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Regulation and metabolic engineering of tanshinone biosynthesis

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We summarize recent discoveries regarding the mechanisms underlying tanshinone biosynthesis and how the process is regulated.

Abstract

Salvia miltiorrhiza (Chinese name: dānshēn) is used in traditional Chinese medicine for the treatment of cardiovascular and cerebrovascular diseases. The tanshinones represent the most important biological active class of compound present in dānshēn extracts. They are synthesized via either the cytoplasmic mevalonate or the plastidial 2-C-methy-D-erythritol 4-phosphate pathway. Here, we summarize recent discoveries regarding the mechanisms underlying tanshinone biosynthesis and how the process is regulated. Tanshinone accumulation *in planta* is affected by a range of elicitors and by the composition of the culture medium. Its production in hairy root cultures can be enhanced by the over-expression of genes encoding 1-deoxy-D-xylulose 5-phosphate synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, geranylgeranyl diphosphate synthase and allene oxide cyclase. The pathway leading to the biosynthesis of the tanshinone precursors miltiradiene and ferruginol, has been engineered in yeast.

Key words: *Salvia miltiorrhiza*; dānshēn; tanshinone biosynthesis; regulation; metabolic engineering.

Abbreviations: human immunodeficiency virus type 1, HIV-1; hepatitis B virus, HBV; tanshinone I, TI; tanshinone IIA, TIIA; tanshinone IIB, TIIB; cryptotanshinone, CT; dyhydrotanshinone I, DTI; expressed sequence tag, EST; transcript-derived fragments, TDFs; isopentenyl diphosphate, IPP; dimethylallyl diphosphate, DMAPP; mevalonate, MVA; 2-C-methy-D-erythritol 4-phosphate, MEP; acetyl-CoA C-acetyltransferase, AACT; 3-hydroxy-3-methylglutaryl-CoA synthase, HMGS; 3-hydroxy-3-methylglutary-CoA, HMG-CoA; 3-hydroxy-3-methylglutaryl-CoA reductase, HMGR; isopentencyl diphosphate, IPP; mevalonate kinase, MK; 5-phosphomevalonate kinase, PMK; mevalonate 5-diphosphate decarboxylase, MDC; glyceraldehyde 3-phosphate, GA-3P; 1-deoxy-D-xylulose 5-phosphate, DXP; DXP synthase, DXS; DXP reductoisomerase, DXR; cytidyl transferase, MCT; 4-(cytidine 5-diphospho)-2-C-methyl-D-erythritol kinase, CMK; 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, MDC; 1-hydroxy-2-methyl-2-(E)-butenyl

4-diphosphate synthase, HDS; 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase, HDR; isopentenyl-diphosphate deltaisomerase, IPPI; geranyl diphosphate synthase, GPPS; geranyl pyrophosphate, GPP; farnesyl diphosphate, FPP; farnesyl diphosphate synthase, FPPS; geranylgeranyl diphosphate synthase, GGPPS; geranylgeranyl diphosphate, GGPP; copalyl diphosphate synthase, CPS; copalyl diphosphate, CPP; *ent*-kaurene synthase-like, KSL; sodium nitroprusside, SNP; nitrogen oxide, (NO); methl jasmonate, MJ; indol-yl-3-acetic acid, IAA; 1-naphthaleneacetic acid, NAA; gibberellic acid, GA3; 6-benzylaminopurine, 6-BA; thidiazuron, TDZ; abscisic acid, ABA; β-aminobutyric acid, BABA; α-animo isobutyric acid, AIB; polyethylene glycol, PEG; reactive oxygen species, ROS.

1. Introduction

Salvia species have been used in herbal medicine in many parts of the world, thanks to their content of a range of medicinally active compounds. The root of *S. miltiorrhiza* (dānshēn in Chinese), for example, is a highly prized ingredient of a number of Asian herbal medicine preparations; it is associated with curative properties against coronary artery disease, angina, myocardial infarction, cerebrovascular disease, hepatitis, chronic renal failure and dysmenorrhea[1,2] As many as 320 dānshēn-containing preparations are manufactured by various pharmaceutical concerns.[3] Some of these have lately become available outside of China, and represent the first traditional Chinese medicines to be approved for clinical trials by the US Food and Drug Administration. Its recognized medicinal value has generated a steady rise in demand for dānshēn, and some 80 Kt per year are currently being produced in China for domestic use and the export trade.[4] A number of *Salvia* spp. are currently exploited in herbal medicine concoctions. *S. aegyptiaca* is an ingredient in formulations directed against numerous complaints, including diarrhoea, gonorrhoea, haemorrhoids, eye diseases, nervous disorders, dizziness and trembling; *S. bucharica* against liver ailment; *S. cavaleriei* against dysentery, boils and superficial wounds; *S. desoleria* against menstrual, digestive and central nervous system diseases; *S. officinalis* against tuberculosis, psoriasis and seborrhoeic eczema; *S. parryi* against various stomach disorders; *S. przewalskii* against cardiovascular problems; and finally *S. yunnanensis* against both the acquired immune deficiency syndrome (AIDS) and hepatitis B viruses.[5−10]

