Chapter 4
Biosynthesis of Sesquiterpene Lactones in Plants and Metabolic Engineering for Their Biotechnological Production

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Abstract In the present chapter, we review some aspects of the biosynthesis of sesquiterpene lactones and its regulation in different medicinal and aromatic plants used in the pharmaceutical industry. In this sense, we describe the mevalonate and the 2-*C*-methyl-D-erythritol 4-phosphate pathways, which generate the corresponding isoprenoid precursors (isopentenyl diphosphate and dimethylallyl diphosphate), as well as the late pathways that lead to sesquiterpene lactone biosynthesis. This chapter also analyses the role of the transcription factors involved in the regulation of sesquiterpene lactone biosynthesis and the different biotechnological approaches that have been developed for sesquiterpene lactone production. In vitro plant cell cultures (comprising micropropagation and plant cell suspension, shoot and root cultures) have emerged as a production platform for many plant secondary metabolites, since they allow their production under controlled conditions and shorter production cycles. The characterisation and isolation of genes involved in the regulation of sesquiterpene lactone biosynthetic pathways have allowed the design of metabolic engineering strategies to increase the production of these metabolites. Moreover, we discuss different strategies to increase sesquiterpene lactone production through genetic engineering. We also focus on the metabolic engineering of the artemisinin biosynthetic pathway in *Artemisia annua*. This metabolic pathway has become a model system not only for the biotechnological production of sesquiterpene lactones but also for the improvement of other plant secondary metabolic pathways. Finally, we analyse the successful expression of the complete artemisinin

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biosynthetic pathway in *Escherichia coli* and *Saccharomyces cerevisiae*, which has led to the efficient accumulation of artemisinic acid in these microorganisms.

Keywords Sesquiterpene lactones · Metabolic engineering · Secondary metabolism · Transcription factors · Plant cell culture · Artemisinin · Yeast · *Escherichia coli*

Abbreviations

AA	Artemisinic acid
AACT	Acetoacetyl-CoA thiolase
ABA	Abscisic acid
ABREs	ABA-responsive elements
ADHI	Alcohol dehydrogenase 1
ADS	Amorpha-4,11-diene synthase
ALDH1	Aldehyde dehydrogenase
AOC	Allene oxide cyclase
AP2/ERF	APETALA2/ethylene response factor
atoB	Acetoacetyl-CoA thiolase gene
*bgl*1	β-Glucosidase gene
bZIP	Basic leucine zipper
CaMV35S	Cauliflower mosaic virus promoter
CDP-ME	4-Diphosphocytidyl-2-*C*-methyl-D-erythritol
CDP-ME2P	4-(Cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol phosphate
CMK	CDP-ME kinase
COS	Costunolide synthase
CPR1	Cytochrome P450 reductase
CYB5	Cytochrome b5
CYP71AV1	Cytochrome P450 monooxygenase
DBR2	Artemisinic aldehyde Δ11 (13) reductase
DMAPP	Dimethylallyl diphosphate
DXR	1-Deoxy-D-xylulose 5-phosphate reductoisomerase
DXS	1-Deoxy-D-xylulose 5-phosphate synthase
EMSA	Electrophoretic mobility shift assay
ERG10	Acetoacetyl-CoA thiolase gene
ERG12	Mevalonate kinase
ERG13	HMG-CoA synthase
ERG20	Farnesyl diphosphate synthase gene
ERG8	Phosphomevalonate kinase
FDS	Farnesyl diphosphate synthase
FPP	Farnesyl diphosphate
FRET	Fluorescence resonance energy transfer
GA	Germacrene A

GA-3P	Glyceraldehyde-3-phosphate
GAA	4,11 (13)-Trien-12-oic acid
GAH	Germacrene A hydroxylase
GAO	Germacrene A oxidase
GAS	Germacrene A synthase
GMP	Good manufacturing practices
GSH	Glutathione
GST	Glandular secretory trichomes
GSW1	GLANDULAR TRICHOME-SPECIFIC WRKY 1
GUS	β-Glucuronidase reporter gene system
HDR	(E)-4-Hydroxy-3-methylbut-2-enyl diphosphate reductase
HDS	1-Hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase
HMBPP	(E)-4-Hydroxy-3-methylbut-2-enyl diphosphate
HMG-CoA	3-Hydroxy-3-methylglutaryl-CoA
HMGR	HMG-CoA reductase
HMGS	HMG-CoA synthase
IDI	Isopentenyl diphosphate isomerase
idi	Isopentenyl diphosphate isomerase gene
IPP	Isopentenyl diphosphate
ipt	Isopentenyl transferase gene
ispA	Farnesyl diphosphate synthase gene
Ja	Jasmonic acid
MAP	Medicinal and aromatic plants
MCT	2-*C*-Methyl-D-erythritol 4-phosphate cytidylyltransferase
MDS	2-*C*-Methyl-D-erythritol 2,4-cyclodiphosphate synthase
ME −2,4cPP	2-*C*-Methyl-D-erythritol 2,4-cyclodiphosphate
MeJ	Methyl jasmonate
MEP	2-*C*-Methyl-D-erythritol 4-phosphate
MEP	2-*C*-Methyl-D-erythritol 4-phosphate pathway
MSI	Mass spectrometry imaging
MVA	Mevalonate pathway
mvaA	HMG-CoA reductase gene
mvaS	HMG-CoA synthase gene
MVD	Mevalonate diphosphate decarboxylase
MVD1	Mevalonate diphosphate decarboxylase gene
MVDP	Mevalonate-5-diphosphate
MVK	Mevalonate kinase
MVP	Mevalonate-5-phosphate
PCC	Plant cell culture
PDR	Pleiotropic drug resistance
PMK	Phosphomevalonate kinase
PTS	Parthenolide synthase
PTS	Patchoulol synthase
RNAi	RNA interference
SA	Salicylic acid

SQS	Squalene synthase
SS	Santalene synthase
STLs	Sesquiterpene lactones
STPS	Sesquiterpene synthases
TAR1	TRICHOME AND ARTEMISININ REGULATOR 1
TFs	Transcription factors
tHMRG	Truncated HMG-CoA reductase
TP	Terpene synthase

4.1 Introduction

Sesquiterpene lactones (STLs) are terpenoid secondary metabolites that are mainly present in plants. These compounds play important roles in plant physiology as deterrent, defence compounds (phytoalexins), alellochemicals and pollinator attractants (Tholl 2015; Bouvier et al. 2005; Gershenzon and Dudareva 2007). Many STLs are accumulated in specialised tissues like glandular trichomes, oil bodies and resin ducts but also in organs like roots and fruits or even in the whole plant (Nagegowda 2010). STLs also show interesting biological activities such as antibacterial, antimalarial, antifungal, anti-inflammatory and anticancer. In addition, they are used in the cosmetic industry as fragrances (Chadwick et al. 2013; Chaturvedi 2011).

Sesquiterpene lactones are C_{15} terpenoids derived from the universal isoprenoid precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). IPP and DMAPP are synthetized through two independent metabolic routes, the mevalonate (MVA) pathway and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, which are localised in the cytosol and in the chloroplast, respectively (Vranová et al. 2013). The biosynthesis of STLs starts with the cyclization of farnesyl diphosphate (FPP) by a sesquiterpene synthase (SPS). The reaction is followed by a series of oxidations and hydroxylations, carried out by cytochrome P450 enzymes. Finally, the C_{15} structure can be modified by alcohol dehydrogenases, reductases and acyl transferases. The diversity of SPS and CYP and their promiscuous enzyme activities result in a great variety of STL structures (Simonsen 2015).

As other secondary metabolic routes, the biosynthesis of STLs is a complex arrangement of enzymatic reactions which are tightly regulated at different levels. Indeed, the MVA, MEP and STL pathways are controlled at the transcriptional and post-transcriptional/translational levels, including feedback regulation (Vranová et al. 2013). Many environmental factors and stimuli, such as light, abiotic stress and pathogens, have been described to affect the STL accumulation and the expression of related genes. In addition, the STL biosynthesis also presents spatial (specific tissue and organ accumulation) and temporal regulation (plant development) (Vranová et al. 2012).

In order to produce these valuable metabolites at the industrial scale, a full elucidation of the biosynthetic pathway and a deep understanding of its regulation are required. In this sense, STL production could be improved through molecular

biology strategies in order to generate genetically modified plants or plant cell cultures or by introducing the full STL biosynthetic pathway in other organisms such as yeast or bacteria (Majdi et al. 2015).

The synthesis of the antimalarial drug, artemisinin, in *Artemisia annua* has been elucidated, and several genes have been overexpressed in *A. annua* plants, thus improving their artemisinin content. Metabolic engineering of the pathway has been also carried out in yeast and bacteria, which proved that STL can be produced efficiently in these expression platforms (Xie et al. 2016). *A. annua* has become a plant model to study STL biosynthesis in plants, allowing the characterisation and isolation of genes involved in other STL pathways. Recently, the full biosynthetic pathway of parthenolide, an anticancer and antimigraine drug present in *Tanacetum parthenium*, has been elucidated, and the gene overexpression in *Nicotiana benthamiana* resulted in parthenolide accumulation (Liu et al. 2014). These advances have been complemented by new techniques of tissue dissection, which allow the isolation of RNA even from very few cells like glandular secretory trichomes (GST) and the construction of specific cDNA libraries (Olofsson et al. 2012). Furthermore, the low cost of transcriptomic sequencing has increased the database identification of genes involved in secondary metabolite production. The transcriptomic analysis resulted in a powerful tool to identify genes encoding enzymes and transcription factors (TFs) of the sesquiterpenoid lactone pathway in *Thapsia laciniata* (thapsigargin, antitumour activity), *Santalum* spp. (santalene, fragrances) and other important medicinal and aromatic plants (Moniodis et al. 2015; Simonsen 2015).

In the present chapter, we review some aspects of STL biosynthesis and the biotechnological approaches for their production. We specifically focus on those plants of medicinal interest and those used in the industry as aromatics.

4.2 Biosynthesis of the Isoprenoid Precursors in Plants

As mentioned above, two independent biosynthetic pathways coexist in plants and are responsible for the synthesis of the universal terpenoid precursors IPP and DMAPP: the mevalonate pathway (cytosol) and the 2-*C*-methyl-D-erythritol 4-phosphate pathway (plastids). On the other hand, the MVA is present in yeast, while the MEP pathway occurs in bacteria.

4.2.1 Mevalonate (MVA) Pathway

The condensation of two acetyl-CoA molecules catalysed by the enzyme acetoacetyl-CoA thiolase (AACT) is the first step of the mevalonate pathway (MVA). The second enzymatic step involves the HMG-CoA synthase (HMGS), resulting in the production of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). In the third step, the enzyme HMG-CoA reductase (HMGR) converts HMG-CoA to mevalonate (MVA)

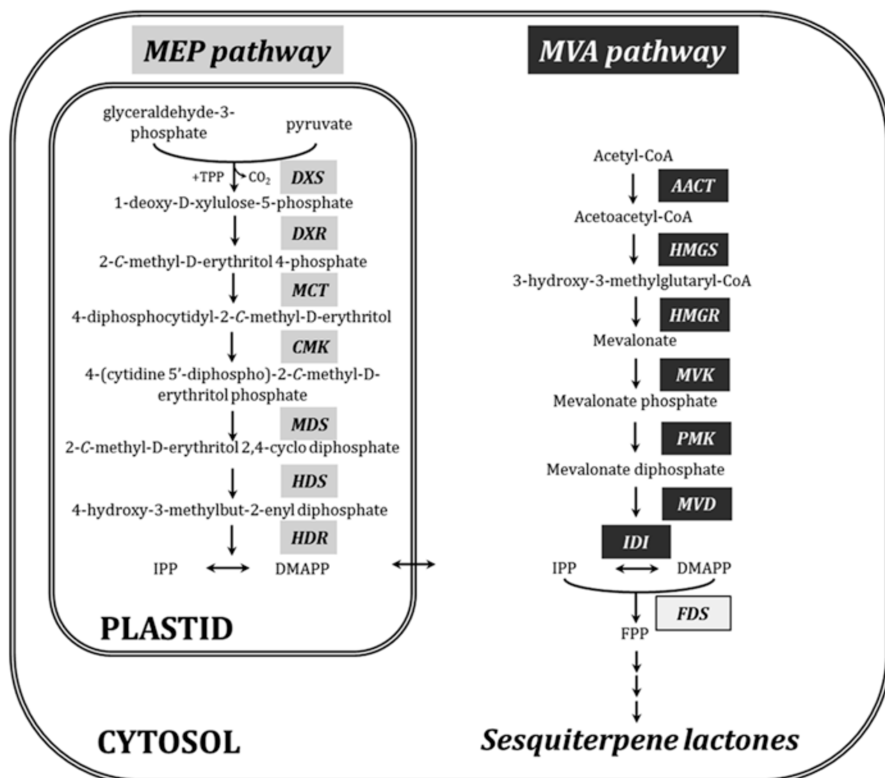

Fig. 4.1 Schematic representation of the biosynthetic pathways involved in IPP and DMAPP production. *AACT* acetoacetyl-CoA thiolase, *HMGS* HMG-CoA synthase, *HMGR* HMG-CoA reductase, *MVK* mevalonate kinase, *PMK* phosphomevalonate kinase, *MVD* mevalonate diphosphate decarboxylase, *IDI* isopentenyl diphosphate isomerase, *DXS* 1-deoxy-D-xylulose 5-phosphate synthase, *DXR* 1-deoxy-D-xylulose 5-phosphate reductoisomerase, *MCT* 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase, *CMK* CDP-ME kinase, *MDS* 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase, *HDS* 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, *HDR* (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase, *FDS* farnesyl diphosphate synthase

in a double reduction step which requires two NADPH. HMGR is a membrane-bound protein associated to the endoplasmic reticulum (ER) that has been extensively studied in different organisms and described as the rate-limiting step in the MVA pathway. In three successive steps catalysed by the enzymes mevalonate kinase (MVK), phosphomevalonate kinase (PMK) and mevalonate diphosphate decarboxylase (MVD), mevalonate is converted to the final product, IPP (Vranová et al. 2013; Tholl 2015; Vranová et al. 2012). IPP is transformed to DMAPP by the IPP isomerase (IDI) which is localised in different cellular compartments such as the cytosol, chloroplasts and mitochondria (Fig. 4.1).

4.2.2 2-C-Methyl-D-erythritol 4-phosphate (MEP) Pathway

The MEP pathway consists of seven enzymatic reactions (Lichtenthaler 1999). The first step is catalysed by the deoxyxylulose phosphate synthase (DXS) and involves the condensation of pyruvate and glyceraldehyde 3-phosphate (GA3P) to produce 1-deoxy-D-xylulose 5-phosphate (DXP). DXP is then converted into 2-*C*-methyl-D-erythritol 4-phosphate (MEP) by the deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). Both DXS and DXR are considered rate-limiting enzymes in the MEP pathway. In four consecutive reactions, MEP is converted into (E)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) by 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase (MCT), CDP-ME kinase (CMK), 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS) and 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (HDS). The last step involves the action of the (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR), which transforms HMBPP into IPP and its isomer DMAPP in a ratio of 5 or 6 to 1 (Vranová et al. 2013; Vranová et al. 2012; Zhao et al. 2013) (Fig. 4.1).

4.2.3 Cross-Talk Between MVA and MEP Pathways

It has been generally assumed that the MVA pathway supplies isoprenoid precursors for the synthesis of sterols, sesquiterpenes and triterpenes while the MEP pathway provides precursors to produce monoterpenes, diterpenes and carotenoids. Despite the spatial separation of both pathways, an exchange of IPP and DMAPP between cytosol and plastids has been observed in the synthesis of different terpenoids. Ram et al. (2010) have demonstrated the key role of HMGR in the MVA pathway. ^{14}C-HMG-CoA was efficiently incorporated into artemisinin; however, the addition of the competitive inhibitor mevinolin inhibited up to 83% the label incorporation into this secondary metabolite. This effect could be reversed by increasing the amount of ^{14}C-HMG-CoA. Moreover, ^{14}C-MVA, which is a product of the HMGR, was also incorporated into artemisinin, and incorporation rates were enhanced by increasing concentrations of the labelled compound. On the other hand, the addition of fosmidomycin, which is an inhibitor of the MEP pathway, reduced the artemisinin accumulation by 14%. The authors concluded that the MVA pathway is a major donor of isoprenoid precursors in the biosynthesis of artemisinin and that HMGR is a rate-limiting step in this route. Another study has demonstrated a decrease in artemisinin accumulation when *A. annua* plants were treated with mevinolin and fosmidomycin, inhibitors of the MVA and MEP pathways, respectively (Towler and Weathers 2007). The results obtained proved that IPP from both pathways contribute to the synthesis of this STL. Additionally, a study performed with $^{13}CO_2$ confirmed that artemisinin was synthesised from IPP and DMAPP precursors provided by both biosynthetic routes (Schramek et al. 2010). In vivo feeding assays performed with deuterium-labelled

precursors (DXP and mevalonic acid lactone) in intact grape berries have demonstrated that both intermediates were incorporated into STL, which accumulated mainly in the exocarp (May et al. 2013). These results have revealed that the cytosolic and plastidial routes provide isoprenoids intermediates for the production of STL and should be considered for further metabolic engineering strategies.

The MVA and MEP pathways are affected by many environmental, spatial and developmental factors, and such influences depend on plant species and culture system used in each experiment (in vitro culture, hairy roots, suspensions, etc.).

