerhoff LLP, Dicke M, Bouwmeester HJ (2005) Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis. Science 309:2070–2072

Khan MY, Aliabbas S, Kumar V, Rajkumar S (2009) Recent advances in medicinal plant biotechnology. Ind J Biotechnol 8:9–22

Kirby J, Keasling JD (2009) Biosynthesis of plant isoprenoids: perspectives for microbial engineering. Annu Rev Plant Biol 60:335–355

Knaeblein J (2004) Biopharmaceuticals expressed in plants. In: Kayser O, Muller RH (eds) Pharmaceutical biotechnology drug discovery and clinical applications. Wiley-VCH, Weinheim, pp 34–56

Knaeblein J (2005) Plant-based expression of biopharmaceuticals. In: Meyers R (ed) Encyclopaedia of molecular cell biology and molecular medicine, vol 10, 2nd edn. Wiley-VCH, Weinheim

Krings U, Lehnert N, Fraatz MA, Hardebusch B, Zorn H, Berger RG (2009) Autoxidation versus biotransformation of alpha-pinene to flavors with Pleurotus sapidus: regioselective hydroperoxidation of alpha-pinene and stereoselective dehydrogenation of verbenol. J Agric Food Chem 57:9944–9950

Kumar S, Hahn FM, Baidoo E, Kahlon TS, Wood DF, McMahan CM, Cornish K, Keasling JD, Daniell H, Whalen MC (2012) Remodeling the isoprenoid pathway in tobacco by expressing the cytoplasmic mevalonate pathway in chloroplasts. Metab Eng 14:19–28

Lange BM, Ahkami A (2013) Metabolic engineering of plant monoterpenes, sesquiterpenes and diterpenes – current status and future opportunities. Plant Biotechnol J 11:169–196

Lesiak K, Zalesna I, Nejc D, Düchler M, Czyz M (2010) Parthenolide, a sesquiterpene lactone from the medical herb feverfew, shows anticancer activity against human melanoma cells *in vitro*. Melanoma Re 20:21–34

Liu CZ, Wang YC, Ouyang F, Ye HC, Li GF (1997) Production of artemisinin by hairy root cultures of *Artemisia annua* L. Biotechnol Lett 19:927–930

Liu B, Wang H, Du Z, Li G, Ye H (2011a) Metabolic engineering of artemisinin biosynthesis in *Artemisia annua* L. Plant Cell Rep 30: 689–694

Liu Q, Majdi M, Cankar K, Goedbloed M, Charnikhova T, Verstappen FW, de Vos RC, Beekwilder J, van der Krol S, Bouwmeester HJ (2011b) Reconstitution of the costunolide biosynthetic pathway in yeast and *Nicotiana benthamiana*. PLoS One 6, e23255

Liu Q, Manzano D, Tanić N, Pesic M, Bankovic J, Pateraki I, Ricard L, Ferrer A, de Vos R, van de Krol S, Bouwmeester H (2014) Elucidation and *in planta* reconstitution of the parthenolide biosynthetic pathway. Metab Eng 23:145–153

Lommen WJ, Schenk E, Bouwmeester HJ, Verstappen FW (2006) Trichome dynamics and artemisinin accumulation during development and senescence of *Artemisia annua* leaves. Planta Med 72: 336–345

Lommen WJ, Elzinga S, Verstappen FW, Bouwmeester HJ (2007) Artemisinin and sesquiterpene precursors in dead and green leaves of *Artemisia annua* L. crops. Planta Med 73:1133–1139

Lu X, Zhang L, Zhang F, Jiang W, Shen Q, Zhang L, Lv Z, Wang G, Tang K (2013) AaORA, a trichome-specific AP2/ERF transcription factor of *Artemisia annua*, is a positive regulator in the artemisinin biosynthetic pathway and in disease resistance to *Botrytis cinerea*. New Phytol 198:1191–1202

Lyss G, Schmidt TJ, Merfort I, Pahl HL (1997) Helenalin, an anti-inflammatory sesquiterpene lactone from Arnica, selectively inhibits transcription factor NF-kappaB. Biol Chem 378:951–961

Ma D, Pu G, Lei C, Ma L, Wang H, Guo Y, Chen J, Du Z, Li G, Ye H, Liu B (2009) Isolation and characterization of AaWRKY1, an *Artemisia annua* transcription factor that regulates the amorpha- 4,11-diene synthase gene, a key gene of artemisinin biosynthesis. Plant Cell Physiol 50:2146–2161