The two major classes of active compounds present in dānshēn are the lipid-soluble tanshinones and the water-soluble phenolic acids. The tanshinones are abietane diterpenes, and were first isolated by Nakao in 1930 from dānshēn roots, from which, in the intervening period, more than 40 other diterpenes have been identified.[11] Tanshinones have been detected throughout the plant, with the exception of the seed. In the root, they accumulate preferentially in the cortex, while in the above ground part of the plant, they are found largely in the epidermis.[12−14] The compounds have been shown to be associated with a range of pharmaceutical

activities. The five most important groups of tanshinone, as defined by their functionality (Fig. 1), are tanshinone I (TI), tanshinone IIA (TIIA), tanshinone IIB (TIIB), cryptotanshinone (CT) and dyhydrotanshinone I (DTI). TI suppresses the growth of breast cancer cells through its interaction with adhesion molecules;[15] TIIA inhibits osteoclast differentiation and affects the bone resorptive activity of differentiated osteoclasts;[16] TIIB exhibits neuro-protective activity in rats;[17] CT inhibits the growth of oral bacteria;[18] and DTI suppresses endothelial cell proliferation, migration, invasion and tube formation.[19]

Traditional methods of tanshinone production are inadequate to meet the rapidly rising demand, largely because yield levels are low and the plants are very slow-growing. Here, the prospects for biotechological intervention aimed at increasing tanshinone production are discussed, focusing on the use of elicitors, the manipulation of culture conditions, and genetic engineering.

2. The tanshinone synthetic pathway in dānshēn roots

The tanshinone biosynthesis pathway is complex and has been only partially elucidated so far. A multi-platform genomics-based approach has recently been initiated, based on the construction of an expressed sequence tag (EST) library, the application of Solexa sequencing and 454 GS-FLX transcriptome pyrosequencing, the use of cDNA microarray technology and cDNA-AFLP profiling.[20–24] The EST library was generated from mRNA extracted from whole plantlets, and comprises 10,228 sequences;[20] the Solexa-based transcriptome sampled from the entire plant life cycle) has defined 56,774 unigenes;[21] the 454 GS-FLX pyrosequencing has generated a set of 64,139 unigenes present in the root and leaf;[22] the cDNA microarray analysis has identified 114 differentially transcribed cDNAs in hairy root cultures;[23] and the cDNA-AFLP profiling, using 128 primer pairs, has revealed that 2300 transcript-derived fragments (TDFs) were differentially expressed among *S. miltiorrhiza* and *S. castanea*.[24] Inspection of a genome draft genome sequence (not currently available in the public domain) has identified 40 terpenoid biosynthesis-related genes, the products of which include enzymes involved in the

biosynthesis of the precursors of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP).[25]