4.2.4 Farnesyl Diphosphate Synthase: Branch Point of Sesquiterpene Lactone Biosynthesis

Farnesyl phosphate (FPP) is generated from IPP and DMAPP by the action of farnesyl diphosphate synthase (FDS) and is the common precursor of all STLs, sterols, triterpenes and prenylated proteins. FPP is the substrate of squalene synthase (SQS), which is the first step in the synthesis of sterols and brassinosteroids, and consequently an important competitive pathway for isoprenoid precursors (Vranová et al. 2013). FDS is localised mainly in the cytosol and the mitochondria. Overexpression of FDS in *A. annua* resulted in higher artemisinin content than non-transformed plant, proving its role as a rate-limiting step in this pathway (Banyai et al. 2010).

4.2.5 Sesquiterpene Lactone Pathway

The first step in the biosynthesis of STLs is the cyclisation of FPP catalysed by sesquiterpene synthases (STPS). STPS are mainly located in the cytosol and are characterised by their plasticity, showing the capacity of multiple substrate utilisation. Santalene synthase (SaSSy) from sandalwood (*Santalum album*), which is used in the industry for its essential oil fragrance, produces a mixture of santalenes (α, ß and epi-ß-santalene) and α-exo-bergamotene (Jones et al. 2008; Diaz-Chavez et al. 2013). Two genes encoding STPS in *Arabidopsis thaliana*, *At5g44630* and *At5g23960*, are considered responsible for at least 20 different STLs present in its floral volatiles (Tholl et al. 2005).

One of the best characterised STPS is germacrene A synthase (GAS), which converts FPP into germacrene A (GA). GA is the precursor of many germacranolide-type STLs. The gene encoding GAS has been isolated from several plant species: among them, *A. annua*, *Barnadesia spinosa* (Nguyen et al. 2016), chicory (de Kraker et al. 1998), *T. parthenium* (Liu et al. 2014), sunflower and lettuce. GA is the backbone skeleton for the biosynthesis of costunolides and parthenolides, both of which are of pharmacological interest for their anticancer activity. In a series of oxidation reactions, GA is converted into germacra-1(10), 4,11 (13)-trien-12-oic

acid (GAA) by germacrene A oxidase (GAO), which is a cytochrome P450-like enzyme. GAA is further oxidised by a costunolide synthase to yield costunolide. Recently, a parthenolide synthase (*TpPTS*) from feverfew (*T. parthenium*) was identified; the enzyme catalyses the epoxidation of C4–C5 double bond of costunolide molecules to yield the final product parthenolide, which has antimigraine and anticancer activities. In that way, the biosynthesis of parthenolide has become the second STL pathway to be fully elucidated (Liu et al. 2014, Yin et al. 2015).

The most important and commercial valuable STL is the antimalarial drug artemisinin, which is produced by *A. annua*. The artemisinin biosynthetic pathway was the first one to be fully characterised (Fig. 4.2) (Wen and Yu 2011). The first reaction is catalysed by the enzyme amorpha-4,11-diene synthase (ADS) that converts FPP into amorpha-4,11-diene (Chang et al. 2000; Mercke et al. 2000). The following

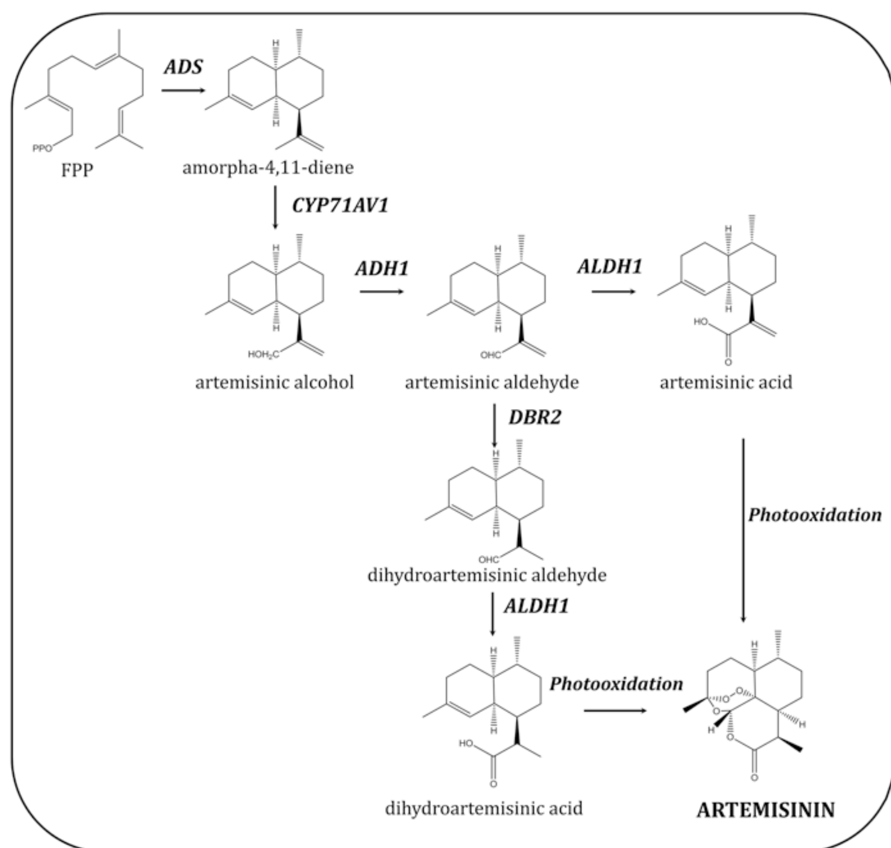

Fig. 4.2 Schematic representation of the artemisinin biosynthetic pathway in Artemisia annua. *ADS* amorpha-4,11-diene synthase, *CYP71AV1* cytochrome P450 monooxygenase, *DBR2* artemisinic aldehyde Δ11 (13) reductase, *ADH1* alcohol dehydrogenase 1, *ALDH1* aldehyde dehydrogenase, *FPP* farnesyl diphosphate

reaction involves a cytochrome P450 monooxygenase (CYP71AV1) and a cytochrome P450 oxidoreductase (CPR) as the native redox partner, which hydroxylates amorpha-4,11-diene to render artemisinic alcohol. The alcohol is then oxidised to artemisinic aldehyde by an alcohol dehydrogenase (ADH1) (Teoh et al. 2006). The artemisinic aldehyde Δ11 (13) reductase (DBR2) catalyses the conversion of artemisinic aldehyde into dihydroartemisinic aldehyde (Zhang et al. 2008), which is finally converted to dihydroartemisinic acid by an aldehyde dehydrogenase (ALDH1) (Teoh et al. 2009). Dihydroartemisinin acid is regarded as the immediate precursor of artemisinin. The reaction seems to be a non-enzymatic photooxidation process, although this subject remains under debate (Wen and Yu 2011; Turconi et al. 2014). Artemisinic aldehyde could also be transformed to artemisinic acid (AA) by ALDH1 as an alternative pathway to artemisinin production (Fig. 4.2).

4.3 Sesquiterpene Lactone Biosynthetic Pathway Characterisation and Regulation in Medicinal and Aromatic Plants

The STL biosynthesis in plants constitutes a complex network of metabolic pathways that compete for common precursors. In addition, it is also strictly regulated by many environmental, spatial and temporal factors. The biosynthetic enzymatic steps that lead to artemisinin, costunolide and parthenolide production have been fully characterised. Moreover, many TFs that control the transcription of biosynthetic genes have been isolated, which allowed a better comprehension on how these STLs are produced. These findings have constituted a great contribution to the understanding and characterisation of other STL biosynthetic pathways in plants. However, the regulation is specific for each STL pathway. Therefore, it is important to recognise the different factors that regulate the upstream biosynthetic pathways (MVA and MEP) as well as those regulating the STL-specific downstream pathway in each STL-producing plant.

Recently, techniques like mass spectrometry imaging (MSI) and fluorescence resonance energy transfer (FRET)-based nanosensors have allowed the identification of cells and tissues that specifically accumulate secondary metabolites. For instance, MSI studies have shown that the accumulation of STLs in *Helianthus annuus* occurs in the capitate glandular trichomes that are present in the leaves. In this sense, these techniques facilitate transcriptome analyses of different MAPs, thus increasing the bulk of knowledge on STL pathways and their regulation.

Ginkgo biloba is one of the most ancient tree species of the plant kingdom and has been used for centuries in traditional Chinese medicine. The main bioactive compounds in the leaf extract are ginkgolides (diterpenes) and bilobalides (sesquiterpenes). A genomic and transcriptomic study allowed the identification of the sequence for a terpene synthase (*GbTP2*) which catalyses the conversion of FPP into (-)-α-bisabolene. The transcript levels of *GbTPS2* were higher in roots and mature leaves than in immature leaves, shoots apices, stem and seeds (Parveen et al. 2015).

Santalum spicatum (sandalwood) and other species produce oil that is rich in sesquiterpenes with a high commercial value. The oil is produced in the xylem tissue of mature trees. A study of the xylem transcriptome has shown the presence of most of the transcripts from the MVA pathway, while the transcripts of only two enzymes of the MEP route (DXS and DXR) were found (Moniodis et al. 2015). These results indicate that the MVA pathway is the main supplier of precursors for STL biosynthesis. HMGR seems to be a key step in the pathway, since it showed the highest transcript levels. High transcript levels of at least three terpene synthases (TPS) were found in the xylem, santalene synthase (SS), α-bisabolol synthase and sesquisabinene B synthase. SS is involved in the biosynthesis of santalol, the main STL present in sandalwood oil. Another abundant transcript found in the transcriptome was the one that encodes a cytochrome P450 of the CYP76 family (*CYP76F39*). This enzyme shows santalene hydroxylase activity and its sequence has 96% identity with the one described in *S. album*. In another study performed in *S. album*, the authors compared the expression pattern of *FDS* and *SS* in mature and immature wood samples. They found a strong correlation between *FDS* and *SS* expression levels with santalol accumulation in mature wood, which suggests that both genes are critical in santalol biosynthesis and are regulated at the developmental level (Jones et al. 2011; Rani et al. 2013).

Xanthium strumarium is a traditional medicinal Chinese herb that accumulates the STLs xanthanolides mainly in glandular trichomes. The plant has been used to treat different illnesses such as leucoderma, tuberculosis, herpes, cancer, ulcers and rheumatism. Xanthanolides and other STLs have been associated with most of the biological activities described in *X. strumarium*. A comparative transcriptome study between leaves and glandular trichomes of *X. strumarium* has been carried out. The genes of the MVA and MEP pathways, *IDI* and *FDS*, showed higher expression levels in trichomes than in leaves. These results suggest the existence of a trichome specificity of STL biosynthesis and the participation of MVA and MEP pathways as suppliers of isoprenoid precursors for their synthesis (Li et al. 2016). The same research group performed a microRNA comparative analysis between leaves and trichomes in order to establish the putative post-transcriptional regulation of the STL biosynthesis in *X. strumarium*. MicroRNAs are small non-coding nucleotides that interact with mRNAs by base complementarity resulting in mRNA degradation or translational inhibition of the target genes. The authors found at least two microRNA sequences that target two key enzymes of the upstream terpenoid pathway: HMGR from the MVA pathway and DXS from the MEP pathway. A microRNA sequence targeted to isopentenyl diphosphate isomerase (IDI) was also detected in the trichome library (Fan et al. 2015).

Valerian roots are used as anxiolytics and sedatives; these biological activities are attributed to several STLs such as valerenic acid and its precursors, valerenadiene and valerenal. Two genes encoding terpene synthases were isolated from a RNA sequencing analysis from roots of *Valeriana officinalis*, *VoTPS1* and *VoTPS2* (Pyle et al. 2012). The heterologous expression in yeast has revealed that TPS1 catalyses the conversion of FPP to germacrene C/D and TPS2 generates valerena-4,7(11)-diene. In a further study, Yeo et al. (2013) have found two genes encoding

TPS, namely, *VoTPS*1 and *VoTPS7*. The former has the same enzymatic activity than the TPS2 found in the previous work, and the latter catalyses the conversion to germacrene C/D. The expression profile of *VoTPS1/7* showed that their transcripts are specifically found in roots. In hairy root cultures, the expression levels of two genes encoding TPS were analysed, and it was found that the expression levels of *VoFDS*, *VoGCS* and *VoVDS* were similar to those found in non-transformed roots (Ricigliano et al. 2016). A putative cytochrome c P450 enzyme (CYP71D442) involved in the biosynthesis of STLs has shown the same profile. In methyl jasmonate (MeJ)-treated hairy roots, the transcripts of *VoFDS*, *VoGCS* and *VoVDS* were upregulated after 12, 25 and 36 h of treatment. On the other hand, the *VoCYP71D442* transcript levels were not induced by MeJ, suggesting that this P450 enzyme is not related to the biosynthesis of valerenic acid (Ricigliano et al. 2016).

Thapsia garganica and related species produce thapsigargin mainly in roots and fruits. This STL has a potent biological activity against solid tumours. Due to its low solubility, a derivative prodrug is currently being assessed in clinical trials (Andersen et al. 2015). Two encoding genes for STPS, *TgTPS1* and *TgTPS2*, were isolated from a cDNA library and RNA sequencing from roots of *T. garganica*. Both were expressed at high levels in roots. The heterologous expression of both sequences in yeast revealed that TPS1 produced cadinene while in TPS2-expressing yeast, the main product was 6β-hydroxy germacra-1(10),4-diene (kunzeaol) (Pickel et al. 2012). The authors proposed that the latter might be the first enzymatic reaction that leads to the biosynthesis of thapsigargin (Drew et al. 2013). A transcriptomic analysis in *T. laciniata* roots has revealed the presence of several putative genes involved in the biosynthesis of STLs: 5 sesquiterpene synthases, 16 cytochromes P450 of the CYP71 family and ALDH and ADH (similar to those present in *A. annua*).

Globe artichoke (*Cynara cardunculus*, *Cynara scolymus*) is a traditional food crop that has also been used with therapeutic purposes in ancient medicine. Globe artichoke accumulates a variety of STLs that are responsible for the classical bitter taste of the plant. Cynaropicrin, which is the main STL present, has shown anti-inflammatory activity and cytotoxicity against cancer cells. Cynaropicrin is formed via the biosynthesis of costunolide, and the three enzymes GAS, GAO and COS have been identified and characterised (Menin et al. 2012). GAS has been found in the cytosol, while GAO and COS were located in the ER. A RT-qPCR analysis of the transcripts has revealed that the genes are highly expressed in mature leaves, as compared with old leaves. These genes were also found to be expressed in callus tissues and the receptacles. The expression pattern correlates with cynaropicrin accumulation, which occurs in the glandular trichomes present in the leaves. A study of the *CcGAO* and *CcCOS* promoters has shown the presence of L1-box *cis*-elements, similar to those found in the genes that are specifically expressed in *Arabidopsis* trichomes (Eljounaidi et al. 2014).

Chicory (*Cichorium intybus*) accumulates STLs that are responsible for the bitterness of the leaves and the stems used in food. Chicory has been used in traditional medicine to promote digestion and appetite. Chicory STLs have shown anticancer, antileukemic, sedative and analgesic activities. de Kraker et al. (2002) have elucidated the complete pathway that leads to the synthesis of costunolide from

FPP. Three enzymes are involved: GAS, germacrene A hydroxylase (GAH) and COS. Costunolide is also the intermediate for the biosynthesis of other STLs such as leucodin and dihydrocostunolide, with these reactions being probably catalysed by a cytochrome P450 enzyme and enoatereductase, respectively. The authors have determined that GAH can also convert amorpha-4,11-diene into amorpha-4,11-dien-12-ol, which is a precursor in the synthesis of artemisinic acid and artemisinin. A transcriptomic analysis of two different chicory cultivars, namely, "Molfetesse" and "Galatina", has been performed, and it was found that two TFs showed correlation with the expression levels of GAS/GAO and the STL content. One of these TFs belongs to the MYB TF family and its levels correlate positively with GAS/GAO expression. The other is a bHLH TF and its expression correlates negatively with that of GAS/GAO. It has been proposed that these two TFs might regulate the biosynthesis of STL in chicory (Testone et al. 2016).

In *T. parthenium*, the accumulation of STL seems to be regulated by MeJ and salicylic acid (SA). Plants treated with MeJ and SA resulted in the upregulation of genes of the MVA and MEP pathways. The transcript levels of *HMGR*, *DXR* and *HDR* increased upon MeJ and SA addition. MeJ-treated plants showed higher expression levels of these genes than SA-treated plants and in both parthenolide accumulation was higher than in control plants (3.1- and 1.96-fold, respectively) (Majdi et al. 2015). The earlier pathways of terpene biosynthesis also presented a spatial and temporal regulation. The genes involved in these pathways were highly expressed in glandular trichomes and flowers; only the expression levels of HMGR were higher in young leaves than in older leaves. The transcript levels of GAS, GAO, COS and PTS, enzymes involved in parthenolide biosynthesis, were induced by the action of MeJ and SA; however this induction was not done coordinately. The expression of *GAS* showed a strong correlation with parthenolide accumulation, which means that GAS could be a crucial step in parthenolide biosynthesis. This correlation was also observed in glandular trichomes and flowers where this STL reached its maximum concentration (Majdi et al. 2011, 2014).

