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Genetic background of selected sesquiterpene lactones biosynthesis in *Asteraceae*. A review

Podłoże genetyczne biosyntezy wybranych laktonów seskwiterpenowych u *Asteraceae*. Praca przeglądowa

Summary. *Asteraceae* family is a rich source of many sesquiterpene lactones (STLs). These secondary metabolites exhibit multidirectional activity including anti-tumor, anti-inflammatory or antimicrobial, just to name a few. Promising approach of metabolic engineering offers a way of increasing the production of STLs by reconstruction of their biosynthetic pathway in a hetero-logous system. Moreover, their production in host plants might be increased through overexpression of biosynthetic genes and/or transcription factors (TFs) positively regulating the pathway. Either of the strategies requires extensive knowledge on the genetic background of STLs biosynthesis pathway. This review summarizes molecular investigations concerning biosynthesis of these medicinally essential metabolites.

Key words: sesquiterpene lactones, biosynthesis pathway genes, transcription factor

INTRODUCTION

Sesquiterpene lactones (STLs) constitute a group of secondary metabolites that can be found mainly in the species belonging to *Asteraceae* family. They are a class of C_{15} terpenoids that contain a lactone ring. Due to their chemical structure STLs are divided into major groups of germacranolides, eudesmanolides, xanthanolides, guaianolides and pseudoguaianolides [Li et al. 2016a]. These compounds are derivatives of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are synthetized through the mevalonate (MVA) pathway and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. The IPP and DMAPP are converted by farnesyl diphosphate synthase (FDS, also designated as FPPS – farnesyl pyrophosphate synthase) into farnesyl diphosphate (FDP, also designated as FPP – farnesyl pyrophosphate), which subsequently undergoes cycli-

sation catalyzed by a sesquiterpene synthase. This is followed by successive hydroxylation/oxidation performed by cytochrome P450 enzymes and (depending on the structure) modification steps catalyzed by alcohol dehydrogenases, reductases and acyltransferases (some of which thus far remain unknown) [Padilla-Gonzalez et al. 2016, Adekenov 2017]. All of these steps undergo a complex regulation, including this occurring at transcriptional level mediated by transcription factors (TFs) [Wang et al. 2021].

STLs display a vast spectrum of pharmacological activities. Numerous studies confirmed their anti-inflammatory, anticancer, antiviral, antiplasmodial, antifungal, antimicrobial and hepatoprotective properties. They have a potential to be used in the treatment and/or prevention of broad range of diseases and disorders, for instance malaria, migraine, fever, rheumatoid arthritis, insomnia, diabetes, various types of cancer (among others leukemia, breast, colon and lung cancer) as well as many inflammation-related disorders [Moujir et al. 2020, Matos et al. 2021].

STLs are mainly found in the leaves and the flowers of *Asteraceae* species, however, at rather low levels. Their low bioavailability is the main limiting factor for widespread use of their pharmacological potential. Therefore, elucidation of STLs biosynthesis pathways and their regulatory mechanism is of great significance. A more complete knowledge on their biosynthetic genes and TFs that control their expression provide opportunities for increasing their production and lowering synthesis costs. This may be achieved through various biotechnological approaches, such as metabolic engineering (both in plants or in microorganisms) [Majdi et al. 2016]. However, in order to do so, extensive molecular characterization of the process is required. Here, a comprehensive review on the genetic background of STLs biosynthesis in *Asteraceae* is given, starting from the earliest to the most recent findings on this topic.