The tanshinones are synthesized from the central five-carbon intermediate IPP, which is itself generated via either the cytoplasmic mevalonic acid (MVA) pathway or the plastidial 2-C-methy-D-erythritol 4-phosphate (MEP) pathway. Acetyl-CoA C-acetyltransferase (AACT) is the first enzyme in the MVA pathway. This enzyme catalyses the formation of acetoacetyl-CoA from two molecules of acetyl-CoA. The enzyme 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) catalyses the condensation of acetyl-CoA and acetoacetyl-CoA to form 3-hydroxy-3-methylglutary-CoA (HMG-CoA), which is subsequently reduced to yield MVA in the presence of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). Mevalonate kinase (MK) phosphorylates MVA into mevalonate-5-phosphate (MVAP), and a further phosphorylation into mevalonate diphosphate (MVAPP) is catalysed by 5-phosphomevalonate kinase (PMK). The transformation of MVAPP to IPP involves the enzyme MVAPP decarboxylase (MDC). The initial step in the MEP pathway is the condensation of pyruvate and glyceraldehyde 3-phosphate (GA-3P) to form 1-deoxy-D-xylulose 5-phosphate (DXP), catalysed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS). In the presence of DXP reductoisomerase (DXR), DXP is reduced to MEP and subsequently is transformed through the action of the enzyme 2-C-methyl-D-erythritol-4-phosphate cytidyl transferase (MCT) into 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-ME). Later steps in the pathway comprise the mediated by CDP-ME kinase (CMK)-mediated phosphorylation of CDP-ME into CDP-ME-2-phosphate (CDP-MEP), which provides the substrate for the action of 2-C- methyl-D-erythritol-2,4-cyclodiphosphate synthase (MDS) to form 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (cMEPP). cMEPP in turn is converted by the enzyme 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate (HMBPP) synthase (HDS) into HMBPP, and finally, HMBPP reductase (HDR) catalyses the formation of IPP and dimethylallyl-diphosphate (DMAPP). The conversion of IPP to DMAPP and the equilibrium concentrations of IPP and DMAPP are controlled by the enzyme isopentenyl-diphosphate deltaisomerase (IPPI). Geranyl diphosphate synthase (GPPS)

condenses IPP to DMAPP to form geranyl pyrophosphate (GPP). The condensation of IPP and GPP to form farnesyl diphosphate (FPP) is mediated by farnesyl diphosphate synthase (FPPS). Geranylgeranyl diphosphate synthase (GGPPS) condenses IPP with FPP to form geranylgeranyl diphosphate (GGPP).[26,27] Copalyl diphosphate synthase (CPS), a class II diterpene cyclase, converts GGPP to copalyl diphosphate (CPP), whereas *ent*-kaurene synthase-like (KSL), a class I diterpene cyclase, produces the abietane miltiradiene class of diterpenes.[28] Miltiradiene is transformed to ferruginol though the action of the cytochrome P450 monooxygenase CYP76AH1 (Fig. 2).[29]

A set of 14 of the dānshēn genes involved in the MVA and MEP pathways, namely *SmAACT1*, *SmHMGS*, *SmHMGR1*, *SmHGMR2*, *SmHGMR3*, *SmDXR*, *SmCMK*, *SmMDS*, *SmHDR1*, *SmGGPPS*, *SmFPPS*, *SmCPS1*, *SmKSL1* and *SmCYP76AH1* has been isolated in recent years (Table 1).[23,30−42] Kai *et al*.[43] successfully demonstrated that the activity of *SmHMGR*, *SmDXS2*, *SmFPPS*, *SmGGPPS* and *SmCPS* is important for the accumulation of tanshinones in hairy root cultures of dānshēn, and have suggested that these five genes could function as rate-limiting genes in the tanshinone biosynthesis pathway. The cyclization of GGPP into diterpenoids is achieved by the action of a number of bifunctional synthases, including the miltiradiene synthases SmCPS and SmKSL. Since SmKSL co-precipitates with SmCPS *in vitro*, the assumption is that these two enzymes interact directly with one another *in vivo*, possibly through the formation of an enzyme complex. Protein modeling has demonstrated that the active sites in an SmKSL-SmCPS complex are more closely associated with one another than are those in SmCPS-SmKSL.[44]

3. Regulation of tanshinone biosynthesis in dānshēn

3.1. Biotic elicitors

Song *et al*.[45] have shown that an elicitor produced by *Armillaria mellea* is able to induce tanshinone accumulation in a dānshēn crown gall culture. Certain oligosaccharins are effective in both promoting hairy root growth and stimulating tanshinone biosynthesis.[46] Yeast elicitor (the carbohydrate fraction of yeast extract)

increased the accumulation of tanshinones in dānshēn hairy root and cell suspension cultures.[43,47−53] Yeast elicitor has a marked positive effect on the growth of hairy roots [48], although this result could not be confirmed by Ge and Wu.[50] This discrepancy may, however, been due to differences in the amount of time allowed. A fungal elicitor derived from a konjac endophytic fungus has been observed to inhibit hairy root growth, but at the same time to enhance the biosynthesis of DTI and CT.[54] Both an extract of the mycelium and the polysaccharide fraction of the endophytic fungus *Trichoderma atroviride* can promote hairy root growth and stimulate the production of tanshinones.[55] Yeast extract, certain oligogalacturonides and particularly an elicitor produced by the fungus *Fusarium oxysporum*, can all increase tanshinone yield.[46] Wu *et al*.[56] have reported that the tanshinone content of hairy roots can be increased by at least 12 fold by co-cultivation with live *Bacillus cereus* cells, although the growth of the hairy roots was significantly inhibited. The polysaccharide fraction of *Bacillus cereus* stimulates tanshinone accumulation in hairy roots by about seven-fold, while the protein fraction promotes hairy root growth.[57] *Streptomyces pactum* Act12 has a certain promotional effect on the growth of hairy root at an appropriate concentration and increases the accumulation level of tanshinone in hairy roots.[58] A chito-oligosaccharide plant growth regulator is known to significantly enhance the accumulation of TIIA, as well as to promote plant and root growth.[59]