The first attempts to study the regulation of artemisinin biosynthesis were carried out in different plant systems such as in vitro cultures (suspension, hairy roots and seedlings) and plants. Different abiotic and biotic elicitors and other factors such as light and plant development were demonstrated to affect artemisinin production. MeJ, chitosan and fungal pathogen homogenates induced artemisinin production in plant and in in vitro cultures. An increase in artemisinin accumulation was observed in hairy root cultures of *A. annua* treated with *Piriformospora indica* homogenates and MeJ. The addition of *P. indica* extracts induced the transcription of several genes involved in artemisinin biosynthesis: *AaHMRG* (MVA), *AaDXS* and *AaDXR* (MEP) and *AaADS*, *AaCYP71AV1*, *AaALH1* and *AaDBR2* (STL pathway). Similar results were obtained with MeJ elicitation, although *AaDXS* was downregulated (Ahlawat et al. 2014). The addition of both elicitors induced the transcription of all mentioned genes with the exception of *AaDXS* and *AaDBR2*. *AaHMRG* showed the highest increase of transcript levels in all the treatments assayed, which corroborates its role as a rate-limiting step. Lei et al. (2011) have observed that the foliar application of chitosan induced the accumulation of artemisinin and dihydroartemisinin compared

with non-treated plants. Chitosan also activated the transcription of STL pathway genes *AaADS*, *AaCYP71AV1* and *AaDBR2*, even though they showed a different expression response over time. The upstream pathways of STL biosynthesis were also positively affected by chitosan. The expression levels of *HMGR* (MVA) increased 2 h after the addition of chitosan, while the levels of FDS transcripts were slightly increased. The *DXS*, *DXR* and *HDR* genes of the MEP pathway were all induced by chitosan. Light was another environmental factor that improved artemisinin accumulation in hairy roots. Increase of light intensity and the period of light irradiation resulted in an increase in the artemisinin content (Liu et al. 2002). The production of artemisinin is also affected by sugars. In fact, *A. annua* seedlings grown in the presence of glucose showed a higher artemisinin content than seedlings grown on sucrose or fructose. Glucose induced the expression of genes encoding the first enzymes involved in the MVA and MEP pathways. *HMRG*, *FDS*, *DXS* and *DXR* were induced after glucose feeding, as compared to control seedlings growing on sucrose. In the same way, the expression levels of *ADS* and *CYP71AV1* were higher than control seedlings. Based on these results, the authors suggested that the carbon source can act as signal involved in the regulation of artemisinin biosynthesis. Arsenault et al. (2010a) have analysed artemisinin production, trichome density and gene expression in the phase of growth that goes from vegetative through reproductive. They found that there was a good correlation between higher levels of trichome density in leaves and artemisinin accumulation. Leaves from the reproductive phase showed a higher density of trichomes per area as well as artemisinin content than those of the vegetative phase. When artemisinin and artemisinic acid (100 μg/ml) were sprayed over vegetative leaves, transcript levels of *CYP* were decreased by both treatments while the expression of ADS was negatively affected only by AA. These results suggested that the expression of ADS and CYP71AV1 is regulated by a negative feedback control mediated by AA and, to a lesser extent, by artemisinin (Arsenault et al. 2010b).

Jasmonate (Ja) and MeJ have been shown to elicit the production of secondary metabolites in many plant species, and the response is usually regulated by jasmonate-responsive APETALA2/ethylene response factor (AP2/ERF) TFs (Shen et al. 2016a, b). It has been observed that MeJ induced artemisinin production in plants and plant cell tissue cultures of *A. annua*. A screening from a GST cDNA library resulted in the isolation of two encoding sequences presenting AP2 conserved domains, *AaERF1* and *AaERF2*. After MeJ treatment, both ERF1 and ERF2 were localised in the nucleus and their expression patterns correlated with those of *ADS* and *CYP71AV1*, which are two genes involved in artemisinin biosynthesis. Moreover, yeast one-hybrid and electrophoretic mobility shift assay (EMSA) analyses showed that both TFs bind to conserved sequence motifs of *ADS* and *CYP71AV1* promoters. The overexpression of both TFs resulted in plants with higher artemisinin and artemisinic acid contents in mature leaves of 3-month-old plants, which correlated with higher transcript levels of *ADS* and *CYP71AV1*, as compared to control plants. On the other hand, the silencing of *AaERF1* and *AaERF2* by a RNA interference (RNAi) approach led to lower levels of STL accumulation as well as decreased *ADS* and *CYP71AV1* transcript levels. The experiments undoubtedly proved that AP2/ERF TFs regulate artemisinin accumulation and *ADS* and *CYP71AV1* expression in MeJ-treated plants (Yu et al. 2012).

Lu et al. (2013b) have isolated another AP2/ERF-type TF named *AaORA* from a cDNA library of *A. annua* leaves, which showed high homology sequence with ORCA3, an APE/ERF TF from *Catharanthus roseus*. The profile expression of *AaORA* in different plant tissues was similar to those of *ADS*, *CYP71AV1* and *DBR2*, showing higher transcript levels in bud flowers than in leaves, stems and roots. *AaORA* was specifically expressed in glandular trichomes. The overexpression of *AaORA* induced the transcription of higher levels of *ADS*, *CYP71AV1* and *DBR2* mRNAs, as compared to control lines. Since the *AeERF1* but not the *AaERF2* gene was also upregulated in transgenic plants, the authors hypothesised that AaORA might trigger artemisinin production through AaERF1.

Ji et al. (2014) have observed that *ADS* and *CYP71AV1* promoters also contained an E-box element with a putative binding site for bHLH TF and searched for candidate sequences in the GST cDNA library mentioned above. The screening resulted in the isolation of an ORF sequence encoding an AabHLH1 TF. The expression pattern of *AabHLH1* revealed that it was highly expressed in flowers and less expressed in leaves, stems and roots. The authors also studied the effect of abscisic acid (ABA) and chitosan on *AabHLH* and *ADS* expression. They observed that the foliar application of ABA (100 mg/l) and chitosan (150 mg/l) induced *AabHLH* and *ADS* expression coordinately. An EMSA analysis confirmed that AabHLH1 binds to E-boxes present in *ADS* and *CYP71AV1* promoters. Furthermore, the overexpression of *AabHLH1* performed by a transient expression experiment showed that the transcript levels of *ADS* and *CYP71AV1* were increased. The transient expression assays revealed that the upstream *AaHMGR* gene of the MVA pathway was also activated. The results obtained showed that bHLH1 TF positively regulates artemisinin biosynthesis.

WRKY TFs are involved in the regulation of different processes in plants such as defence, embryogenesis and secondary metabolite biosynthesis. Ma et al. (2009a) have explored the GST of *A. annua* cDNA library and found a specific cDNA sequence expressed in GST (*AaWRKY1*) that showed homology with other WRKY TFs. In *A. annua*, high expression levels of *AaWRKY1* were found in GST, while in flowers, leaves and roots, the expression was much lower. When *A. annua* leaves were treated with MeJ and chitosan, the expression of *AaWRKY1* was rapidly induced, and, in both cases, it preceded the upregulation of *ADS*. An EMSA analysis showed that AaWRKY1 binds to the W-box motif also present in the *AaADS* promoter. The transient expression of *AaWRKY1* in *A. annua* plants induced the expression of other genes of the STL pathway such as *CYP71AV1*, *HMGR* and *DBR2*. This finding allowed speculating that their promoters also contain W-boxes, although this hypothesis was not corroborated by the authors. The *FDS* expression was not induced by the transient expression of *AaWRKY1*. It has also been observed that the overexpression of *AaWRKY1* in tobacco upregulated the gene expression of the 5-epi-aristolochene synthase (EAS4), which is a STPS (Ma et al. 2009b).

The basic leucine zipper TF (bZIP) family has been described before to play an important role in ABA signalling regulation in plants. They bind to *cis*-element motifs named ABA-responsive elements (ABREs). By means of a sequence database study, Zhang et al. (2015) have identified a bZIP TF (*AabZIP1*) probably involved in ABA signalling and highly expressed in GST. The levels of *AabZIP1* mRNA were found to be higher in flowers and flower buds than in leaves, stem and roots. Different

types of abiotic stress such as drought and saline triggered the expression of high levels of *AabZIP1* in the same fashion as ABA-treated plants, thus corroborating the participation of this TF in the regulation of stress responses. It has also been demonstrated that AabZI1P binds to *ADS* and *CYP71AV1* promoters. Transgenic plants overexpressing *AabZIP1* have shown higher mRNA levels of different genes involved in the artemisinin pathway such as *ADS*, *CYP71AV1*, *DBR2* and *ALDH1*. The authors also observed that ABA induced the upregulation of both *AaERF1* and *AaERF2*, which suggests a possible cross-talk between ABA and Ja signalling.

Recently, the TRICHOME AND ARTEMISININ REGULATOR 1 (TAR1), which is another TF from the AP2/ERF family, has been isolated from GST of *A. annua*. TAR1 plays an important role in trichome development and artemisinin biosynthesis. As expected, TAR1 showed high expression levels in bud flowers and young leaves and particularly in GSTs and in apical meristems. Transgenic plants that constitutively overexpressed TAR1 accumulated more artemisinin, DAA and AA than their non-transgenic counterparts. Moreover, TAR1 activated the expression of *ADS* and *CYP71AV1* but not that of the genes encoding the key enzymes of the MVA (HMGR) and MEP (DXS and DXR) pathways (Tan et al. 2015). An EMSA analysis has revealed that TAR1 was able to bind *cis*-element present in *ADS* and *CYP71AV1* promoters. The authors observed that silencing of TAR1 by RNAi caused a decrease in the artemisinin content in *A. annua* plants, which correlated with the low expression levels of *TAR1*, *ADS*, *CYP71AV1* and *DBR2*. On the other hand, the expression of *DXS* and *DXR* (MEP pathway) was increased. TAR1-RNAi plants also showed abnormal trichome development (Tan et al. 2015).

In summary, several TFs positively regulate artemisinin biosynthesis; they mediate plant development and the response to different stimuli such as phytohormones (ABA, MeJ) and biotic (pathogens and chitosan) and abiotic types of stress (drought and saline). They mainly activate the expression of two key enzymes of the STL pathway, ADS and CYP71AV1 and, to a lesser extent, DBR2 and ALDH1. ADS and CYP71 have been previously described as rate-limiting enzymes of the artemisinin biosynthetic pathway, and they seem to be tightly regulated by these TFs. Although there has been a great advance in the understanding of how these TFs regulate artemisinin biosynthesis, the interaction between them seems to be a complex regulation network that remains unknown.

4.4 Biotechnological Approaches for the Production of Sesquiterpene Lactones: Plant Cell Culture

Many medicinal and aromatic plants are usually slow-growing species, and their products are harvested from wild or agricultural crops with low yields. They are also affected by environmental conditions such as drought, climate changes, pathogens, fires and other natural disasters. Due to these factors, their supply for the industry may become irregular and non-homogenous (Atanasov et al. 2015). In addition, numerous MAP species are difficult to cultivate massively using a conventional

agricultural program, and 20% of them are considered to be in danger of extinction (Kolewe et al. 2008). The chemical synthesis of most of these secondary metabolites, especially STLs, is feasible but commercially non-viable (Wilson and Roberts 2012). The increased demand for these products requires a production platform that can satisfy a continuous supply, a homogeneous production, GMP requirements and a low environmental impact (Rea et al. 2011). In vitro plant cell culture (PCC) has emerged as an alternative production system for many plant secondary metabolites since it can meet all the requirements mentioned above. PCC includes micropropagation, suspension cells and organ cultures (shoots and roots). Micropropagation is a well-established industrial production system for many ornamental, vegetable, forestry and fruit species (Pence 2011). Plant cell suspension cultures (undifferentiated cells) also offer a continuous and homogenous product supply in short growth cycles. The PCC process can be developed in traditional industrial bioreactors used for microorganism or mammalian cell cultures (Atanasov et al. 2015; Eibl and Eibl 2008; Eibl et al. 2009). In many cases, undifferentiated cell cultures usually present low productivities because secondary metabolite accumulation is associated with tissue or organ differentiation. Besides, these cultures are genetically unstable (Mora-Pale et al. 2013). Therefore, the establishment of organ culture shoots and roots constitutes an interesting alternative. These organ cultures usually accumulate secondary metabolites at the same level and pattern as the parent plant. Organ root cultures are genetically and biochemically stable and have become an attractive system to produce secondary metabolites, since the biosynthesis of many of them occurs in the roots. Two kinds of root cultures can be employed: adventitious and hairy roots (Talano et al. 2012). Adventitious root cultures are established after excision of the root from the entire plant and the addition of phytohormones in the culture medium. CBN (Korea) is a company that produces adventitious roots of *Panax ginseng* (ginseng saponins) at a scale of 10 m^3 (Baque et al. 2012). Hairy root cultures are obtained by infection of plant explants with *Agrobacterium rhizogenes*. These cultures can produce high amounts of secondary metabolites and grow at high rates without the addition of growth regulators (Talano et al. 2012). The Swiss company ROOTec Bioactives AG (currently part of Green2Chem, Belgium) has developed a technology for the production of plant natural compounds based on hairy root cultures. The production of secondary metabolite by PCC can also be improved by the manipulation of culture conditions that include inoculum size and age, minerals, carbon source, light, addition of precursors, pH and temperature. Elicitation is another strategy to enhance the production of secondary metabolites. Elicitors are stress conditions or molecules that activate secondary metabolite biosynthesis in plants. MeJ, chitosan, SA, coronatine, yeast extracts and heavy metals, among others, have been extensively used to improve secondary metabolite productivities (Murthy et al. 2014; Wilson and Roberts 2012). These approaches can be combined with in situ product removal (ISPR) strategies that consist of the addition of a second phase in the culture medium. The second phase can be either liquid or solid; in that way, the compounds of interest are incorporated into the second phase and are easily separated from the culture medium (Wilson and Roberts 2012). Moreover, feedback inhibition by product accumulation can be avoided.

Table 4.1 Different biochemical strategies used to increase artemisinin production in plant cell and tissue cultures

Cell culture	Treatment	Artemisinin production	Reference
Hairy roots	Chitosan MeJ Yeast extract	1.8 mg/g dry weight 1.5 mg/g dry weight 0.9 mg/g dry weight	Putalun et al. (2007)
Hairy roots	Cerebroside elicitor Cerebroside elicitor + SNP	2.1 mg/g dry weight (16.3 mg/l) 2.2 mg/g dry weight (22.4 mg/l)	Wang et al. (2009)
Hairy roots	Oligosaccharide elicitor Oligosaccharide elicitor + SNP	1.3 mg/g dry weight (12 mg/l) 2.2 mg/g dry weight (28.5 mg/l)	Zheng et al. (2008)
Suspensions	Precursor addition Yeast elicitor Combined	56 mg/l 76 mg/l 115 mg/l	Baldi and Dixit (2008)
Suspensions	Cyclodextrin Cyclodextrin + MeJ	25.2 μmol/g dry weight 27.5 μmol/g dry weight	Durante et al. (2011)
Suspensions	MeJ Miconazole (inhibitor)	14.4 μg/g dry weight 13 μg/g dry weight	Caretto et al. (2011)
Hairy roots	MeJ *P. indica* extracts *P. indica* extracts + MeJ FPP (precursor)	(13.3 mg/l) (15.6 mg/l) (19.0 mg/l) (10.3 mg/l)	Ahlawat et al. (2014)

A. annua plant cell and tissue cultures have been extensively studied as a continuous source of artemisinin, and, in order to produce it at industrial scale, many research groups have employed different plant cell platforms to achieve this goal (Table 4.1). Suspension cell, shoot and hairy root cultures have been used to produce the antimalarial drug. These cultures have been combined with different strategies to improve artemisinin productivities, and some of the variables analysed are summarised in Table 4.1. Elicitation has been the most common, simple and successful approach to improve artemisinin production. Chitosan, MeJ, oligosaccharides and fungal extracts have demonstrated to increase the artemisinin content, as compared to non-treated cultures; in general the artemisinin contents reached values of 1–2.5 mg/g dry weight and concentrations that varied from 10 to 120 mg/l (Putalun et al. 2007; Wang et al. 2009; Zheng et al. 2008; Ahlawat et al. 2014; Durante et al. 2011). The addition of precursors of the artemisinin pathway as well as inhibitors of the competitive steroid pathway has also resulted in higher levels of artemisinin accumulation (Baldi and Dixit 2008; Caretto et al. 2011).

Hairy root cultures have been grown in bioreactors with different configurations (Table 4.2) such as bubble column, airlift, modified stirred tank and mist reactor. Mist bioreactor has shown a better performance than the others for both growth and secondary metabolite production (Patra and Srivastava 2014, 2016; Souret et al. 2003). Moreover, *A. annua* shoot cultures grown in a mist reactor also resulted in an enhanced biomass yield and artemisinin production (3.3- and 1.4-fold) than those grown in a bubble column and multiplate flow reactor (Liu et al. 2003).

Hairy root cultures of *V. officinalis* were also the PCC system chosen for the production of valerenic acid. Transformed roots elicited with MeJ and a *Fusarium graminearum* extract resulted in an increased valerenic acid production that was

Table 4.2 Bioreactor configurations employed in artemisinin production by plant cell and tissue cultures

Cell culture	Bioreactor	Artemisinin Production	Reference
Hairy roots	Bubble column (3.0 l) Mist reactor (3.0 l)	0.27 mg/g dry weight 1.12 mg/g dry weight	Patra and Srivastava (2016)
Hairy roots	Bubble column (1.5 l) Mist reactor (1.5 l)	0.14 μg/g fresh weight 0.29 μg/g fresh weight	Souret et al. (2003)
Hairy roots	Shake flask Modified stirrer tank (1.5 l)	(3.4 mg/l) (4.3 mg/l)	Patra and Srivastava (2016)
Hairy roots	Bubble column (1.5 l) Mist reactor (1.5 l)	2.94 μg/g dry weight 0.98 μg/g dry weight	Kim and Keasling (2001)
Shoots	Modified airlift Multiplate flow reactor Mist reactor (0.4 l)	3.5 mg/g dry weight (14.6 mg/l) 3.2 mg/g dry weight (34.4 mg/l) 1.8 mg/g dry weight (48.2 mg/l)	Liu et al. (2003)

6-fold (2.3 mg/g dry weight) and 12-fold (3.02 mg/g dry weight) higher, respectively, than non-elicited roots (0.24 mg/g dry weight). The addition of Ca^{2+} and Mg^{2+} as abiotic elicitors led to an increase in valerenic acid accumulation that was 8-fold (1.83 mg/g dry weight) and 4.2-fold (1.1 mg/g dry weight) higher than control cultures (0.23 mg/g dry weight). The authors considered the use of these salts as a cost-effective strategy for large-scale production (Torkamani et al. 2014a, b).