ARTEMISININ

Artemisinin belongs to STLs produced by *Artemisia annua* and many other *Artemisia* species (e.g. *A. makrocephala*, *A. vachanica, A. vulgaris*) [Numonov et al. 2019]. According to Majdi [2016] it is the most widely used sesquiterpene drug in the world. Due to artemisinin and its derivatives being effective in the treatment of malaria and viral infections (and also their potential anticancer activity) [Wani et al. 2021], numerous studies focused on its biosynthesis elucidation (Fig. 1). Formation of FPP catalyzed by FPPS is recognized as the first step in artemisinin biosynthetic pathway [Ma et al. 2015]. The coding sequence of *FPPS* from *A. annua* was firstly reported by Matsushita et al. [1996]. Among subsequent studies concentrating on artemisinin biosynthesis from a genetic point of view are those carried out by Chang et al. [2000] and Mercke et al. [2000]. The authors successfully isolated the full-length cDNA sequence of amorpha-4,11-diene synthase (*ADS*), which catalyzes the next step in artemisinin biosynthesis, that is the cyclization of FPP. Another key enzyme involved in the process is a multifunctional sesquiterpene oxidase CYP71AV1, which was cloned and characterized by Teoh et al. [2006]. Soon after the role and coding sequences of artemisinic aldehyde Δ11(13) double bond reductase (*DBR2*) [Zhang et al. 2008], aldehyde dehydrogenase 1 (*ALDH1*) [Teoh et al. 2009] and alcohol dehydrogenease (*ADH1*) [Polichuk et al. 2010] from *A. annua* also became known.

As glandular trichomes in plants produce numerous secondary metabolites, artemisinin included, Soetaert et al. [2013] compared its transcriptomic data to data obtained from

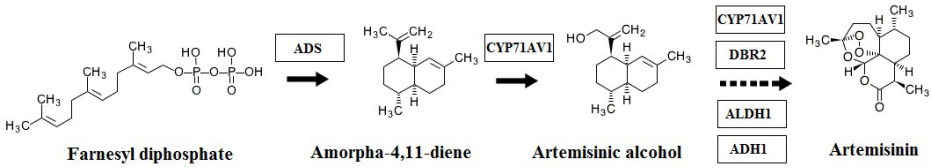


Fig. 1. Overview of artemisinin biosynthesis. ADS, amorpha-4,11-diene synthase; CYP71AV1, cytochrome P450 monooxygenase; DBR2, artemisinic aldehyde Δ11(13) double bond reductase; ALDH1, aldehyde dehydrogenase 1; ADH1, alcohol dehydrogenease. Solid and dashed arrows represent single- and multistep reactions, respectively.

filamentous trichomes of *A. annua.* The study showed that most of the genes involved in artemisinin biosynthesis (apart from *FDS*) were significantly upregulated in glandular trichomes. However, all of the corresponding transcripts were also detected in filamentous trichomes.

Integrated approach which combined metabolomics, transcriptomics and gene function analyses undertook by Ma et al. [2015] resulted in the identification of sequences encoding eight enzymes involved in artemisinin biosynthesis (*FDS*, *ADS*, *CPR*, *CYP71AV1*, *DBR2*, *ALDH1*, *CYB5* and *ADH1*) in cDNA libraries of six *A. annua* tissues (e.g. leaves of various spatial positions, the inflorescence branches, head inflorescence buds, fully open head inflorescence). The researchers compared the gene expression patterns to metabolite levels and demonstrated its association during plant development. Moreover, the coding sequences of genes involved in both MVA and MEP pathways, which precede the artemisinin biosynthesis, were also obtained within this study.

Further studies regarding artemisinin focused on TFs and their role in its production regulation. One of identified trichome-specific TFs in *A. annua* is *AaORA*, which belongs to AP2/ERF family. *AaORA* was confirmed to act as a positive regulator in artemisinin biosynthesis. It was shown to display similar expression patterns as *ADS*, *CYP71AV1* and *DBR2*. Moreover, its overexpression resulted in upregulation of these genes transcription, which consequently led to increased metabolite biosynthesis. Conversely, in *AaORA* RNAi transgenic lines, observed decrease in *AaORA* transcript level was accompanied by reduced transcript levels of above-mentioned genes as well as lower artemisinin content [Lu et al. 2013].