Majdi M, Karimzadeh G, Malboobi MA, Omidbaigi R, Mirzaghaderi G (2010) Induction of tetraploidy to feverfew (*Tanacetum parthenium* Schulz. Bip): morphological, physiological, cytological and phytochemical changes. Hortscience 45:16–21

Majdi M, Liu Q, Karimzadeh G, Malboobi MA, Beekwilder J, Cankar K, Bouwmeester H (2011) Biosynthesis and localization of parthenolide in glandular trichomes of feverfew (*Tanacetum parthenium* l. Schulz bip.). Phytochemistry 72:1739–1750

Majdi M, Karimzadeh G, Malboobi MA (2014) Spatial and developmental expression of key genes of terpene biosynthesis in *Tanacetum parthenium*. Biologia Plantarum 58:379–384

Majdi M, Abdollahi MR, Maroufi A (2015) Parthenolide accumulation and expression of genes related to parthenolide biosynthesis affected by exogenous application of methyl jasmonate and salicylic acid in *Tanacetum parthenium*. Plant Cell Reports 34:1909–1918

Malarz J, Stojakowska A, Kisiel W (2007) Effect of methyl jasmonate and salicylic acid on sesquiterpene lactone accumulation in hairy roots of *Cichorium intybus*. Acta Physiol Plant 29:127–132

Martin VJ, Pitera DJ, Withers ST, Newman JD, Keasling JD (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat Biotechnol 21:796–802

Martin GDA, Durrant MC, Reese PB (2008) A predictive cytochrome P450 monooxygenase functional model for generic hydroxylation by *Rhizopus oryzae* ATCC 11145. J Theor Comput Chem 7:421–433

Miles CS, Ost TW, Noble MA, Munro AW, Chapman SK (2000) Protein engineering of cytochromes P-450. Biochim Biophys Acta 1543: 383–407

Morant M, Bak S, Moller BL, Werck-Reichhart D (2003) Plant cytochromes P450: tools for pharmacology, plant protection and phytoremediation. Curr Opin Biotechol 14:151–162

Muntendam R, Melillo E, Ryden A, Kayser O (2009) Perspectives and limits of engineering the isoprenoid metabolism in heterologous hosts. Appl Microbiol Biotechnol 84:1003–1019

Musharraf SG, Najeeb A, Khan S, Pervez M, Ali RA, Choudhary MI (2010) Microbial transformation of 5α-hydroxycaryophylla-4(12), 8(13)-diene with *Macrophomina phaseolina*. J Mol Catal B: Enzym 66:156–160

Newman JD, Marshall J, Chang M, Nowroozi F, Paradise E, Pitera D, Newman KL, Keasling JD (2006) High level production of amorpha-4,11-diene in a two-phase partitioning bioreactor of metabolically engineered *Escherichia coli*. Biotechnol Bioeng 95:684–691

Nguyen DT, Gopfert JC, Ikezawa N, Macnevin G, Kathiresan M, Conrad J, Spring O, Ro DK (2010) Biochemical conservation and evolution of germacrene A oxidase in Asteraceae. J Biol Chem 285:16588–16598

Olofsson L, Lundgren A, Brodelius PE (2012) Trichome isolation with and without fixation using laser microdissection and pressure catapulting followed by RNA amplification: expression of genes of terpene metabolism in apical and sub-apical trichome cells of *Artemisia annua* L. Plant Sci 183:9–13

Olsson ME, Olofsson LM, Lindahl AL, Lundgren A, Brodelius M, Brodelius PE (2009) Localization of enzymes of artemisinin biosynthesis to the apical cells of glandular secretory trichomes of *Artemisia annua* L. Phytochemistry 70:1123–1128

Paradise EM, Kirby J, Chan R, Keasling JD (2008) Redirection of flux through the FPP branch-point in *Saccharomyces cerevisiae* by down-regulating squalene synthase. Biotechnol Bioeng 100:371–378

Parshikov IA, Muraleedharan KM, Avery MA, Williamson JS (2004) Transformation of artemisinin by *Cunninghamella elegans*. Appl Microbiol Biotechnol 64:782–786

Parshikov IA, Netrusov AI, Sutherland JB (2012) Microbial transformation of antimalarial terpenoids. Biotechnol Adv 30:1516–1523