Chicory hairy root cultures were found to produce 1.4% dry weight of 8-deoxylactucin glucoside. This amount is two orders of magnitude higher than that produced by roots of chicory plants (Malarz et al. 2002). The accumulation of this STL seems to be affected by light, since hairy roots incubated in the dark produced lower levels (0.88% dry weight) than those of hairy roots incubated under light conditions (1.37% dry weight). In conclusion, hairy roots of chicory could be an alternative system for the production of 8-deoxylactucin glucoside, although these cultures presented a different pattern of STLs after many years of subculture. In *Rudbeckia hirta* hairy roots and suspension cell cultures, light has also been essential to induce the production of pulchelin E (Luczkiewicz et al. 2002). The contents of pulchelin E were higher in transformed roots (14.0 mg/g dry weight) than in suspension cultures (9.0 mg/g dry weight).

Despite all the progress made in the field of secondary metabolite production by PCC, few processes have reached an industrial scale. The main reasons could be the low intrinsic productivities of PCC and the fact that the biosynthetic pathways that lead to secondary metabolite production and their regulation have not been fully elucidated yet.

4.5 Metabolic Engineering

Metabolic engineering comprises several strategies that use DNA technology to modify metabolic networks (Paddon and Keasling 2014). Among these strategies, it is possible to (1) overexpress one or various enzymes of the metabolic pathway (generally those involved in rate-limiting steps), (2) downregulate enzymes at

metabolic branching points in order to increase the carbon flux towards the selected pathway, (3) overexpress TFs involved in the regulation of the biosynthetic pathway (in order to increase the expression of several enzymes at the same time) and (4) overexpress other proteins that can indirectly affect STL accumulation (e.g. oncogenes, biosynthetic enzymes of STL-regulating molecules, etc.) (Paddon and Keasling 2014; Majdi et al. 2016).

4.5.1 Metabolic Engineering in Plants and Plant Cell Culture

Liu et al. (2014) have been able to isolate and characterise the full biosynthetic pathway that led to parthenolide production. As mentioned above, four genes are responsible for parthenolide accumulation from the universal precursor, FPP in *T. parthenium*: *TpGAS*, *TpGAO*, *TpCOS* and *TpPTS*. The transient expression of the first three genes in *N. benthamiana* plants resulted in the accumulation of costunolide (9.6 μg/g fresh weight). The LC-QTOF-MS analysis of the transformed leaves has revealed that costunolide was found in two conjugated forms with cysteine and glutathione (GSH). In a further experiment, a transient expression of the four genes (*TpGAS*, *TpGAO*, *TpCOS* and *TpPTS*) with *AtHMGR* resulted in the production of free parthenolide to a level up to 2 ng/g fresh weight. The low levels presented were due to the conjugation of parthenolide with cysteine (1368 ng/g fresh weight) and GSH (88 ng/g fresh weight). The co-expression of *AtHMGR* was necessary to increase the carbon flux towards the STL biosynthesis. In *T. parthenium*, costunolides and parthenolides are accumulated in their free forms in glandular trichomes. The presence of the conjugated forms of both STLs in *N. benthamiana* may be a detoxification mechanism. Since parthenolides and costunolides accumulate specifically in trichomes, the authors proposed directing the expression of the full pathway to trichomes to avoid formation of conjugates. In order to improve parthenolide production, the authors suggested that chicory or lettuce could be better heterologous platforms since they naturally accumulate costunolide derivatives in special structures like laticifers located all over the plant. It is interesting to mention that the conjugated versions of parthenolides, i.e. parthenolide-cysteine and parthenolide-GSH, have also shown anticancer activities against different tumours albeit at lower levels than the free parthenolide.

Recently, metabolic engineered hairy roots of *V. officinalis* that overexpress *VoFDP* and *VoVDS* have been established. These genes encode two enzymes involved in valerenic acid and valerenal biosynthesis. The transgenic lines obtained showed higher transcript levels of *VoFDP* and *VoVDS* than control lines although there were differences in the mRNA levels among them. Transgenic lines overexpressing *VoFDP* presented higher levels of valerenadiene, valerenal and ß-caryophyllene, which is another STL that competes for the common substrate FPP. The higher STL levels could be due to the increment in FPP cytosolic supply. Recombinant hairy roots overexpressing *VoVDS* showed 1.5- to 4-fold increased valerenic acid contents than the control line and, to a lesser extent, in valerenal and valerenadiene (Ricigliano et al. 2016). On the other hand, the production of ß-caryophyllene was not affected.

MeJ elicitation of all transgenic lines produced an increment of STL content, as compared to non-elicited cultures. These results revealed that other enzymatic steps or regulation processes could be limiting STL accumulation.

It is known that the STL biosynthesis and the reaction catalysed by FDS that is responsible for the FPP supply occur in the cytosol. The overexpression of STPs in the cytosol has been the general strategy to increase STL accumulation in transgenic plants, although in many cases, this strategy did not result in higher STL contents. Wu et al. (2006) have hypothesised that the low amounts of STLs registered could be due to a tight regulation at different levels of the biosynthetic pathway. With the aim of corroborating this hypothesis, they redirected the overexpression of FDS (avian source) and the enzyme patchoulol synthase (PTS) to chloroplasts of *Nicotiana tabacum (*Wu et al. 2006). PTS is a sesquiterpene synthase that converts FPP into patchoulol, a main compound of the patchouli essential oil fragrance. Several expression vectors were constructed; *FDS* and *PTS* genes were cloned under the control of strong constitutive promoters with or without a plastid targeting signal sequence (tp). Plastid-targeted enzymes resulted in transgenic plants with 10–30 μg/g fresh weight of patchoulol contents, while the cytosolic engineering of the pathway in plant cells never exceeded 10–12 μg/g fresh weight. The expression of these enzymes in plastid also resulted in the presence of many new STLs that have never been described in *Nicotiana* plants. The same strategy was carried out with *FDS* and *ADS* from *A. annua*, improving amorpha-4,11-diene accumulation to levels of 25 μg/g fresh weight, which are several orders of magnitude higher than those found in transgenic plants with ADS targeted to cytosol (Wu et al. 2006). This redirection of the carbon flux of isoprenoid precursors is an interesting approach to increase natural products accumulation in plants.

4.5.2 Metabolic Engineering of the Artemisinin Biosynthetic Pathway

Since the content of artemisinin in *A. annua* is extremely low (between 0.01% and 0.8% of the plant dry weight), several strategies, briefly described in Sect. 4.4, have been applied to enhance artemisinin accumulation. Moreover, metabolic engineering for artemisinin production became a model system not only for the production of STLs but also for other plant secondary metabolic pathways.

4.5.2.1 Overexpression of Genes Involved in the Artemisinin Biosynthetic Pathway

Several authors have reported the overexpression of one or various enzymes involved in artemisinin biosynthesis, not only the specific enzymes responsible for artemisinin synthesis but also those involved in the pathways that generate the precursors of its production (Table 4.2).

One of the enzymes that were overexpressed was the FDS. There is an early report of the expression of a foreign copy of *FDS* (from *Gossypium arboreum*) in hairy roots of *A. annua*, by *A. rhizogenes* genetic transformation. This strategy resulted in an enhanced accumulation of artemisinin in transgenic hairy roots, as compared to control hairy roots (between ~2.5 and 3.0 mg/g dry weight, as compared to ~0.75 mg/g dry weight, respectively) (Chen et al. 1999). The same enzyme expressed in *A. annua* plants resulted in increased levels of artemisinin (~8–10 mg/g dry weight) in five transgenic lines, as compared to those in control lines (~ 3 mg/g dry weight) (Chen et al. 2000). Other authors have reported the overexpression of FDS from *A. annua*, resulting in three transgenic lines with higher artemisinin content (~0.8–0.9% dry weight), as compared to control lines (~0.65% dry weight) (Han et al. 2006). Another enzyme that has been a target for metabolic engineering was HMGR. Some authors have reported the expression of HMGR from *C. roseus* L. in *A. annua* plants, resulting in higher artemisinin content (up to 0.6 mg/g dry weight, as compared to untransformed lines rendering 0.37 mg/g dry weight) (Nafis et al. 2011). Other authors have reported increases between 17.1 and 22.5%, as compared to control lines (Aquil et al. 2009). In a more recent report, *HMGR* and *FDS* were overexpressed in *A. annua* plants. At least five of the clones analysed showed a higher artemisinin content, as compared to non-transgenic plants. The highest artemisinin accumulation was near 9 mg/g dry weight (1.8-fold increase, as compared to controls) and was accompanied by higher *HMGR* and *FDS* transcript levels, as compared to controls (2.80-fold and 3.68-fold, respectively) (Wang et al. 2011).

Ma et al. have evaluated the terpenoid metabolic profile in a transgenic *A. annua* plant that overexpressed *ADS*. They found that this line showed higher accumulation of artemisinin and artemisinic acid (both ~1.2 mg/g dry weight), as compared to GUS-expressing plants (~0.6 and ~0.8 mg/g dry weight, respectively) (Ma et al. 2009).

Successful results were obtained either overexpressing genes from the general pathway or genes for the specific artemisinin biosynthetic pathway. For instance, the overexpression of *DXR* and co-overexpression of *CYP71A1* and *CPR* in *A. annua* plants have been reported. All transgenic lines showed higher artemisinin content, between 0.62 and 1.21 mg/g dry weight for plants overexpressing *DXR*, as compared to 0.52 mg/g dry weight obtained in control lines (1.21- to 2.35-fold increases), and between 1.46 and 2.44 mg/g dry weight in plants overexpressing *CYP71AV1* and *CPR*, as compared to 0.91 mg/g dry weight of control lines (1.61- to 2.69-fold increases) (Xiang et al. 2012). In another report dealing with the co-overexpression of *CYP71AV1* and *CPR*, four transgenic lines showed higher artemisinin content (~0.9–1.0 mg/g dry weight), as compared to controls (~0.7 mg/g dry weight), and were accompanied by higher transcript levels of the overexpressed genes, although the increases were different among lines and among genes (Shen et al. 2012).

In the investigation carried out by Alam et al., *HMGR* and *ADS* were co-expressed in *A. annua* plants. Higher levels of *HMGR* and *ADS* transcripts were found in transgenic lines, and in three selected ones, the artemisinin content was ~1.6–1.8 mg/g dry weight, as compared with the 0.2 mg/g dry weight detected in control plants, which represents a ~7-fold increase (Alam and Abdin 2011).

Some authors have addressed this subject by multiple gene engineering, by the co-overexpression of three enzymes, one from the FDS pathway and two from the specific artemisinin pathway. For instance, *FDS*, *CYP71AV1* and *CPR* were overexpressed in *A. annua* plants, resulting in higher expression levels of these genes, although to a different extent among them and among the different transgenic lines obtained. Six transgenic lines showed a significant increase in the artemisinin content, from 1.40 mg/g fresh weight (~1.7-fold) up to 2.98 mg/g fresh weight (~3.6-fold), as compared to 0.83 mg/g fresh weight obtained with the control line (Chen et al. 2013).

Another interesting work on artemisinin production was that performed by Lu et al., who were able to overexpress *ADS*, *CYP71AV1* and *CPR* genes in *A. annua* plants. Although the mRNA levels corresponding to the three genes varied between the eight transgenic lines tested, all of them showed increased artemisinin content, as compared to the control line (between 1.5- and 2.4-fold increases) (Lu et al. 2013a).

Substrate channelling is a relatively new strategy analysed to increase artemisinin production. This strategy is based on the fact that the immediate transference of the product of one enzyme to the next one in a biosynthetic pathway can improve reaction rates. In a recent work, *ADS* has been expressed as a fusion protein with FDS in two high artemisinin-producing varieties of *A. annua* (var. Chongqing and var. Anamed), under the control of either CaMV35S or *CYP71AV1* promoter. In both lines, the fusion gene rendered higher transcript levels under the control of the CaMV35S promoter (constitutive) than under the *CYP71AV1* promoter (tissue-specific). As compared to control lines, the transcript levels of *ADS* were higher than those of *FDS*, possibly because *FDS* is highly expressed in wild-type plants. The artemisinin content was increased up to 2–2.5 times in both varieties (around 2.5% dry weight) when the fusion gene was specifically expressed in GST (i.e. under *CYP71AV1* promoter), whereas the CaMV35S promoter was not as effective in improving the artemisinin content (Han et al. 2016).

4.5.2.2 Blockage of Metabolic Competing Routes

Another strategy that has been employed to enhance artemisinin production was the downregulation of metabolic routes that compete with the artemisinin biosynthetic pathway for a common precursor. In the case of artemisinin biosynthesis, FPP becomes a branch point, since it is the precursor of sterol and sesquiterpene biosynthesis. This approach was carried out by Wang et al. who evaluated the suppression of the expression of squalene synthase (SQS), the first enzyme in sterol biosynthesis, by expressing an antisense copy of this enzyme in *A. annua* plants. In two transgenic lines, *SQS* expression was reduced, as compared to the control; and the artemisinin content was increased by 22–23%, as compared to the control line (Wang et al. 2012). Zhang et al. have also evaluated the effect of *SQS* suppression by hairpin-RNA-mediated RNAi technique. The expression of *SQS* was significantly suppressed in some of the transgenic lines obtained (up to 60%), resulting in a decrease in the levels of the sterols campesterol, stigmasterol, *β*-sitosterol and

ergosterol, whereas the artemisinin content was increased up to 31.4 mg/g dry weight (~3.14-fold higher than that of control plants) before the flowering state (Zhang et al. 2009). In another work, β-caryophyllene synthase (CPS) was suppressed by antisense technology. This enzyme is one of the sesquiterpene synthases that competes for the FPP pool. The antisense fragment of *CPS* cDNA was delivered into *A. annua* by *A. tumefaciens*, and the transgenic lines were evaluated in terms of the expression of related genes and the accumulation of related metabolites. As expected, *CPS* expression, as well as the β-caryophyllene content, was reduced in all transgenic lines, whereas the *HMGR*, *FDS*, *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* expressions were enhanced. This was attributed to the high FPP availability, which induced the expression of these genes. The artemisinin production was increased in transgenic lines up to 54.9%, as compared to control lines, and was accompanied by both an increase in dihydroartemisinic acid production (probably due to the higher expression of artemisinin-related enzymes) and a decrease in artemisinic acid content (Chen et al. 2011).

In this sense, Lv et al. have evaluated the effects of the downregulation of the four enzymes that compete with ADS for FPP in plants: CPS, BFS, GAS (STPS) and SQS (Table 4.2). In all the different transgenic lines obtained, artemisinin was increased, as compared to the control line (77, 77, 103 and 71% in anti-CPS, anti-BFS, anti-GAS and anti-SQS plants, respectively). Similar results were obtained for the dihydroartemisinic acid content (increases of 132, 54, 130 and 223% in anti-CPS, anti-BFS, anti-GAS and anti-SQS plants, respectively). The highest artemisinin content in transgenic lines was ~12 mg/g dry weight (Lv et al. 2016b).

4.5.2.3 Overexpression of Transcription Factors

As mentioned above, the expression of a TF that regulates a whole metabolic pathway is a strategy to increase the amount of a certain compound. As described in Sect. 4.3, several TFs have been described to be involved in the regulation of the artemisinin biosynthesis. Most of them belong to the AP2/ERF family, such as AaORA, AaERF 1 and AaERF2 and TAR1 (Tan et al. 2015).

The characterisation of AaERF1 and AaERF2 has allowed determining that these TFs interact with specific motifs in *ADS* and *CYP71AV1* promoters. Their overexpression in *A. annua* enhanced the accumulation of artemisinin and artemisinic acid (by 19–67% and 11–76% in plants overexpressing *AaERF1* and by 24–51% and 17–121% in plants overexpressing *AaERF2*, respectively) (Yu et al. 2012). The overexpression of *AaORA* led to an increase in its own transcript levels (between 4–14-fold), as well as those of *ADS*, *CYP71AV1*, *DBR2* and *AaERF1* (around 6–13-, 5–17-, 4–13-and 4–12-fold, respectively). The production of artemisinin and dihydroartemisinic acid was higher in transgenic plants, as compared to control plants (by 40–53% and 22–35%, respectively) (Lu et al. 2013b). On the other hand, the overexpression of TAR1 led to an increase of the corresponding RNA levels (by 12- to 23-fold), and higher artemisinin, dihydroartemisinic acid and artemisinic acid content in leaves (22–38%, 69–130% and 28–164%, respectively) and in flower buds (34–57%, 22–79% and 12–61%, respectively) (Tan et al. 2015).

The AabZIP1 TF has been overexpressed in *A. annua*, resulting in an increase of the corresponding mRNA levels, as compared to those of wild-type (between 20- and 35-fold). The increases in the transcript levels of *ADS* and *CYP71AV1* (6- to 8-fold) were accompanied by an increment in artemisinin and dihydroartemisinic acid production in the transgenic lines by 0.7- to 1.5-fold and 0.3- to 0.8-fold, as compared to the control line, respectively (Table 4.3) (Zhang et al. 2015).