Zhou et al. [2020] identified *AaTAR2* (*TRICHOME AND ARTEMISININ REGULATOR 2*), which belongs to MYB TFs family, as playing a crucial role in both trichome formation and artemisinin production. *AaTAR2*-supressed plants were reported to exhibit expression downregulation of key enzymes involved in artemisinin biosynthesis. Moreover, they had reduced metabolite content as well as reduced number of glandular trichomes. On the contrary, *AaTAR2*-overexpression lines showed increased artemisinin content, upregulated levels of corresponding transcripts and elevated number of trichomes.

Chen et al. [2017] reported that artemisinin biosynthesis is positively regulated by the WRKY TF *AaGSW1* (*GLANDULAR TRICHOME-SPECIFIC WRKY 1*). Its overexpression resulted in increased artemisinin content and elevated transcript levels of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1*. It was also reported that *AaGSW1* directly promoted the expression of *CYP71AV1* by binding to its promoter, whereas other genes were activated through indirect means (e.g. positive regulation of *AaORA* expression).

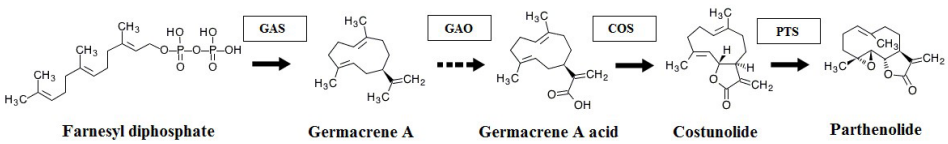
Apart from TFs promoting artemisinin biosynthesis, one acting as a negative regulator of the pathway was also identified. Wu et al. [2021] reported that *AaMYB15*, an R2R3-MYB class TF, acts as *AaORA* repressor. By suppressing *AaORA* transcriptional activity it indirectly downregulated the expression of key genes involved in artemisinin biosynthesis. The study showed that *AaMYB15* overexpression led to decreased transcript levels of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* along with reduced artemisinin content.

PARTHENOLIDE AND ITS PRECURSOR – COSTUNOLIDE

Parthenolide is the major STL that can be found in *Tanacetum parthenium*. Additionally to being used in the treatment of migraine, it was found to have anti-inflammatory and anti-cancer activity [Agatonovic-Kustrin and Morton 2018]. The initial step in parthenolide biosynthesis (Fig. 2) is the cyclization of FDP catalyzed by germacrene A synthase (GAS). The first study dedicated to isolation and characterization of *TpGAS* in *T. parthenium* was conducted by Majdi et al. [2011]. The research showed that high expression of *TpGAS* found in glandular trichomes was closely correlated with high concentrations of parthenolide. Subsequent enzymes involved in the pathway include germacrene A oxidase (GAO), which converts germacrene A to germacrenoic acid and costunolide synthase (COS), which participates in costunolide formation. Finally, parthenolide synthase (PTS) catalyzes the last step of parthenolide biosynthesis. All the above-mentioned biosynthetic genes were successfully identified in *T. parthenium* by Liu et al. [2014]. The study showed that reconstitution of complete parthenolide biosynthetic pathway in *Nicotiana benthamiana* through transient heterologous gene expression resulted in production of the metabolite.

The influence of plant growth regulators (namely methyl jasmonate – MJ and salicylic acid – SA) on parthenolide production in *T. parthenium* was investigated by Majdi et al. [2015]. The study showed that expression of all biosynthetic genes (*TpGAS*, *TpGAO*, *TpCOS* and *TpPTS*) was upregulated by exogenous application of MJ and SA. Transcription of selected genes from upstream pathways (MVA and MEP) was also enhanced. Obtained results indicated that changes in parthenolide accumulation after the phytohormones treatment corresponded to observed changes in *TpGAS* transcript level. Therefore, GAS is believed to play a crucial role in parthenolide biosynthesis regulation.