Payne T, Clement J, Arnold D, Lloyd A (1999) Heterologous myb genes distinct from GL1enhance trichome production when overexpressed in *Nicotiana tabacum*. Development 126:671–682

Pazouki L, Memari HR, Kännaste A, Bichele R, Niinemets Ü (2015) Germacrene A synthase in yarrow (*Achillea millefolium*) is an enzyme with mixed substrate specificity: gene cloning, functional characterization and expression analysis. Front Plant Sci 6:111. doi:10.3389/fpls.2015.00111

Perez-Rodriguez M, Jaffe FW, Butelli E, Glover BJ, Martin C (2005) Development of three different cell types is associated with the activity of a specific MYB transcription factor in the ventral petal of *Antirrhinum majus* flowers. Development 132:259–370

Picman AK (1986) Biological activities of sesquiterpene lactones. Biochem Sys Ecol 14:255–281

Pitera DJ, Paddon CJ, Newman JD, Keasling JD (2007) Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. Metab Eng 9:193–207

Quesada-Vargas R, Ruiz ON, Daniell H (2005) Characterization of heterologous multi-gene operons in transgenic chloroplasts: transcription, processing and translation. Plant Physiol 138:1746–1762

Rafi M, Bai N, Ho CT, Rosen RT, White E, Perez D, Dipaola RS (2005) A sesquiterpenelactone from *Inula britannica* induces anti-tumor effects dependent on Bcl-2 phosphorylation. Antican Res 25:313–318

Ralley L, Enfissi EMA, Misawa N, Schuch W, Bramley PM, Fraser PD (2004) Metabolic engineering of ketocarotenoid formation in higher plants. Plant J 39:477–486

Rea PA (1999) MRP subfamily ABC transporters from plants and yeast. J Exp Bot 50:895–913

Rea PA (2007) Plant ATP-binding cassette transporters. Annu Rev Plant Biol 58:347–375

Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, Chang MC, Withers ST, Shiba Y, Sarpong R, Keasling JD (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440:940–943

Robbins MP, Paolocci F, Hughes JW, Turchetti V, Allison G, Arcioni S, Morris P, Damiani F (2003) Sn, a maize *bHLH* gene, modulates anthocyanin and condensed tannin pathways in *Lotus corniculatus*. J Exp Bot 54:239–248

Rodriguez E, Towers GHN, Mitchell JC (1976) Biological activities of sesquiterpene lactones. Phytochemistry 15:1573–1580

Rydén AM (2010) Oxygen dependent biosynthesis of artemisinin precursors in yeast, In PhD Thesis. University of Groningen, Department of Pharmaceutical Biology

Singh ND, Kumar S, Daniell H (2015) Expression of β-glucosidase increases trichome density and artemisinin content in transgenic *Artemisia annua* plants. Plant Biotechnol J. doi:10.1111/pbi.12476

Souret FF, Kim Y, Wyslouzil BE, Wobbe KK, Weathers PJ (2003) Scale-up of *Artemisia annua* L. hairy root cultures produces complex patterns of terpenoid gene expression. Biotechnol Bioeng 83:653–667

Spakowicz DJ, Strobel SA (2015) Biosynthesis of hydrocarbons and volatile organic compounds by fungi: bioengineering potential. Appl Microbiol Biotechnol 99:4943–4951

Steliopoulos P, Wust M, Adam KP, Mosandl A (2002) Biosynthesis of the sesquiterepene germacrene D in *Solidago canadensis*: 13C and 2H labeling studies. Phytochemistry 60:13–20

Stojakowska A, Kisiel W (1997) Production of parthenolide in organ cultures of feverfew. Plant Cell Tiss Org Cult 47:159–162

Takahashi S, Yeo Y, Greenhagen BT, McMullin T, Song L, Maurina-Brunker J, Rosson R, Noel JP, Chappell J (2007) Metabolic engineering of sesquiterpene metabolism in yeast. Biotechnol Bioeng 97:170–181

Tang K, Shen Q, Yan T, Fu X (2014) Transgenic approach to increase artemisinin content in *Artemisia annua* L. Plant Cell Rep 33:605–615

Teoh KH, Polichuk DR, Reed DW, Nowak G, Covello PS (2006) *Artemisia annua* L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. FEBS Lett 580:1411–1416