AaWRKY1 is another TF that has been isolated from *A. annua*. Its overexpression (50- to 90-fold increased expression) led to the increase in RNA levels of *ADS* and *CYP71AV1* (1.5- to 3.0-fold and 4.4- to 14.0-fold, respectively) as well as a higher artemisinin content (1.3- to 2.0-fold), as compared to non-transgenic control plants (Jiang et al. 2016).

AaMYC2 is a TF activated by Ja, which is a positive regulator of artemisinin biosynthesis. As a consequence of its overexpression in *A. annua* plants, transcript levels of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* were increased (1.7- to 8.4-fold, 2.0- to 5.9-fold, 1.2- to 2.9-fold and 1.2- to 2.5-fold, respectively). While the artemisinic acid content was decreased, the artemisinin and dihydroartemisinic acid accumulation was enhanced (by 23–55% and 17–217%, respectively) (Shen et al. 2016a, b).

When the GLANDULAR TRICHOME-SPECIFIC WRKY 1 (AaGSW1) was isolated from *A. annua* and characterised, it was found that its overexpression led to an increase in the transcript levels of *CYP71AV1* (3- to 6-fold) and *ALDH1* (4- to 7-fold). Interestingly, the expression of *ADS* and *DBR2* was enhanced (3- to 5- and 2- to 3-fold, respectively), which was attributed to an increased *AaORA* expression (by 4-fold). Whereas artemisinin and dihydroartemisinic acid were higher in transgenic plants (increases of 55–100 and 50–60%, respectively), artemisinic acid was decreased (by 30–90%, respectively) (Chen et al. 2017).

Lv et al. have reported the overexpression of AaNAC1, which is a transcription factor belonging to the NAC superfamily in *A. annua*. The selection of this TF (from *A. annua* glandular trichome transcriptome) was made on the basis of its abundance and due to the fact that it was induced by SA and MeJ, which are known elicitors of artemisinin production (see Sects. 4.3 and 4.4). In some transgenic lines, the overexpression of *AaNAC1* was accompanied by a significant increase in the mRNA levels of *ADS*, *DBR2* and *ALDH1* but not of *CYP71AV1*, whereas those of *HMGR* remained unchanged. Nevertheless, the content of artemisinin and dihydroartemisinic acid was significantly increased in all transgenic lines (between 14% and 79% and between 30% and 150%, respectively) (Lv et al. 2016a). In a recent work, the *AaMYB1* TF was identified in *A. annua* and characterised. Plants overexpressing *AaMYB1* (either under the *CYP71AV1* or the CaMV35S promoter) showed an increase in artemisinin and dihydroartemisinic acid contents, when compared to control plants (~10 mg/g dry weight artemisinin and ~1.5 mg/g dry weight dihydroartemisinic acid). The accumulation of both compounds were higher in 35S::*AaMYB1* plants (systemic expression; up to ~20 mg/g dry weight artemisinin and up to ~5 mg/g dry weight dihydroartemisinic acid), as compared to p*CYP71AV1*::AaMYB1 plants (trichome expression; up to ~15 mg/g dry weight artemisinin and up to ~3 mg/g dry weight dihydroartemisinic acid). Genes related to artemisinin biosynthesis were upregulated in both 35S::*AaMYB1* and p*CYP71AV1*::AaMYB1 plants, especially *CYP71AV1* and *ADS* (Matías-Hernández et al. 2017).

Table 4.3 Metabolic engineering of the artemisinin biosynthetic pathway carried out in Artemisia annua plants or plant cell cultures

Gene(s)	Result	Reference
Overexpression of genes involved in artemisinin biosynthesis		
FDS gene (from *G. arboreum)* in *A. annua* hairy roots	Increased artemisinin content (between ~2.5 and 3.0 mg/g dry weight), as compared to control hairy roots (~0.75 mg/g dry weight)	Chen et al. (1999)
FDS gene (from *G. arboreum)* in *A. annua* plants	Higher artemisinin content (~8–10 mg/g fresh weight), as compared to control lines (~ 3 mg/g dry weight)	Chen et al. (2000)
FDS in *A. annua* plants	Increased artemisinin content in three transgenic lines, as compared to control (~0.8–0.9 and ~0.65% dry weight, respectively)	Han et al. (2006)
HMGR (from *C. roseus* L) in *A. annua* plants	Higher artemisinin content, up to 0.6 mg/g dry weight, as compared to untransformed lines (0.37 mg/g dry weight)	Nafis et al. (2011)
HMGR (from *C. roseus* L) in *A. annua* plants	Increase in artemisinin content, between 17.1 and 22.5%, as compared to control lines	Aquil et al. (2009)
HMGR and *FDS* genes in *A. annua* plants	Increased artemisinin content in nine transgenic lines, between 7 and 9 mg/g dry weight (up to 1.8-fold increase), as compared to control Higher *HMGR* and *FDS* transcript levels, as compared to control (up to 2.80-fold and 3.68-fold, respectively)	Wang et al. (2011)
ADS gene in *A. annua* plants	Higher artemisinin and artemisinic acid accumulation (both ~1.2 mg/g dry weight), as compared to control (~0.6 and ~0.8 mg/g dry weight, respectively)	Ma et al. (2009)
DXR, *CYP71AV1* and *CPR* in *A. annua* plants	Higher artemisinin content in plants overexpressing *DXR* (1.21- to 2.35-fold increases) Higher artemisinin content in plants overexpressing *CYP71AV*1 and *CPR* (1.61- to 2.69-fold increases)	Xiang et al. (2012)
CYP71AV1 and *CPR* in *A. annua* plants	Higher artemisinin content in four transgenic lines (~0.9–1.0 mg/g dry weight), as compared to control (~0.7 mg/g dry weight) Higher transcript levels of *CYP71AV1* and *CPR* (variations between lines and between genes)	Shen et al. (2012)
HMGR and *ADS* genes in *A. annua* plants	Increased artemisinin content (~7-fold increase) in three selected transgenic lines Higher levels of *HMGR* and *ADS* transcripts in transgenic lines	Alam and Abdin (2011)
FDS, *CYP71AV1* and *CPR* in *A. annua* plants	Higher artemisinin content (between 1.40 and 2.98 mg/g fresh weight), as compared to control (0.83 mg/g fresh weight) Higher expression levels of *FDS*, *CYP71AV1* and *CPR* (variable between genes and transgenic lines)	Chen et al. (2013)
ADS, *CYP71AV1* and *CPR* genes in *A. annua* plants	1.5- and 2.4-fold increases in artemisinin content	Lu et al. (2013a)

(continued)

Table 4.3 (continued)

Gene(s)	Result	Reference
ADS::FDS fusion gene in *A. annua* plants, under CaMV35S or *CYP71AV1* promoters	Higher transcript level of *ADS::FDS* gene under CaMV35S than under the *CYP71AV1* promoter Increased artemisinin content (2–2.5 times; around 2.5% dry weight) in plants expressing *ADS::FDS* fusion gene under *CYP71AV1* promoter	Han et al. (2016)
Inhibition of competing metabolic routes		
Suppression of *SQS* expression (antisense technology)	Increased artemisinin content (by 22–23%, as compared to that of control)	Wang et al. (2012)
Suppression of *CPS* expression (antisense technology)	Increase in artemisinin content (up to 54.9%, as compared to control lines) Higher expression of *HMGR*, *FDS*, *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* genes	Chen et al. (2011)
Suppression of *SQS* expression (hairpin-RNA-mediated RNAi)	Increased artemisinin content in some transgenic lines, up to 31.4 mg/g dry weight (~3.14-fold increase) Reduced SQS expression in some transgenic lines obtained (up to 60%)	Zhang et al. (2009)
Downregulation of *CPS*, *BFS*, *GAS*) and *SQS* in *A. annua* plants	Increased artemisinin content (77, 77, 103 and 71% in anti-CPS, anti-BFS, anti-GAS and anti-SQS plants, respectively) Increased dihydroartemisinic acid content (132, 54, 130 and 223% in anti-CPS, anti-BFS, anti-GAS and anti-SQS plants, respectively)	Lv et al. (2016b)
Overexpression of transcription factors		
AaERF1 and *AaERF2* in *A. annua* plants	Increased mRNA levels of *ADS* and *CYP71AV1* (2- to 8-fold and 1.2- to 5-fold, respectively) Enhanced artemisinin and artemisinic acid accumulation (19–67% and 11–76%, respectively, in plants overexpressing *AaERF1* and by 24–51% and 17–121%, respectively, in plants overexpressing *AaERF2*)	Yu et al. (2012)
AaORA in *A. annua* plants	Increased transcript levels of *ADS*, *CYP71AV1*, *DBR2* and *AaERF1* (around 6–13-, 5–17-, 4–13-and 4–12-fold, respectively) Higher artemisinin and dihydroartemisinic acid content in transgenic plants (by 40–53% and 22–35%, respectively)	Lu et al. (2013b)
TAR1 in *A. annua* plants	Higher artemisinin, dihydroartemisinic acid and artemisinic acid content in leaves (22–38%, 69–130% and 28–164%, respectively) and in flower buds (34–57%, 22–79% and 12–61%, respectively)	Tan et al. (2015)
AabZIP1 in *A. annua* plants	Increased transcript levels of *ADS* and *CYP71AV1* (6- to 8-fold) and of *DBR2* and *ALDH1* Increased artemisinin and dihydroartemisinic acid content (by 0.7- to 1.5-fold and 0.3- to 0.8-fold, as compared to control, respectively)	Zhang et al. (2015)

(continued)

Table 4.3 (continued)

Gene(s)	Result	Reference
AaWRKY1 gene in *A. annua* plants	Increase in mRNA levels of *ADS* and *CYP71AV1* Increase in artemisinin content (between 1.3- to 2.0-fold)	Jiang et al. (2016)
AaMYC2 in *A. annua* plants	Higher artemisinin (by 23–55%) and dihydroartemisinic acid content (17–217%) and lower artemisinic acid Increased expression of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1*	Shen et al. (2016a, b)
AaGSW1 in *A. annua* plants	Increased artemisinin and dihydroartemisinic acid content (55–100 and 50–60%, respectively). Lower levels of artemisinic acid (by 30–90%) Increased transcript levels of *AaORA*, *CYP71AV1*, *ALDH1*, *ADS* and *DBR2*	Chen et al. (2017)
AaNAC1 in *A. annua* plants	Increase in mRNA levels of *ADS*, *DBR2* and *ALDH1* Increase in artemisinin and dihydroartemisinic acid content (up to 79% and 150%, respectively)	Lv et al. (2016a)
AaMYB1 in *A. annua* plants	Increase in artemisinin and dihydroartemisinic acid contents (up to ~20 and ~5 mg/g dry weight, respectively), when compared to control Higher accumulation when *AaMYB1* was under CaMV35S promoter than *CYP71AV1* promoter Higher transcript levels *ADS* and *CYP71AV1* and, to a lesser extent, of *DBR2*, *FDS* and *ALDH1* in both 35S::AaMYB1 and *pCYP71AV1*::AaMYB1 plants	Matías-Hernández et al. (2017)
Expression of unrelated genes		
ipt gene from *A. tumefaciens* in *A. annua* plants	Increased artemisinin content (between 30% and 70%) in transformed plants Higher cytokinin levels, increased growth of axillary buds, increased chlorophyll content and lower root mass	Sa et al. (2001)
*bgl*1 from *T. reesei* in *A. annua* plants	Artemisinin content varied depending on the position and age of the leaf (increase up to 66.5%) Higher density of glandular trichomes on both leaf and flower surfaces	Singh et al. (2016)
Overexpression of *AaICS1* in *A. annua* plants	Increased artemisinin content (up to 1.9-fold), as compared to control lines	Wang et al. (2016)
Overexpression of *AaPYL9* in *A. annua* plants	Higher increase in artemisinin content upon ABA treatment in transgenic plants, as compared to control (between 74% and 95% and 33%, respectively) Higher increase in *ADS*, *FDS* and *CYP71AV1* expression levels in transgenic plants after ABA treatment	Zhang et al. (2013)
Overexpression of *AOC* in *A. annua* plants	Increased content of artemisinin (by 38–97%), artemisinic acid (by 172–675%) and dihydroartemisinic acid (by 125–248%) Higher transcript levels of *FDS*, *CYP71AV1* and *DBR2*	Lu et al. (2014)

(continued)

Table 4.3 (continued)

Gene(s)	Result	Reference
rol B and *rol C* genes in *A. annua* plants	Increased accumulation of artemisinin (between 2.7- and 9.2-folds), artesunate (between 4- and 12.6-folds) and dihydroartemisinin (1.2–3-folds) in plants expressing *rol B* Increased accumulation of artemisinin (4–4.6-folds), artesunate (between 4.4- and 9.1-folds) and dihydroartemisinin (1.5–2-folds) in *rol C*-expressing plants Higher mRNA levels of *ADS*, *CYP71AV1*, *ALDH1* and *TFAR1*. Higher glandular trichome density	Dilshad et al. (2015)
rol ABC genes in *A. annua* and *A. dubia* plants	Accumulation of 33.12 (~9-fold increase) and 2.32 mg (~21-fold increase) artemisinin per g dry weight in *A. annua* and *A. dubia* shoots, respectively Increased expression of *ADS*, *CYP71AV1* and *ALDH1* genes in both species	Kiani et al. (2016)
AtCRY1 in *A. annua* plants	Increased artemisinin content (by 30–40%) Increased expression of *ADS*, *CYP71AV1* and *FDS*	Hong et al. (2009)

4.5.2.4 Miscellaneous Metabolic Engineering Strategies in Artemisinin-Producing Plants

A different strategy used to increase artemisinin accumulation was to express genes that could indirectly affect its production. For instance, phytohormones are involved in plant growth and physiological changes and also regulate secondary metabolite production. One of the strategies evaluated to increase the artemisinin production was the expression in *A. annua* of an isopentenyl transferase from *A. tumefaciens*. This enzyme is involved in cytokinin biosynthesis in transformed cells and is thought to enhance cytokinin levels in transformed plants. Apart from the increase in the artemisinin content in transformed plants (between 30% and 70%), the levels of iPA and iP were significantly higher and were accompanied by increased growth of axillary buds, increased chlorophyll content and lower root mass (Sa et al. 2001).

Another work that was aimed at increasing the artemisinin accumulation by modifying phytohormone production was that of Singh et al. (2016). The authors evaluated the effect of expressing a β-glucosidase from *Trichoderma reesei*, fused to the vacuole-targeting sequence of chitinase at the C terminus, in *A. annua* plants. This strategy was based on the fact that phytohormones are usually accumulated as glycosylated (inactive) conjugates inside the vacuoles and that their release and conversion into the active form involve the hydrolytic cleavage by glucosidases. Although a higher density of glandular trichomes on both leaf and flower surfaces was observed in transformed plants, the artemisinin content varied depending on the position and age of the leaf, but on average, it increased up to 66.5% when compared to untransformed plants.

Since SA is a phytohormone that has a positive effect on the artemisinin production, the isochorismate synthase from *A. annua* (*AaICS1*, an enzyme involved in SA synthesis) was isolated, characterised and overexpressed. These transgenic plants

showed higher artemisinin content, up to 1.9-fold, as compared to control plants, thus proving to be an interesting alternative to enhance the artemisinin accumulation (Wang et al. 2017).

On the other hand, a report demonstrates an enhanced artemisinin accumulation as a consequence of an increased ABA sensitivity mediated by the overexpression of an ABA receptor (*AaPYL9*). When ABA was applied, both control and transgenic lines showed increased expression of *ADS*, *FDS* and *CYP71AV1*, but not *HMGR*, although in transgenic lines the changes in *ADS*, *FDS* and *CYP71AV1* expression were more dramatic. Regarding the artemisinin content, similar levels were observed in both lines before ABA treatment, whereas after the hormone application, there was a rise in the artemisinin content in transgenic plants, as compared to control plants (between 74–95% and 33%, respectively) (Zhang et al. 2013).

Given the fact that exogenous Ja enhances artemisinin accumulation, Lu et al. have evaluated the overexpression of allene oxide cyclase (AOC), the key enzyme in the biosynthesis of Ja. Transgenic plants resulted in a significant increase in Ja levels, which was accompanied by increases in artemisinin (by 38–97%), dihydroartemisinic acid (by 125–248%) and artemisinic acid (by 172–675%) contents. Moreover, higher transcript levels of *FDS*, *CYP71AV1* and *DBR2* were observed (1.7- to 4.3-fold, 5.8- to 17-fold and 1.5- to 5.1-fold, respectively), whereas no significant changes were observed in the expression of *ADS*, *CPR* and *ALDH1* (Lu et al. 2014).

It is well known that hairy root cultures can produce a high and stable level of secondary metabolites and that this effect is a consequence of the expression of *rol* genes, which are responsible for the phenotypic changes on growth and metabolism. The individual effects of *rolB* and *rolC* expression were evaluated in *A. annua* plants, resulting both in an increase of artemisinin, artesunate and dihydroartemisinin, although the highest production of these metabolites was observed in a line expressing the *rolB* gene. In both cases, higher levels of *ADS*, *CYP71AV1* and *ALDH1* transcripts were detected, as well as a higher glandular trichome density and higher mRNA levels of *TFAR1* (trichome-specific fatty acyl-CoA reductase 1, which is supposed to be involved in trichome development) (Dilshad et al. 2015).