3.2. Abiotic elicitors

Supplementation of growth medium with various metal ions can promote tanshinone biosynthesis in dānshēn hairy roots and seedlings.[43,49,51,52,60−65] both Cu^{2+} and Zn^{2+} have a positive effect on biomass accumulation,[60] while Ag^{+} and Co^{2+} have a negative one.[51,61] Mn^{2+} has a dose-dependent impact on seedling growth.[62] The provision of 100 μM sodium nitroprusside (SNP), which supplies a nitrogen oxide moiety, also enhances tanshinone production.[63] Some plant growth regulators, such as methl jasmonate (MJ), indol-yl-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), gibberellic acid (GA3), 6-benzylaminopurine (6-BA), thidiazuron (TDZ) and abscisic acid (ABA), induced tanshinone accumulation in dānshēn hairy roots and

seedlings.[43,49,54,66–72] Gupta *et al*.[69] have shown that hairy root growth is stimulated by the inclusion of both TDZ and 6-BA, while according to Sun *et al*.[67], GA3 promotes the growth of the aerial part of the dānshēn plant, but inhibits the growth of the root. The above-ground and underground biomass of dānshēn increased with the increasing of IAA concentration and then decreased. The impact of MJ and ABA on the growth of dānshēn is inconclusive; according to Ge and Wu,[50] MJ stimulates the growth of hairy roots, but this conclusion was not borne out in other experiments.[63,66] Gupta *et al*.[69] were unable to demonstrate any effect of supplying hairy roots with ABA, while Sheng and Zhu claimed that the phytohormone has a negative impact.[72] The provision of β-aminobutyric acid (BABA), α-animo isobutyric acid (AIB) or sodium nitroprusside all increase the level of tanshinone production in hairy root cultures, as well as enhancing hairy root growth.[50,52,69] Shi *et al*.[73] and Wu and Shi[53] have shown that supplementing hairy root cultures with sorbitol also enhances their tanshinone content, as does that of polyethylene glycol (PEG)-6000, although in the latter case, the supplement suppresses biomass accumulation.[70,71,74]

3.3. Other treatments

Moisture stress favors the accumulation of TI, TIIA, CT and DTI. Severe drought reduces the shoot and root biomass of dānshēn plants, although an episode of mild drought appears to have the opposite effect.[75,76] Tanshinone content responds negatively to an increase in nitrogen availability.[77,78] Han and Liang have shown that that the greater the quantity of available phosphorus, the higher the TIIA content of the plant.[78]

3.4. Combinations of elicitors and treatments

Some biotic elicitors and abiotic elicitors have been shown to act synergistically to regulate tanshinone biosynthesis. The exposure of hairy root dānshēn cultures to Ag^{+} prior to supplementation by yeast extract amplifies the enhancing effect of the supplement.[49] When yeast extract and Ag^{+} are supplied simultaneously, the accumulation of TI is encouraged; combining yeast extract and Co^{2+} favors TIIA

content, and the combination of yeast extract with AIB promotes CT accumulation.[52] Both the provision of sucrose and the replacement of the medium prior to the addition of Ag^+ reverses the growth inhibition, significantly increasing biomass concentration and tanshinone yield.[61] Ge and Wu were able to demonstrate that combining yeast extract with BABA or MJ increases the production of tanshinones, but only when hormones are given a few days before the provision of yeast extract.[50] Yeast extract and sorbitol appear to act synergistically, since the provision of both simultaneously has a larger effect than the provision of either additive on its own.[53,72] Combinations of yeast extract and the various elicitors do not inhibit the growth of dānshēn hairy roots; in fact, the combination yeast extract plus sorbitol even promotes the expansion of the root biomass.[43,49,50,52,53,73] Combining a fungal elicitor with MJ similarly increases tanshinone content to higher levels than either the fungal elicitor or MJ on its own.[54] A combination of low level IAA and GA also promoted increase in both dānshēn biomass and tanshinone content.[67] The total content of tanshinone IIA in hairy roots is stimulated by the presence of 0.2 mg/L NAA and 3.0 mg/L 6-BA.[68] Finally, spraying the leaves with a solution of Cu^{2+} and Zn^{2+} appears to stimulate the production of tanshinones in the root.[60]