Ting HM, Wang B, Rydén AM, Woittiez L, Van Herpen T, Verstappen FWA, Van der Krol A (2013) The metabolite chemotype of *Nicotiana benthamiana* transiently expressing artemisinin biosynthetic pathway genes is a function of CYP71AV1 type and relative gene dosage. New Phytologist 199:352–366

Tsuruta H, Paddon CJ, Eng D, Lenihan JR, Horning T, Anthony LC, Regentin R, Keasling JD, Renninger NS, Newman JD (2009) High-level production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in *Escherichia coli*. PLoS One 4, e4489

Urban P, Mignotte C, Kazmaier M, Delorme F, Pompon D (1997) Cloning, yeast expression, and characterization of the coupling of two distantly related *Arabidopsis thaliana* NADPH-cytochrome P450 reductases with P450 CYP73A5. J Biol Chem 272:19176–19186

van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289:295–297

van Herpen TWJM, Cankar K, Nogueira M, Bosch D, Bouwmeester HJ, Beekwilder J (2010) *Nicotiana benthamiana* as a production platform for Artemisinin precursors. PLoS One 5, e14222. doi:10.1371/journal.pone.0014222

Verma D, Daniell H (2007) Chloroplast vector systems for biotechnology applications. Plant Physiol 145:1129–1143

Vickers CE, Bongers M, Liu Q, Delatte T, Bouwmeester H (2014) Metabolic engineering of volatile isoprenoids in plants and microbes. Plant, Cell and Environment 37:1753–1775

Wallaart TE, Pras N, Quax WJ (1999a) Isolation and identification of dihydroartemisinic acid hydroperoxide from *Artemisia annua*: a novel biosynthetic precursor of artemisinin. J Nat Prod 62:1160–1162

Wallaart TE, van Uden W, Lubberink HG, Woerdenbag HJ, Pras N, Quax WJ (1999b) Isolation and identification of dihydroartemisinic acid from *Artemisia annua* and its possible role in the biosynthesis of artemisinin. J Nat Prod 62:430–433

Wallaart TE, Pras N, Beekman AC, Quax WJ (2000) Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chemotypes. Planta Medica 66:57–62

Wang H, Song Y, Shen H, Liu Y, Li Z, Wang H, Chen J, Liu B, Ye H (2012) Effect of antisense squalene synthase gene expression on the increase of Artemisinin content in *Artemisia anuua*. Transgenic Plants - Advances and Limitations, PhD. Yelda Ozden Çiftçi (Ed.), ISBN: 978-953-51-0181-9

Withers ST, Keasling JD (2007) Biosynthesis and engineering of isoprenoid small molecules. Appl Microbiol Biotechnol 73:980–990

Wu S, Chappell J (2008) Metabolic engineering of natural products in plants; tools of the trade and challenges for the future. Curr Opin Biotechnol 19:145–152

Wu S, Schalk M, Clark A, Miles RB, Coates R, Chappell J (2006) Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. Nat Biotechnol 24:1441–1447

Xu YH, Wang JW, Wang S, Wang JY, Chen XY (2004) Characterization of *Ga*WRKY1, a cotton transcription factor that regulates the sequiterpene synthase gene (+)-™-cadinene synthase. Plant Physiol 134:507–515

Yazaki K (2005) Transporters of secondary metabolites. Curr Opin Plant Biol 8:301–307

Yin H, Zhuang YB, Li EE, Bi HP, Zhou W, Liu T (2015) Heterologous biosynthesis of costunolide in *Escherichia coli* and yield improvement. Biotechnol Lett 37:1249–1255

Yu ZX, Li JX, Yang CQ, Hu WL, Wang LJ, Chen XY (2012) The jasmonate responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in *Artemisia annua* L. Mol Plant 5:353–365

Yuuya S, Hagiwara H, Suzuki T, Ando M, Yamada A, Suda K, Kataoka T, Nagai K (1999) Guaianolides as immunomodulators. Synthesis and biological activities of dehydrocostus lactone, mokko lactone, eremanthin, and their derivatives. J Nat Prod 62:22–30

Zhan J, Guo H, Dai J, Zhang Y, Guo D (2002) Microbial transformations of artemisinin by *Cunninghamella echinulata* and *Aspergillus niger*. Tetrahedron Lett 43:4519–4521

Zhang Y, Nowak G, Reed DW, Covello PS (2011) The production of artemisinin precursors in tobacco. Plant Biotechnol J 9:445–454