In a more recent publication, *rol abc* genes were expressed in *A. annua* and *A. dubia* plants, via *A. tumefaciens* genetic transformation. The expressions of *ADS*, *CYP71AV1* and *ALDH1* genes were significantly higher in transformed plants of both species, although the magnitude of the increase varied among genes. Regarding artemisinin accumulation, while it was highly increased in transformed *A. annua* shoots, as compared to control shoots (33.12 mg/g dry weight and 3.94 mg/g dry weight, respectively), the increase observed in *A. dubia* shoots was less pronounced (2.32 mg/g dry weight vs. 0.11 mg/g dry weight in control lines) (Kiani et al. 2016).

Finally, since environmental conditions affect the production of secondary metabolites, cryptochrome 1 from *Arabidopsis thaliana* (*AtCRY1*), which is a key receptor that perceives light signals, was expressed in *A. annua* plants. Apart from phenotypic changes (colouring of aerial organs, lower height), the artemisinin content of the transgenic lines tested was increased by 30–40%, and it was accompanied by an increase in the transcript levels of *ADS* and *CYP71AV1*, but those of *FDS* were increased in only one of the transgenic lines (Hong et al. 2009) (Table 4.3).

4.5.2.5 Production of Artemisinin in Plant Systems

Several advantages make tobacco an ideal host for artemisinin production: fast growth, the production of high amount of biomass and the fact that there are well-established transformation protocols. The first reports in tobacco dealt with the expression of ADS, the first specific step in artemisinin biosynthesis. Transgenic lines were able to express a functional enzyme and yielded 0.2–1.7 ng/g fresh weight of amorpha-4,11-diene (Wallaart et al. 2001).

The effect of compartmentalisation on the carbon flux and terpenoid accumulation was studied by either the cytosolic or the plastid expression of ADS together with an avian FDS in tobacco. While amorpha-4,11-diene production only reached 0.5 ng/g fresh weight in plants expressing only cytosolic ADS, the plastid co-expression of ADS and FDS led to a drastic increase in amorpha-4,11-diene accumulation (up to 425 mg/g fresh weight), highlighting the advantages of compartmentalisation for critical enzymatic steps (Wu et al. 2006).

Zhang et al. have assayed the production of artemisinin precursors by co-expressing different enzymes of the artemisinin biosynthetic pathway. Plastid expression of FDS and ADS has led to the accumulation of amorpha-4,11-diene (from barely undetectable levels up to 4 μg/g fresh weight). However, when FDS, ADS and CYP71AV1 were co-expressed (the two first in plastids and the other in the cytosol), only artemisinic alcohol and the precursor amorpha-4,11-diene were detected (no accumulation of artemisinic aldehyde or acid was observed). The co-expression of these enzymes together with ALDH1 or both ALDH1 and DBR2 failed to achieve the accumulation of the desired artemisinic or dihydroartemisinic acids, which was attributed to either an impaired oxidation from the corresponding aldehyde in tobacco or the reversion by endogenous enzymes (Zhang et al. 2011).

With a similar objective, transient agroinfiltration experiments were performed in *N. benthamiana*, by using combinations of *HMGR*, *FDS*, *ADS* and *CYP71AV1*. To avoid gene silencing and the use of different promoters, some gene constructs included only one promoter and ribosomal skipping sequences and mitochondrial targeting sequences for compartmentalisation. The co-expression of *FDS*, a truncated version of *HMGR* (tHMGR) and *ADS*, resulted in higher amorpha-4,11-diene accumulation, as compared to the expression of *ADS* alone. The co-expression of FDS, tHMGR and ADS, together with CYP71AV1, resulted in a drastic decrease in amorpha-4,11-diene levels, and although the desired products were not detected, the formation of an unknown compound (up to ~ 40 μg/g fresh weight) was observed. That compound was later identified as artemisinic acid-12-β-diglucoside, being the only metabolite of amorpha-4,11-diene produced and the proof of the functionality of CYP71AV1 in *N. benthamiana* leaves (van Herpen et al. 2010). In a further study, these authors confirmed the production of artemisinin-related compounds as glycosylated derivatives. They analysed the expression and gene sequences of *DBR2*, *ALDH1* and *CYP71AV1* from two chemotypes of *A. annua* (high artemisinin production, HAP, and low artemisinin production, LAP). The authors found that the chemotype was apparently defined by both the type of CYP71AV1 (from HAP or LAP plants) and the relative proportion of DBR2 and ALDH1. By performing transient

expression experiments in *N. benthamiana* leaves that combined the expression of *CYP71AV1* with *DBR2* (together with *ADS* and *FDS*, both targeted to the mitochondria and *tHMGR*), a variety of free and glycosylated compounds were detected, including free dihydroartemisinic and artemisinic alcohols and aldehydes (with barely no acids being detected), and mainly conjugated dihydroartemisinic and artemisinic acids. The additional expression of *ALDH1* resulted in an increase in glycosylated artemisinic and dihydroartemisinic acids content (Ting et al. 2013). In order to increase artemisinic and dihydroartemisinic acid content in the apoplast of *N. benthamiana* leaves, these researchers studied the effect the expression of different pleiotropic drug resistance (PDR) transporters and lipid transfer proteins (LTP), isolated from *A. annua*, together with the expression of the biosynthetic pathway of artemisinin. The combined expression of *AaLTP3* and *AaPDR2* led to an increase in the artemisinin and arteannuin B accumulation in the apoplast of *N. benthamiana* leaves (Wang et al. 2016).

Finally, the successful production of artemisinin in tobacco has been reported. This was achieved by constructing a mega-vector that carried a truncated version of *HMGR* from yeast and *CPR*, *CYP71AV1*, *DBR2* and either *ADS* (cytosolic) or *mtADS* (targeted to mitochondria), all these from *A. annua*. Artemisinin accumulation was detected in transgenic lines expressing the cytosolic version of ADS (between 0.45 and 0.98 μg/g fresh weight); however, the accumulation levels were even higher when ADS was targeted to mitochondria (between 5.0 and 6.8 μg/g fresh weight). The success of this strategy was attributed to several factors, including the expression of HMGR to increase the carbon flux through the MVA pathway (which resulted in an increased availability of precursors), the co-expression of *CPR* together with *CYP71AV1* and the use of intense light conditions during tobacco cultivation that might contribute to the spontaneous conversion of dihydroartemisinic acid to artemisinin. Although the levels of artemisinin were lower than those produced by *A. annua*, the process can be further optimised, thus resulting in a promising platform for artemisinin production (Farhi et al. 2011).

4.5.2.6 Heterologous Production of Artemisinin in Microorganisms

While several authors have focused on enhancing the artemisinin production in plants, others have started to work on the heterologous production in microbial systems. The Semi-synthetic Artemisinin Project focused on the alternative production of artemisinin or its precursors by metabolic engineering of microorganisms (Paddon and Keasling 2014). Microbial hosts, such as *Escherichia coli* or *Saccharomyces cerevisiae* are well-known hosts for protein expression. They exhibit several advantages over plant systems, such as ease for genetic manipulation and transformation, fast growth in inexpensive culture media, higher productivities and absence of feedback inhibition due to secretion to the culture media (Majdi et al. 2016). Several authors have reported either the direct production of artemisinin or its precursor, artemisinic acid, followed by a chemical conversion to artemisinin (Paddon and Keasling 2014; Keasling 2012).

Production in *E. coli* This microorganism has been employed as a microbial cell factory for the production of target sesquiterpenes or their precursor compounds. In the latter case, specific sesquiterpenes can be obtained from those precursors by synthetic organic chemistry (Majdi et al. 2016). As mentioned above, the Semi-synthetic Artemisinin Project was initiated in 2004 with the aim of producing an artemisinic precursor in an engineered microorganism. *E. coli* was the first microorganism chosen during the initial stages of the project development (Paddon and Keasling, 2014). Several groups had previously engineered the MEP pathway in order to increase the production of isoprenoids in *E. coli* (Farmer and Liao, 2001; Kajiwara et al. 1997; Kim and Keasling, 2001). Increasing the flux through the pathway by balancing the pool of GA3P and pyruvate or overexpressing DXS and IPP isomerase led to an increase in isoprenoids production; however, they were low (at the range of mg/l), most probably due to limitations on the control mechanisms that inhibited synthesis in the native host.

However, and based on previous reports, the Semi-synthetic Artemisinin Project was able to significantly improve the isoprenoids production by a heterologous expression of the *S. cerevisiae* MVA pathway in *E. coli*. The heterologous expression of the *S. cerevisiae* MVA pathway was carried out by introducing two plasmids into *E. coli*: the MevT plasmid, which contained the mevT operon constituted by *atoB*, *ERG13* and *tHMG1* genes, and the MevB plasmid, which contained the mevB operon formed by *idi*, *ispA*, *MVD1*, *ERG8* and *ERG1*2 genes. The mevT operon (also called as the "top pathway") is needed for the conversion of acetyl-CoA to mevalonate, while the mevB operon (also called as the "bottom pathway") is necessary to convert mevalonate into FPP (Fig. 4.3). Both plasmids were coupled with the expression of a synthetic codon-optimised version of the *A. annua* ADS, which resulted in the accumulation of the artemisinin precursor amorpha-4,11-diene. This engineered *E. coli* could produce 112 mg/l of amorpha-4,11-diene (Martin et al. 2003). Nevertheless, the productivity of this strain has been underestimated due to the volatile nature of amorpha-4,11-diene, which evaporates from the fermenter. The presence of amorpha-4,11-diene in the exhaust gas was corroborated by a NMR spectroscopy analysis. The application of an in situ product removal strategy by employing a two-phase partitioning bioreactor with a dodecane organic phase allowed improving the amorpha-4,11-diene production to a final concentration that reached 0.5 g/l (Newman et al. 2006).

On the other hand, despite the high levels of amorpha-4,11-diene produced through the optimisation of the fermentation conditions, it was found that the accumulation of intermediates limited the carbon flux and inhibited cell growth (Pitera et al. 2007). This was due to the fact that the expression of the three enzymes encoded by the mevT operon was imbalanced, which led to the accumulation of HMG-CoA that inhibited growth. A synthetic combinatorial approach showed that the reduced expression of HMGS and the overexpression of a truncated HMGR could reduce growth inhibition and HMG-CoA accumulation. Consequently, higher titres of mevalonate were produced. Therefore, it was demonstrated that the carbon flux balancing through the heterologous MVA pathway is a key determinant to optimise isoprenoid biosynthesis in engineered *E. coli* (Pitera et al. 2007).

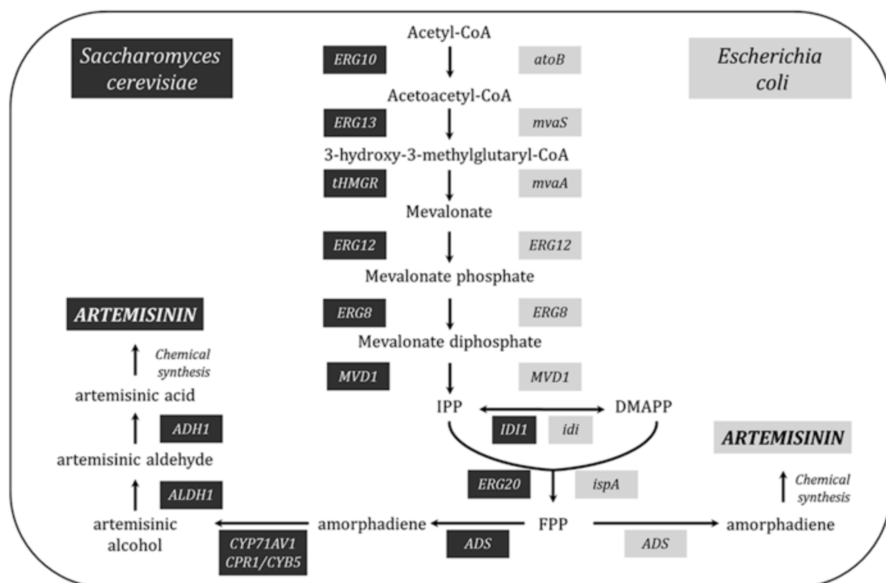

Fig. 4.3 Metabolic engineering strategies to produce arteminisin in *Saccharomyces cerevisiae* and *Escherichia coli. ERG10 and atoB* acetoacetyl-CoA thiolase, *ERG13 and mvaS* HMG-CoA synthase, *mvaA* HMG-CoA reductase, *tHMRG* truncated HMG-CoA reductase *ERG12* mevalonate kinase, *ERG8* phosphomevalonate kinase, *MVD1* mevalonate diphosphate decarboxylase, *IDI1 and idi* isopentenyl diphosphate isomerase, *ERG20 and ispA* farnesyl diphosphate synthase, *ADS* amorpha-4,11-diene synthase, *CYP71AV1* cytochrome P450 monooxygenase, *CYB5* cytochrome b5, *CPR1* cytochrome P450 reductase, *ADH1* alcohol dehydrogenase 1, *ALDH1* aldehyde dehydrogenase, *FPP* farnesyl diphosphate. (Figure adapted from Paddon and Keasling (2014))

Considering that the expression of mevT operon led to an imbalance in the carbon flux through the MVA, further efforts were made to improve the production of amorpha-4,11-diene by increasing the mevalonate production. Yeast genes for HMGS and HMGR (the second and third enzymes in the pathway) were replaced with equivalent genes from *Staphylococcus aureus*, which allowed doubling the concentrations of amorpha-4,11-diene (Fig. 4.3). Moreover, amorpha-4,11-diene titres were further increased by developing a novel nitrogen- and carbon-limited fed-batch fermentation process. This optimisation led to an average titre of 27.4 g/l amorpha-4,11-diene. Subsequent chemical conversion of amorpha-4,11-diene to artemisinin would allow achieving the 25 g/l estimated concentration that is required for the commercial feasibility of the artemisinin semi-synthetic production (Tsuruta et al. 2009).

Production of Artemisinin in Engineered Yeasts Since P450 enzymes from eukaryotic origin (such as CYP71AV1) cannot be correctly expressed in *E. coli* (Paddon and Keasling 2014), *S. cerevisiae* was considered as a host for artemisinin production. The advantages of this system over *E. coli* are the existence in

S. cerevisiae of a MVA pathway that produces FPP, and, as it is a eukaryotic organism, the cell environment is more suitable for the expression of plant enzymes (Majdi et al. 2016). Moreover, this system is more industrially robust and less susceptible to phage contamination (Krivoruchko and Nielsen 2015).

One of the first reports of heterologous sesquiterpene production in yeast dealt with the expression of epi-cedrol synthase and the production of epi-cedrol from the endogenous FPP pool. In order to increase epi-cedrol yields, further modifications included the changing of the mating type and the expression of *UPC2–1* (a semi-dominant mutant version of *UPC2*, which is a transcription factor previously demonstrated to increase the carbon flux towards the sterol biosynthesis), a truncated version of HMGR (tHMGR) and FDS. The highest accumulation of epi-cedrol was obtained in a MATa strain that co-expresses UPC2–1, tHMGR and epi-cedrol synthase (Jackson et al. 2003). A few years later, the production of amorpha-4,11-diene was achieved in *S. cerevisiae* CEN.PK113-5D. In this case, *ADS* from *A. annua* was expressed under a galactose-inducible promoter, either after homologous recombination into the host genome or delivered by a suitable yeast expression vector. Although higher amounts of amorpha-4,11-diene were obtained in the plasmid-transformed yeast, as compared to the genomic-transformed one (600 and 100 μg/l, respectively), after a 16-day batch, yields were low, possibly due to an FPP shortage (Lindahl et al. 2006).

The production of artemisinic acid in *S. cerevisiae* was first reported by Ro et al. These authors were able to express an engineered MVA pathway in *S. cerevisiae* (a derivative from the S288C strain), together with the specific enzymes from artemisinin biosynthesis ADS, CYP71AV1 and CPR from *A. annua*. Since the expression of ADS alone rendered low levels of amorpha-4,11-diene, several modifications were made in the MVA pathway in order to increase FPP formation. By combining the overexpression of two copies of a truncated form of HMGR (tHMGR), the downregulation of *ERG9* (a gene encoding SQS, which competes with ADS for FPP) by a methionine repressible promoter (PMET3); the overexpression of *UPC2–1*, which is an activated allele of *UPC2* (a global transcription factor which positively affects the expression of some enzymes from the MVA pathway); and the overexpression of the gene encoding FDS (*ERG20*), the authors achieved a 500-fold increase in amorpha-4,11-diene production (~150 mg/l), as compared to previous reports. Further expression of CYP71AV1 and CPR in this strain led to a high extracellular accumulation of artemisinic acid, up to 115 mg in a 1 liter aerated bioreactor (Ro et al. 2006).

In order to increase amorpha-4,11-diene accumulation in *S. cerevisiae*, Shiba et al. engineered a pyruvate dehydrogenase bypass for enhancing the supply of acetyl-CoA to the MVA pathway. By co-expressing endogenous acetaldehyde dehydrogenase and a mutant version of acetyl-CoA synthetase from *Salmonella enterica*, the carbon flux through the MVA pathway was increased, and consequently, higher amounts of amorpha-4,11-diene were obtained (Shiba et al. 2007).