Costunolide, the immediate precursor of parthenolide and intermediate in biosynthesis of many other STLs, is reported to exhibit a wide range of biological activities, includ-



GAS – germacrene A synthase, GAO – germacrene A oxidase, COS – costunolide synthase, PTS – parthenolide synthase. Solid and dashed arrows represent single- and multistep reactions, respectively

Fig. 2. Overview of parthenolide biosynthesis

ing anti-inflammatory, anti-allergic, anti-diabetic, anti-carcinogenic and neuroprotective properties [Kim and Choi 2019]. Apart from molecular characterization in *T. parthenium,* the genes related to costunolide biosynthesis have been isolated from other species belonging to *Asteraceae* family, such as *Lactuca sativa* (*GAS* identified by Bennett et al. [2002], *GAO* identified by Nguyen et al. [2010], *COS* identified by Ikezawa et al. [2011]), *Helianthus annuus* (*GAS* identified by Göpfert et al. [2009], *GAO* identified by Nguyen et al. [2010], *COS* identified by Frey et al. [2020]) or *Saussurea lappa* (all genes identified by Bains et al. [2019]).

The study of Thakur et al. [2020] provided insight into transcriptional modulation of costunolide biosynthesis in *S. lappa*. Comparative transcriptomics suggested the putative role of TFs belonging to MYB family in regulation of *SlCOS1* expression, which is responsible for the last step of costunolide generation. Further research performed in this species focused on the final step of MVA pathway, that is the generation of the common precursor for sesquiterpenoid biosynthesis – isopentenyl diphosphate (IPP) catalyzed by diphosphomevalonate decarboxylase (DPD). This step is considered to have a potential for costunolide pathway engineering. The study revealed the involvement of *SlDOF2*, *SlbHLH3* and *SlWRKY2* TFs from Dof, bHLH and WRKY families in positive regulation of *SlDPD* [Thakur et al. 2021].

XANTHATIN AND OTHER XANTHANOLIDES

Xanthatin, xanthumin (Fig. 3) and 8-epixanthatin belong to the STLs classified as xanthanolides, which are synthesized in the glandular trichomes of *Xanthium strumarium*. They exhibit antitumor, antimicrobial and antifungal activities [Fan et al. 2019]. Xanthanolides are reported to derive from the universal sesquiterpene precursor FDP, which is used by sesquiterpene synthase for the formation of specialized sesquiterpene skeleton. Attempts of elucidating the biogenesis of xanthanolides undertaken by Li et al. [2016a] primarily focused on the sesquiterpene synthase gene identification. Analysis of the glandular trichome transcriptome of *X. strumarium* enabled isolation of three sesquiterpene synthases (*XsTPS1*, *XsTPS2* and *XsTPS3*), of which one (*XsTPS3*) was confirmed to participate in the formation of germacrene A – the suggested intermediate in the biosynthesis of xanthanolides. Following research of Li et al. [2016b] enabled identification of other putative genes related to xanthanolides biosynthesis. A number of candidate genes involved in subsequent steps of xanthanolides formation were proposed, including cytochrome CYP71 P450s, dehydrogenases and acetyltransferases genes. However, further research confirming their role in the pathway is required. Also, over one hundred TFs abundantly expressed in the trichomes were detected, mostly from AP2/ERF and WRKY family, providing a starting point for the better understanding of the regulatory mechanism of the pathway.

LACTUCIN AND ITS DERIVATIVES

Lactucin (Fig. 3), lactucopicrin or 8-deoxylactucin are the exemplary guaianolides that can be found in *Cichorium intybus*, *Cichorium endivia* and *Lactuca serriola*. They are reported to act as analgesics, sedatives and sleep-inducing agents [Moujir et al. 2020].