The research group involved in the Semi-synthetic Artemisinin Project published the development of an industrial fermentation process for the production of artemisinin in yeast. They were able to improve artemisinic acid accumulation up to 2.5 g/l (a ~25-fold increase, as compared to that reported by Ro et al. 2006). The development of a fed-batch process using a defined medium with galactose as both carbon source and as inducer allowed the production of 1.3 g/l of artemisinic acid. The addition of methionine for downregulating *ERG9* (controlled by the *MET3* repressible promoter) increased the artemisinic acid content, up to 1.8 g/l. Finally, the development of an algorithm that controlled both the feed and the agitation led to the maximum production of artemisinic acid (2.5 g/l) (Lenihan et al. 2008). In a following publication, researchers from Amyris Inc. and the University of California developed a process to produce amorpha-4,11-diene from *S. cerevisiae* and a three-step chemical conversion to obtain dihydroartemisinic acid (Westfall et al. 2012). Based on the CEN.PK2 strain, the authors constructed a completely new one, since the CEN.PK2 strain physiology is better characterised and it has high sporulation efficiency, as compared to the previously employed S288C strain. The chromosomal modifications included the overexpression of every enzyme on the MVA pathway and the repression of *ERG9* by the repressible *MET3* promoter. Strains transformed with a plasmid carrying ADS achieved high accumulation of amorpha-4,11-diene (~1,2 g/l), whereas the same strain transformed with a plasmid carrying *ADS*, *CYP71AV1* and *CPR* produced artemisinic acid, but to a lower extent (~200 mg/l). In a previous report, they had shown that artemisinic acid accumulation led to plasmid instability and the induction of PDR genes in yeasts (Ro et al. 2008). The authors then focused on the amorpha-4,11-diene production, which was improved up to ~40 g/l with further modifications including the deletion of *GAL80* (negative regulator of the galactose regulon) to avoid the need for galactose addition and culture medium optimization. Finally, they developed a chemical process for the conversion of amorpha-4,11-diene to dihydroartemisinic acid, with an overall yield of 48.4% (Westfall et al. 2012).

In one of the latest publications of the researchers involved in the Semi-synthetic Artemisinin Project, the artemisinic acid accumulation in yeasts was reported to reach 25 g/l. In order to achieve this result, Y337 strain (Westfall et al. 2012) was improved by a proper balance between *CYP71AV1* and *CPR1* expression (a reduced *CPR1* expression increased cell viability) (Fig. 4.3), together with the expression of three new genes isolated from *A. annua* (apart from *ADS*): *CYB5* (encoding cytochrome b5, which enhances the reaction rate of cytochromes P450), *ALDH1* (artemisinic aldehyde dehydrogenase) and *ADH1* (a putative alcohol dehydrogenase). The process was further improved by the development of an extractive fermentation using isopropyl myristate (10% v/v) and a feedback-controlled ethanol pulse-feed process (Paddon et al. 2013).

In a very recent report, the disruption of *PAH1* gene, which encodes phosphatidic acid phosphatase (responsible for the generation of neutral triglycerides from phosphatidic acid) in *S. cerevisiae* through CRISPR/Cas9 technology, provoked a drastic ER proliferation. The co-expression of ADS, CYP71AV1 and CPR from *A. annua* in this yeast strain led to a 2-fold increase in artemisinic acid, as compared to the wild-type strain (Arendt et al. 2017).

4.5.2.7 Metabolic Engineering in Moss

Over the last years, the moss *Physcomitrella patens* has emerged as a biotechnological platform for recombinant protein production in the biopharmaceutical industry. A biotechnological company that employs this biological platform was founded in 1999 for biotherapeutic production. Moreover, this platform has recently been considered for the heterologous production of plant secondary metabolites. In mosses, the haploid phase dominates the growth cycle (protonema), which can be cultured easily under sterile conditions in different suitable containers such as Petri dishes, Erlenmeyer flasks and bioreactors. Mosses grow photoautotrophically in simple media containing inorganic salts without vitamins, sugars or phytohormones. These medium characteristics assure a low risk of contamination, high volume and cost-effective media that enable a simply scaling-up process. Moss tissue cultures are biochemically and genetically stable over time, and they have proved to be very resistant to stress conditions. Genes can be efficiently introduced in *P. patens* tissues by homologous recombination. In addition, these cultures can be preserved under liquid nitrogen for years (Reski et al. 2015; Simonsen et al. 2009).

P. patens have been genetically modified in order to overexpress two sesquiterpene synthases, patchoulol synthase (PTS) and santelene synthase (SS). These enzymes are involved in the first steps in the biosynthesis of patchoulol and β-santalene, respectively. They were expressed in the cytosol and in plastids of moss tissue cultures. In cytosol, these enzymes were also co-expressed with a truncated version of the enzyme HMGR (tHMGR). In this tHMGR, the regulatory domain was deleted in order to accomplish enzyme deregulation, which led to increased levels of isoprenoid precursors (Donald et al. 1997). *P. patens* overexpressing PTS in the cytosol accumulated patchoulol to a level of 0.2–0.8 mg/g dry weight. The co-expression of PTS with the tHMGR version resulted in an increase of patchoulol content that reached 1.34 mg/g dry weight. Transgenic lines expressing PTS in plastid accumulated 0.2 mg/g dry weight of patchoulol. *P. patens* overexpressing SS and tHMGR in the cytosol rendered a santelene content of 0.003–0.022 mg/g dry weight, while in cell lines expressing SS alone santelene was not detected. When SS was targeted to chloroplasts, the santelene content was 0.039–0.035 mg/g dry weight, which was the highest yield ever achieved in moss tissues (Zhan et al. 2014). The authors proposed that these results offer the possibility to produce other STLs such as parthenolides or thapsigargin in *P. patens*, although productivities should be increased.

4.6 Conclusion

The combination of techniques such as mass spectrometry imaging (MSI), fluorescence resonance energy transfer (FRET)-based nanosensors and laser microdissection together with metabolic analyses (GC-MS, LC-MS, NMR) has allowed identifying and localising the synthesis of secondary metabolites, not only at the level of organs and tissues but also at the cellular level. In addition, it has also allowed performing a more specific analysis of the transcriptome of the cells and tissues producing STLs,

leading to the isolation of new genes involved in their biosynthesis. Provided the biosynthetic pathway is partially or fully elucidated, many putative heterologous hosts can be genetically modified in order to produce the desire metabolites. For instance, metabolic engineering has allowed establishing transgenic plants in *A. annua*, which presented higher concentrations than the plants traditionally used in artemisinin production. It is not always possible to apply this technology to all STL-producing plants, since transformation protocols for some of them have not been developed yet. The alternative may be the use of fast-growing and high biomass producer crops whose agronomic practices are well known. The metabolic engineering in heterologous hosts may have undesirable effects such as the accumulation of STLs in their conjugated forms with cysteine, sugars and glutathione, as is the case of the production of parthenolides in *N. benthamiana*. Some factors must be taken into account for transgenic plant development, such as the availability of lands that are normally intended for crop cultures, public acceptance, environmental impact and the investment required to obtain the approvals of the governmental agencies.

The in vitro cultivation of plant cells and organs and the potential use of *P. patents* constitute an alternative to the natural production of STLs since they offer a controlled system and shorter production cycles. The main disadvantage is still their low productivities and the initial investment costs to build an industrial plant equipped with bioreactors. The bulk of knowledge accumulated over decades about the biochemistry, physiology, genetics and scale-up fermentation of microorganisms has allowed yeast and *E. coli* to be considered the first choice to design metabolic engineering cells to produce either STLs or their precursors. Metabolic engineered yeasts have probably shown better performances than *E. coli* because they are able to express more efficiently the cytochrome P450 enzymes involved in STL biosynthesis.

Recently, Sanofi Pasteur has acquired the rights for the production of artemisinin in recombinant yeasts, a development of the Dr. Keasling group from the University of California, but the fall in the market price of artemisinin below $ 250 per kg caused the process to be economically unachievable (Peplow 2016).

In summary, there are many biotechnological systems available for the industrial production of STLs; however, all of them must be economically competitive when compared to the production and extraction of the metabolites from their natural sources.

References

Ahlawat S, Saxena P, Alam P et al (2014) Modulation of artemisinin biosynthesis by elicitors, inhibitor, and precursor in hairy root cultures of *Artemisia annua* L. J Plant Interact 9:811–824

Alam P, Abdin MZ (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(10):1919–1928

Andersen TB, López CQ, Manczak T et al (2015) Thapsigargin from *Thapsia* L. to mipsagargin. Molecules 20(4):6113–6127

Aquil S, Husaini AM, Abdin MZ et al (2009) Overexpression of the HMG-CoA reductase gene leads to enhanced artemisinin biosynthesis in transgenic *Artemisia annua p*lants. Planta Med 75(13):1453–1458

Arendt P, Miettinen K, Pollier J et al (2017) An endoplasmic reticulum-engineered yeast platform for overproduction of triterpenoids. Metab Eng 40:165–175

Arsenault PR, Vail D, Wobbe KK et al (2010a) Reproductive development modulates gene expression and metabolite levels with possible feedback inhibition of artemisinin in *Artemisia annua*. Plant Physiol 154(2):958–968

Arsenault PR, Vail DR, Wobbe KK et al (2010b) Effect of sugars on artemisinin production in *Artemisia annua* L.: transcription and metabolite measurements. Molecules 15(4):2302–2318

Atanasov AG, Waltenberger B, Pferschy-Wenzig EM et al (2015) Discovery and resupply of pharmacologically active plant-derived natural products: a review. Biotechnol Adv 33(8):1582–1614

Baldi A, Dixit VK (2008) Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*. Bioresour Technol 99(11):4609–4614

Banyai W, Kirdmanee C, Mii M et al (2010) Overexpression of farnesyl pyrophosphate synthase (FDS) gene affected artemisinin content and growth of *Artemisia annua* L. Plant Cell Tissue Organ Cult 103:255–265

Baque MA, Moh SH, Lee EJ et al (2012) Production of biomass and useful compounds from adventitious roots of high-value added medicinal plants using bioreactor. Biotechnol Adv 30(6):1255–1267

Bouvier F, Rahier A, Camara B (2005) Biogenesis, molecular regulation and function of plant isoprenoids. Prog Lipid Res 44(6):357–429

Caretto S, Quarta A, Durante M et al (2011) Methyl jasmonate and miconazole differently affect arteminisin production and gene expression in *Artemisia annua* suspension cultures. Plant Biol (Stuttg) 13(1):51–58

Chadwick M, Trewin H, Gawthrop F et al (2013) Sesquiterpenoids lactones: benefits to plants and people. Int J Mol Sci 14(6):12780–12805

Chang YJ, Song SH, Park SH et al (2000) Amorpha- 4,11-diene synthase of *Artemisia annua*: cDNA isolation and bacterial expression of a terpene synthase involved in artemisinin biosynthesis. Arch Biochem Biophys 383:178–184

Chaturvedi D (2011) Sesquiterpene lactones: structural diversity and their biological activities. In: Tiwari V, Mishra B (eds) Opportunity, challenge and scope of natural products in medicinal chemistry. Research Signpost, Kerala, pp 313–334

Chen DH, Liu CJ, Ye HC et al (1999) Ri-mediated transformation of *Artemisia annua* with a recombinant farnesyl diphosphate synthase gene for artemisinin production. Plant Cell Tissue Organ Cult 57(3):157–162

Chen DH, Ye HC, Li GF (2000) Expression of a chimeric farnesyl diphosphate synthase gene in *Artemisia annua* L. transgenic plants via *Agrobacterium tumefaciens*-mediated transformation. Plant Sci 155(2):179–185

Chen JL, Fang HM, Ji YP et al (2011) Artemisinin biosynthesis enhancement in transgenic *Artemisia annua* plants by downregulation of the β-Caryophyllene synthase gene. Planta Med 77(15):1759–1765

Chen Y, Shen Q, Wang Y et al (2013) The stacked over-expression of *FDS*, *CYP71AV1* and *CPR* genes leads to the increase of artemisinin level in *Artemisia annua* L. Plant Biotechnol Rep 7(3):287–295

Chen M, Yan T, Shen Q et al (2017) Glandular trichome-specific wrky 1 promotes artemisinin biosynthesis in *Artemisia annua*. New Phytol 214(1):304–316

de Kraker JW, Franssen MCR, de Groot A et al (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 et al (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

Diaz-Chavez ML, Moniodis J, Madilao LL et al (2013) Biosynthesis of sandalwood oil: *Santalum album* CYP76F cytochromes P450 produce santalols and bergamotol. PLoS One 8(9):e75053. https://doi.org/10.1371/journal.pone.0075053

Dilshad E, Cusido RM, Palazon J et al (2015) Enhanced artemisinin yield by expression of *rol* genes in *Artemisia annua*. Malar J 14:424

Donald KA, Hampton RY, Fritz IB (1997) Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme a reductase on squalene synthesis in *Saccharomyces cerevisiae*. Appl Environ Microbiol 63(9):3341–3344

Drew DP, Dueholm B, Weitzel C et al (2013) Transcriptome analysis of *Thapsia laciniata* Rouy provides insights into terpenoid biosynthesis and diversity in Apiaceae. Int J Mol Sci 14(5):9080–9098

Durante M, Caretto S, Quarta A et al (2011) β-Cyclodextrins enhance artemisinin production in *Artemisia annua* suspension cell cultures. Appl Microbiol Biotechnol 90(6):1905–1913

Eibl R, Eibl D (2008) Design of bioreactors suitable for plant cell and tissue cultures. Phytochem Rev 7:593–598

Eibl R, Werner S, Eibl D (2009) Disposable bioreactors for plant liquid cultures at litre-scale. Eng Life Sci 9:156–164

Eljounaidi K, Cankar K, Comino C et al (2014) Cytochrome P450s from *Cynara cardunculus* L. CYP71AV9 and CYP71BL5, catalyze distinct hydroxylations in the sesquiterpene lactone biosynthetic pathway. Plant Sci 223:59–68

Fan R, Li Y, Li C, Zhang Y (2015) Differential microRNA analysis of glandular trichomes and young leaves in *Xanthium strumarium* L. reveals their putative roles in regulating terpenoid biosynthesis. PLoS One 10(9):e0139002. https://doi.org/10.1371/journal.pone.0139002

Farhi M, Marhevka E, Ben-Ari J et al (2011) Generation of the potent anti-malarial drug artemisinin in tobacco. Nat Biotechnol 29(12):1072–1074

Farmer WR, Liao JC (2001) Precursor balancing for metabolic engineering of lycopene production in *Escherichia coli*. Biotechnol Prog 17:57–61

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

Han JL, Liu BY, Ye HC et al (2006) Effects of overexpression of the endogenous farnesyl diphosphate synthase on the artemisinin content in *Artemisia annua* L. J Integr Plant Biol 48(4):482–487

Han J, Wang H, Kanagarajan S et al (2016) Promoting artemisinin biosynthesis in *Artemisia annua* plants by substrate channeling. Mol Plant 9(6):946–948

Hong GJ, Hu WL, Li JX et al (2009) Increased accumulation of artemisinin and anthocyanins in *Artemisia annua* expressing the *Arabidopsis* blue light receptor *CRY1*. Plant Mol Biol Report 27(3):334–341

Jackson BE, Hart-Wells EA, Matsuda SPT (2003) Metabolic engineering to produce sesquiterpenes in yeast. Org Lett 5(10):1629–1632

Ji Y, Xiao J, Shen Y et al (2014) Cloning and characterization of AabHLH1, a bHLH transcription factor that positively regulates artemisinin biosynthesis in *Artemisia annua*. Plant Cell Physiol 55(9):1592–1604

Jiang W, Fu X, Pan Q et al (2016) Overexpression of *AaWRKY1* leads to an enhanced content of artemisinin in *Artemisia annua*. Biomed Res Int 2016:7314971. https://doi.org/10.1155/2016/7314971

Jones CG, Keeling CI, Ghisalberti EL et al (2008) Isolation of cDNAs and functional characterisation of two multi-product terpene synthase enzymes from sandalwood, *Santalum album* L. Arch Biochem Biophys 477(1):121–130

Jones CG, Moniodis J, Zulak KG et al (2011) Sandalwood fragrance biosynthesis involves sesquiterpene synthases of both the terpene synthase (TPS)-a and TPS-b subfamilies, including santalene synthases. J Biol Chem 286(20):17445–17454

Kajiwara S, Fraser PD, Kondo K et al (1997) Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. Biochem J 324:421–426

Keasling JD (2012) Synthetic biology and the development of tools for metabolic engineering. Metab Eng 14(3):189–195

Kiani BH, Suberu J, Mirza B (2016) Cellular engineering of *Artemisia annua* and *Artemisia dubia* with the *rol ABC* genes for enhanced production of potent anti-malarial drug artemisinin. Malar J 15(1):252. https://doi.org/10.1186/s12936-016-1312-8

Kim SW, Keasling JD (2001) Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. Biotechnol Bioeng 72:408–415

Kolewe ME, Gaurav V, Roberts SC (2008) Pharmaceutically active natural product synthesis and supply via plant cell culture technology. Mol Pharm 5(2):243–256

Krivoruchko A, Nielsen J (2015) Production of natural products through metabolic engineering of *Saccharomyces cerevisiae*. Curr Opin Biotechnol 35:7–15

Lei C, Ma D, Pu G et al (2011) Foliar application of chitosan activates artemisinin biosynthesis in *Artemisia annua* L. Ind Crop Prod 33:176–182

Lenihan JR, Tsuruta H, Diola D et al (2008) Developing an industrial artemisinic acid fermentation process to support the cost-effective production of antimalarial artemisinin-based combination therapies. Biotechnol Prog 24(5):1026–1032

Li Y, Chen F, Li Z et al (2016) Identification and functional characterization of sesquiterpene synthases from *Xanthium strumarium*. Plant Cell Physiol 57(3):630–641

Lichtenthaler HK (1999) The 1-deoxy-d-xylulose- 5-phosphate pathway of isoprenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 50:47–65

Lindahl AL, Olsson ME, Mercke P et al (2006) Production of the artemisinin precursor amorpha-4,11-diene by engineered *Saccharomyces cerevisiae*. Biotechnol Lett 28(8):571–580

Liu CZ, Guo C, Wang YC et al (2002) Effect of light irradiation on hairy root growth and artemisinin biosynthesis of *Artemisia annua* L. Process Biochem 38:581–585

Liu CZ, Guo G, Wang YC et al (2003) Comparison of various bioreactors on growth and artemisinin biosynthesis of *Artemisia annua* L. shoot cultures. Process Biochem 39(1):45–49

Liu Q, Manzano D, Tanić N et al (2014) Elucidation and in planta reconstitution of the parthenolide biosynthetic pathway. Metab Eng 23:145–153

Lu X, Shen Q, Zhang L et al (2013a) Promotion of artemisinin biosynthesis in transgenic *Artemisia annua* by overexpressing *ADS*, *CYP71AV1* and *CPR* genes. Ind Crop Prod 49:380–385

Lu X, Zhang L, Zhang F et al (2013b) *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(4):1191–1202

Lu X, Zhang F, Shen Q et al (2014) Overexpression of allene oxide cyclase improves the biosynthesis of artemisinin in *Artemisia annua* L. PLoS One 9(3):e91741. https://doi.org/10.1371/journal.pone.0091741

Luczkiewicz M, Zárate R, Dembińska-Migas W et al (2002) Production of pulchelin E in hairy roots, callus and suspension cultures of *Rudbeckia hirta* L. Plant Sci 163:91–100

Lv Z, Wang S, Zhang F et al (2016a) Overexpression of a novel NAC domain-containing transcription factor gene (*AaNAC1*) enhances the content of artemisinin and increases tolerance to drought and *Botrytis cinerea* in *Artemisia annua*. Plant Cell Physiol 57(9):1961–1971

Lv Z, Zhang F, Pan Q et al (2016b) Branch pathway blocking in *Artemisia annua* is a useful method for obtaining high yield Artemisinin. Plant Cell Physiol 57(3):588–602

Ma C, Wang H, Lu X et al (2009a) Terpenoid metabolic profiling analysis of transgenic *Artemisia annua* L. by comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry. Metabolomics 5(4):497–506

Ma D, Pu G, Lei C et al (2009b) 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, Liu Q, Karimzadeh G et al (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*. Biol Plant 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 Rep 34:1909–1918

Majdi M, Ashengroph M, Abdollahi MR (2016) Sesquiterpene lactone engineering in microbial and plant platforms: parthenolide and artemisinin as case studies. Appl Microbiol Biotechnol 100(3):1041–1059

Malarz J, Stojakowska A, Kisiel W (2002) Sesquiterpene lactones in a hairy root culture of *Cichorium intybus*. Z Naturforsch C 57(11–12):994–997

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

Matías-Hernández L, Jiang W, Yang K et al (2017) *AaMYB1* and its orthologue *AtMYB61* affect terpene metabolism and trichome development in *Artemisia annua* and *Arabidopsis thaliana*. Plant J 90(3):520–534

May B, Lange BM, Wüst M (2013) Biosynthesis of sesquiterpenes in grape berry exocarp of *Vitis vinifera* L.: evidence for a transport of farnesyl diphosphate precursors from plastids to the cytosol. Phytochemistry 95:135–144

Menin B, Comino C, Portis E et al (2012) Genetic mapping and characterization of the globe artichoke (+)-germacrene a synthase gene, encoding the first dedicated enzyme for biosynthesis of the bitter sesquiterpene lactone cynaropicrin. Plant Sci 190:1–8

Mercke P, Bengtsson M, Bouwmeester HJ et al (2000) Molecular cloning, expression, and characterization of amorpha-4,11-diene synthase, a key enzyme of artemisinin biosynthesis in *Artemisia annua* L. Arch Biochem Biophys 381:173–180

Moniodis J, Jones CG, Barbour EL et al (2015) The transcriptome of sesquiterpenoid biosynthesis in heartwood xylem of western Australian sandalwood (*Santalum spicatum*). Phytochemistry 113:79–86

Mora-Pale M, Sanchez-Rodriguez SP, Linhardt RJ et al (2013) Metabolic engineering and *in vitro* biosynthesis of phytochemicals and non-natural analogues. Plant Sci 210:10–24

Murthy HN, Lee EJ, Paek KY (2014) Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. Plant Cell Tissue Organ Cult 118(1):1–16

Nafis T, Akmal M, Ram M et al (2011) Enhancement of artemisinin content by constitutive expression of the HMG-CoA reductase gene in high-yielding strain of *Artemisia annua* L. Plant Biotechnol Rep 5(1):53–60

Nagegowda DA (2010) Plant volatile terpenoid metabolism: biosynthetic genes, transcriptional regulation and subcellular compartmentation. FEBS Lett 584(14):2965–2973

Newman JD, Marshall J, Chang M et al (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 TD, Faraldos JA, Vardakou M et al (2016) Discovery of germacrene a synthases in *Barnadesia spinosa*: the first committed step in sesquiterpene lactone biosynthesis in the basal member of the Asteraceae. Biochem Biophys Res Commun 479(4):622–627

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

Paddon CJ, Keasling JD (2014) Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. Nat Rev Microbiol 12(5):355–367

Paddon CJ, Westfall PJ, Pitera DJ et al (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 496(7446):528–532

Parveen I, Wang M, Zhao J et al (2015) Investigating sesquiterpene biosynthesis in *Ginkgo biloba*: molecular cloning and functional characterization of (E, E)-farnesol and α-bisabolene synthases. Plant Mol Biol 89(4–5):451–462

Patra N, Srivastava AK (2014) Enhanced production of artemisinin by hairy root cultivation of *Artemisia annua* in a modified stirred tank reactor. Appl Biochem Biotechnol 174(6):2209–2222

Patra N, Srivastava AK (2016) Artemisinin production by plant hairy root cultures in gas- and liquid-phase bioreactors. Plant Cell Rep 35(1):143–153

Pence VC (2011) Evaluating costs for the *in vitro* propagation and preservation of endangered plants. In Vitro Cell Dev Biol Plant 47:176–187

Peplow M (2016) Synthetic biology's first malaria drug meets market resistance. Nature 530(7591):389–390

Pickel B, Drew DP, Manczak T et al (2012) Identification and characterization of a kunzeaol synthase from *Thapsia garganica*: implications for the biosynthesis of the pharmaceutical thapsigargin. Biochem J 448(2):261–271

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

Putalun W, Luealon W, De-Eknamkul W et al (2007) Improvement of artemisinin production by chitosan in hairy root cultures of *Artemisia annua* L. Biotechnol Lett 29(7):1143–1146

Pyle BW, Tran HT, Pickel B et al (2012) Enzymatic synthesis of valerena-4,7(11)-diene by a unique sesquiterpene synthase from the valerian plant (*Valeriana officinalis*). FEBS J 279(17):3136–3146

Ram M, Khan MA, Jha P et al (2010) HMG-CoA reductase limits artemisinin biosynthesis and accumulation in *Artemisia annua* L. plants. Acta Physiol Plant 32(5):859–866

Rani A, Ravikumar P, Reddy MD et al (2013) Molecular regulation of santalol biosynthesis in *Santalum album* L. Gene 527(2):642–648

Rea G, Antonacci A, Lambreva M et al (2011) Integrated plant biotechnologies applied to safer and healthier food production: the Nutra-snack manufacturing chain. Trends Food Sci Technol 22:353–366

Reski R, Parsons J, Decker EL (2015) Moss-made pharmaceuticals: from bench to bedside. Plant Biotechnol J 13:1191–1198

Ricigliano V, Kumar S, Kinison S et al (2016) Regulation of sesquiterpenoid metabolism in recombinant and elicited *Valeriana officinalis* hairy roots. Phytochemistry 125:43–53

Ro DK, Paradise EM, Ouellet M et al (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440:940–943

Ro DK, Ouellet M, Paradise EM et al (2008) Induction of multiple pleiotropic drug resistance genes in yeast engineered to produce an increased level of anti-malarial drug precursor, artemisinic acid. BMC Biotechnol 8(1):83

Sa G, Mi M, He-chun Y et al (2001) Effects of *ipt* gene expression on the physiological and chemical characteristics of *Artemisia annua* L. Plant Sci 160(4):691–698

Schramek N, Wang H, Römisch-Margl W et al (2010) Artemisinin biosynthesis in growing plants of *Artemisia annua.* A $^{13}CO_2$ study. Phytochemistry 71(2–3):179–187

Shen Q, Chen YF, Wang T et al (2012) Overexpression of the cytochrome P450 monooxygenase (*cyp71av1*) and cytochrome P450 reductase (*cpr*) genes increased artemisinin content in *Artemisia annua* (Asteraceae). Genet Mol Res 11(3):3298–3309

Shen Q, Lu X, Yan T et al (2016a) The jasmonate-responsive AaMYC2 transcription factor positively regulates artemisinin biosynthesis in *Artemisia annua*. New Phytol 210(4):1269–1281

Shen Q, Yan T, Fu X et al (2016b) Transcriptional regulation of artemisinin biosynthesis in *Artemisia annua* L. Sci Bull 61:18–25

Shiba Y, Paradise EM, Kirby J et al (2007) Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. Metab Eng 9(2):160–168

Simonsen HT (2015) Elucidation of terpenoid biosynthesis in non-model plants utilizing transcriptomic data. Next Generat Seq Appl 2:111

Simonsen HT, Drew DP, Lunde C (2009) Perspectives on using *Physcomitrella patens* as an alternative production platform for thapsigargin and other terpenoid drug candidates. Perspect Medicin Chem 3:1–6

Singh ND, Kumar S, Daniell H (2016) Expression of beta-glucosidase increases trichome density and artemisinin content in transgenic *Artemisia annua* plants. Plant Biotechnol J 14(3):1034–1045

Souret FF, Kim Y, Wyslouzil BE et al (2003) Scale-up of *Artemisia annua* L. hairy root cultures produces complex patterns of terpenoid gene expression. Biotechnol Bioeng 83(6):653–667

Talano MA, Wevar Oller AL, González PS et al (2012) Hairy roots, their multiple applications and recent patents. Recent Pat Biotechnol 6:115–133

Tan H, Xiao L, Gao S et al (2015) Trichome and artemisinin regulator 1 is required for trichome development and artemisinin biosynthesis in *Artemisia annua*. Mol Plant 8(9):1396–1411

Teoh KH, Polichuk DR, Reed DW et al (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

Teoh KH, Polichuk DR, Reed DW et al (2009) Molecular cloning of an aldehyde dehydrogenase implicated in artemisinin biosynthesis in *Artemisia annua*. Botany 87:635–642

Testone G, Mele G, Di Giacomo E et al (2016) Insights into the sesquiterpenoid pathway by metabolic profiling and de novo transcriptome assembly of stem-Chicory (*Cichorium intybus* Cultigroup "Catalogna"). Front Plant Sci 8(7):1676

Tholl D (2015) Biosynthesis and biological functions of terpenoids in plants. Adv Biochem Eng Biotechnol 148:63–106

Tholl D, Chen F, Petri J et al (2005) Two sesquiterpene synthases are responsible for the complex mixture of sesquiterpenes emitted from *Arabidopsis* flowers. Plant J 42(5):757–771

Ting HM, Wang B, Rydén AM et al (2013) The metabolite chemotype of *Nicotiana benthamiana* transiently expressing artemisinin biosynthetic pathway genes is a function of *CYP71AV1* type and relative gene dosage. New Phytol 199(2):352–366

Torkamani M, Abbaspour N, Jafari M et al (2014a) Elicitation of valerenic acid in the hairy root cultures of *Valeriana officinalis* L (Valerianaceae). Trop J Pharm Res 13:943–947

Torkamani M, Jafari M, Abbaspour N et al (2014b) Enhanced production of valerenic acid in hairy root culture of *Valeriana officinalis* by elicitation. Central Eur. J Biol 9(9):853–863

Towler MJ, Weathers PJ (2007) Evidence of artemisinin production from IPP stemming from both the mevalonate and the nonmevalonate pathways. Plant Cell Rep 26(12):2129–2136

Tsuruta H, Paddon CJ, Eng D et al (2009) High-level production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in *Escherichia coli*. PLoS One 4(2):e4489. https://doi.org/10.1371/journal.pone.0004489

Turconi J, Griolet F, Guevel R et al (2014) Semisynthetic artemisinin, the chemical path to industrial production. Org Process Res Dev 18:417–422

van Herpen TWJM, Cankar K, Nogueira M et al (2010) *Nicotiana benthamiana* as a production platform for artemisinin precursors. PLoS One 5(12):e14222. https://doi.org/10.1371/journal.pone.0014222

Vranová E, Coman D, Gruissem W (2012) Structure and dynamics of the isoprenoid pathway network. Mol Plant 5(2):318–333

Vranová E, Coman D, Gruissem W (2013) Network analysis of the MVA and MEP pathways for isoprenoid synthesis. Annu Rev Plant Biol 64:665–700

Wallaart TE, Bouwmeester HJ, Hille J et al (2001) Amorpha-4,11-diene synthase: cloning and functional expression of a key enzyme in the biosynthetic pathway of the novel antimalarial drug artemisinin. Planta 212(3):460–465

Wang JW, Zheng LP, Zhang B et al (2009) Stimulation of artemisinin synthesis by combined cerebroside and nitric oxide elicitation in *Artemisia annua* hairy roots. Appl Microbiol Biotechnol 85(2):285–292

Wang Y, Jing F, Yu S et al (2011) Co-overexpression of the *HMGR* and *FDS* genes enhances artemisinin content in *Artemisia annua* L. J Med Plant Res 5(15):3396–3403

Wang H, Song Y, Shen H et al (2012) Effect of antisense squalene synthase gene expression on the increase of artemisinin content in *Artemisia annua*. In: Çiftçi YÖ (ed) Transgenic plants - advances and limitations. InTech, Rijeka, pp 397–406

Wang B, Kashkooli AB, Sallets A et al (2016) Transient production of artemisinin in *Nicotiana benthamiana* is boosted by a specific lipid transfer protein from *A. annua*. Metab Eng 38:159–169

Wang LY, Zhang Y, Fu XQ et al (2017) Molecular cloning, characterisation and promoter analysis of the isochorismate synthase (*AaICS1*) gene from *Artemisia annua*. J Zhejiang Univ Sci B 18(8):662–673

Wen W, Yu R (2011) Artemisinin biosynthesis and its regulatory enzymes: progress and perspective. Pharmacogn Rev 5(10):189–194

Westfall PJ, Pitera DJ, Lenihan JR et al (2012) Production of amorpha-4,11-diene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. Proc Natl Acad Sci USA 109(3):E111–E118

Wilson SA, Roberts SC (2012) Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. Plant Biotechnol J 10(3):249–268

Wu S, Schalk M, Clark A et al (2006) Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. Nat Biotechnol 24(11):1441–1447

Xiang L, Zeng L, Yuan Y et al (2012) Enhancement of artemisinin biosynthesis by overexpressing *dxr*, *cyp71av1* and *cpr* in the plants of *Artemisia annua* L. Plant Omics J 5(6):503–507

Xie DY, Ma DM, Judd R et al (2016) Artemisinin biosynthesis in *Artemisia annua* and metabolic engineering: questions, challenges, and perspectives. Phytochem Rev 15:1093–1114

Yeo YS, Nybo SE, Chittiboyina AG et al (2013) Functional identification of valerena-1,10-diene synthase, a terpene synthase catalyzing a unique chemical cascade in the biosynthesis of biologically active sesquiterpenes in *Valeriana officinalis*. J Biol Chem 288(5):3163–3173

Yin H, Zhuang YB, Li EE et al (2015) Heterologous biosynthesis of costunolide in *Escherichia coli* and yield improvement. Biotechnol Lett 37:1249–1255

Yu ZX, Li JX, Yang CQ et al (2012) The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in *Artemisia annua* L. Mol Plant 5(2):353–365

Zhan X, Zhang YH, Chen DF et al (2014) Metabolic engineering of the moss *Physcomitrella patens* to produce the sesquiterpenoids patchoulol and α/β-santalene. Front Plant Sci 18(5):636

Zhang Y, Teoh KH, Reed DW et al (2008) The molecular cloning of artemisinic aldehyde D-11(13) reductase and its role in glandular trichome-dependent biosynthesis of artemisinin in *Artemisia annua*. J Biol Chem 283:21501–21508

Zhang L, Jing F, Li F et al (2009) Development of transgenic *Artemisia annua* (Chinese wormwood) plants with an enhanced content of artemisinin, an effective anti-malarial drug, by hairpin-RNA-mediated gene silencing. Biotechnol Appl Biochem 52(3):199–207

Zhang Y, Nowak G, Reed DW et al (2011) The production of artemisinin precursors in tobacco. Plant Biotechnol J 9(4):445–454

Zhang F, Lu X, Lv Z et al (2013) Overexpression of the *Artemisia* orthologue of ABA receptor, AaPYL9, enhances ABA sensitivity and improves artemisinin content in *Artemisia annua* L. PLoS One 8(2):e56697. https://doi.org/10.1371/journal.pone.0056697

Zhang F, Fu X, Lv Z et al (2015) A basic leucine zipper transcription factor, AabZIP1, connects abscisic acid signaling with artemisinin biosynthesis in *Artemisia annua*. Mol Plant 8(1):163–175

Zhao L, Chang WC, Xiao Y et al (2013) Methylerythritol phosphate pathway of isoprenoid biosynthesis. Annu Rev Biochem 82:497–530

Zheng LP, Guo YT, Wang JW et al (2008) Nitric oxide potentiates oligosaccharide-induced artemisinin production in *Artemisia annua* hairy roots. J Integr Plant Biol 50(1):49–55