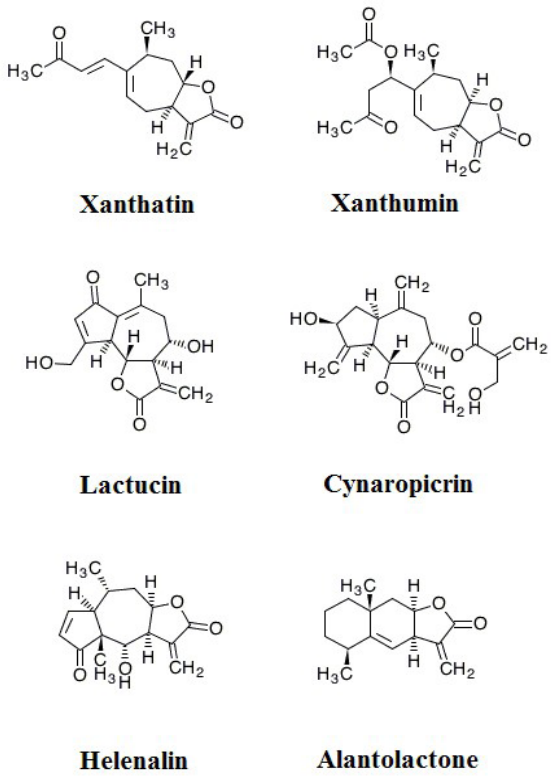


Fig. 3. Structures of addressed sesquiterpene lactones

Lactucin-related compounds derive from costunolide, which is initially transformed by kauniolide synthase (KLS) into kauniolide and then further modified. Genes involved in costunolide generation in *C. intybus* have been already isolated and characterized. Initially, Bouwmeester et al. [2002] identified two isoforms of germacrene A synthase- long (*CiGASlo*) and short one (*CiGASsh*). Bogdanović et al. [2020] evaluated the effect of their silencing by RNAi on the concentration of downstream metabolites. The reduction of guaianolide oxalates content (8-deoxylactucin-15-oxalate, lactucin-15-oxalate and lactucopicrin-15-oxalate) was demonstrated. The coding sequence for the next enzyme involved in the pathway, GAO (primarily annotated as CYP71AV8), was reported by Cankar et al. [2011]. The third gene related to costunolide formation, *COS*, was characterized by Liu et al. [2011]. However, the genes involved in consecutive steps of lactucin biosynthesis (starting from the gene encoding KLS) in either of *Cichorium* or *Lactuca* species remain unknown. So far, the *KLS* has only been characterized in *T. parthenium* [Liu et al. 2018].

Testone et al. [2019] performed transcriptome characterization of *Cichorium endivia* in relation to STLs biosynthesis pathway. Not only did they identify the *GAS*, *GAO*, *COS* genes, but also found positive correlation between their transcription and STLs content, suggesting regulatory mechanisms occurring at transcriptional level. The transcriptional control of *GAS* by five TFs from MYB, MYB related and WRKY families was inferred.

CYNAROPICRIN

Cynaropicrin (Fig. 3) is a guaianolide type STL known for its antitumor (cytotoxic and pro-apoptotic) activity. The compound is present in several species from *Asteraceae* family, such as *Cynara cardunculus*, *Centaurea drabifolia* or *Saussurea calcicola* [Moujir et al. 2020]. Initial reaction leading to cynaropicrin biosynthesis is catalyzed by GAS, coding sequence of which in *C. cardunculus* was first published by Menin et al. [2012]. The study confirmed the role of *CcGAS* in cynaropicrin synthesis by showing correlation between its expression level and the metabolite content. Eljounaidi et al. [2014] succeeded in the identification of genes involved in subsequent germacrene A transformation, that is *CYP71AV9* (*CcGAO*) and *CYP71BL5* (*CcCOS)*. Recently, Puglia et al. [2020] carried out an investigation of *C. cardunculus* transcriptome and found that most of the STLs biosynthetic genes were strongly expressed in the flower heads. They annotated four transcripts for *GAS*, two transcripts for *GAO* and two transcripts for *COS*. Downstream pathway, leading to the cynaropicrin synthesis, is yet to be elucidated.

ARGLABIN

Apart from being the source of artemisinin, genus *Artemisia* is rich in other STLs such as arglabin. This metabolite was first isolated from *Artemisia glabella*, but can be found also in *Artemisia myriantha* and *Artemisia jacutica*. It demonstrates anti-tumor and anti-inflammatory properties [Moujir et al. 2020, Matos et al. 2021]. Recent molecular investigation of arglabin biosynthetic genes performed in *A. glabella* resulted in identification of partial coding sequences of *AgGAS*, *AgGAO* and *AgCOS*. Moreover, their expression in callus tissues and *in vitro* regenerated plants was confirmed. The genes responsible for further enzymatic modifications of costunolide still need to be determined [Adekenova et al. 2021].

FUTURE PERSPECTIVES

A vast range of species belonging to *Asteraceae* family that are a rich source of many valuable STLs lack the basic biochemical and genetic information regarding their biosynthesis. Among them is *Taraxacum officinale*, with its STLs (e.g. taraxacin, taraxacerin) showing hepatoprotective and anti-inflammatory potential [Rasool and Sharma 2014]. Similarly, the biosynthesis of alantolactone (Fig. 3) in *Inula helenium* requires investigation as multiple studies proved its effectiveness in cancer treatment [Babaei et al. 2021]. Helenalin (Fig. 3) and 11,13-dihydrohelenalin, the major active substances of the extracts prepared from *Arnica montana,* a very old medicinal plant, are known to demonstrate anti-inflammatory and antibacterial properties [Drogosz and Janecka 2019]. Nevertheless, their biosynthesis is still poorly understood.

As new compounds belonging to STLs are being discovered [e.g. Li et al. 2021] new possibilities of their application in the treatment of various diseases and disorders arise. Due to their limitless potential usage, it has become essential to gain knowledge on their biosynthesis. Germacrene A is considered a central intermediate in sesquiterpenes synthesis [Xu and Dickschat 2020], therefore many studies undertaken in previously uncharr-

acterized species begin from its molecular investigation. Such was the case of *Achillea millefolium* [Pazouki et al. 2015], *Matricaria chamomilla* [Ling et al. 2020] and many of the abovementioned *Asteraceae* species. Initial investigations lead to further studies resulting in knowledge which subsequently allows for novel approaches in metabolic engineering to be explored. Combinatorial biosynthesis, for instance, which is based on combining biosynthetic genes from different species in a single host enables the synthesis of novel compounds with potentially improved medicinal value [Eljounaidi and Lichman 2020]. This strategy was explored in *Asteraceae* by Kashkooli et al. [2019] and was possible due to previous studies which aimed at elucidation of STLs biosynthesis in *A. annua* and *T. parthenium*. What is more, understanding of molecular basics of sesquiterpene biosynthesis and its regulation opens new perspectives for their production via transgenic approach. The STLs production can be achieved in heterologous systems or modified in host plants by overexpression of genes involved in biosynthetic pathway, overexpression of TFs which are positive regulators of this pathway and silencing of negatively regulating TFs, by suppressing competitive pathway genes or by inhibiting further conversion of the metabolite [Majdi et al. 2016, Wani et al. 2021]. Overall, a more complete knowledge of secondary metabolites synthesis might be used to increase the production of the desired compounds and play a pivotal role in the development of new therapeutic drugs. Nonetheless, even though there is already a significant amount of data in the literature on molecular background of STLs biosynthesis in *Asteraceae*, further investigations are required in order to fully exploit their pharmacological potential.

CONCLUSION

Sesquiterpene lactones, secondary metabolites found in the *Asteraceae* family, display many biological activities and therefore are recognized as medicinally essential compounds. Their importance have led many researchers to undertake efforts focused on elucidating their biosynthesis pathways. Here, fundamental accomplishments concerning STLs production are reviewed from molecular genetics point of view. Findings concerning the biosynthesis of some of the most widely known STLs, including artemisinin, parthenolide and costunolide, are reported. Moreover, recent studies on some of less known STLs, such as lactucin and cynaropicrin, are also discussed. Overall, the elucidation of STLs biosynthesis and characterization of the genes involved in the pathways is of great significance, as this knowledge is crucial for future strategies aiming at STLs production improvement.

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