4. The mechanism of elicitor-induced tanshinone accumulation in dānshēn

The heightened accumulation of tanshinones induced by exogenously supplied MJ involves the activity of at least six enzymes, namely SmAACT, SmHMGS, SmHMGR, SmDXR, SmDXS2, SmGGPPS, SmIPPI and SmCPS. A slightly different group of enzymes - SmHMGR, SmDXS2, SmIPPI, SmFPPS, SmGGPPS and SmCPS - is important for enhancement in tanshinone content triggered by exposure to Ag^+. The beneficial effect of supplementation with yeast extract involves the six enzymes SmHMGS, SmDXR, SmDXS2, SmCMK, SmIPPI and SmCPS, while the combination of yeast extract supplementation and Ag^+ pre-treatment up-regulates the eight genes *SmAACT*, *SmHMGS*, *SmDXR*, Sm*DXS2*, Sm*CMK*, Sm*FPPS*, Sm*GGPPS* and *SmCPS*, and at the same time maintains a consistently high abundance of *SmHMGR* and *SmIPPI* transcript.[43] Yang *et al*.[71] have shown that the abundance of

SmHMGR, *SmDXS* and *SmDXR* transcripts, and the activity of SmHMGR and SmDXS are both stimulated by the presence of PEG, ABA and MJ. The genes *SmHMGR* and *SmDXR* are both up-regulated in the presence of nitric oxide,[63] *SmHMGR*, *SmDXR*, *SmGGPPS*, *SmCPS* and *SmKSL* are up-regulated by supplementation with the polysaccharide fraction of the endophytic fungusi *Trichoderma atroviride* D16.[55] Thus, both the MVA and MEP pathways are activated by MJ, yeast extract, Ag^{+}, PEG, ABA and nitric oxide, and as a result, so is tanshinone biosynthesis. It has been proposed that the exogenous supply of PEG and ABA triggers the release of endogenous MJ via the activation of an ABA signaling pathway and that this additional MJ (and similarly exogenously supplied MJ) provides the signal directing the increased production of tanshinones via the MEP pathway.[71] Ge and Wu have suggested that the induction in tanshinone accumulation is response to supply of yeast extract plus Ag^{+} results from increased flux through the MEP pathway,[49] but that it may also involve an element of crosstalk with the MVA pathway, which is known to be an important determinant of cell growth.[79] Cu^{2+}, Zn^{2+}, MJ, PEG and ABA-induced tanshinone production is ROS-mediated, whereas that induced by nitric oxide is ROS-independent.[63,65,70]

5. Metabolic engineering of tanshinone biosynthesis

The over-expression of *SmHMGR2* increases the level of SmHMGR2 activity and enhances the production of tanshinones and squalene in cultured hairy roots,[38] while similarly, the over-expression of *SmGGPPS* and/or *SmHMGR* and/or *SmDXS* increases tanshinone production.[80] Of the three enzymes involved, SmGGPPS has the greatest effect on tanshinone production and SmHMGR the least. Simultaneously over-expressing *SmHMGR* and *SmGGPPS* results in a particularly high level of tanshinone production. The over-expression of *SmAOC* significantly enhances the yield of TIIA.[81] Zhou *et al*.[44] have proposed a modular pathway engineering strategy to assemble a heterologous miltiradiene pathway in yeast. Miltiradiene is the precursor of tanshionones in dānshēn (Fig. 2). Fusion of *SmCPS* and *SmKSL*, and also of of *BTS1* (encoding GGPP synthase) and *ERG20* (FPP synthase) significantly

improves the yield of miltiradiene. The best performing transgenic yeast strain proved able to generate 365 mg/L miltiradiene. Guo *et al.*[29] have further shown that the incorporation of genes encoding CYP76AH1 and phyto-CYP reductase in miltiradiene-producing yeast results in measurable amounts of ferruginol.

6. Conclusions and future prospects

The mechanistic basis of tanshinone biosynthesis has not yet been fully elucidated. The genes encoding a number of enzymes, included in this pathway in particular DXS, HDR, AACT, HMGR, GGPPS, CPS and KSL are members of multi-gene families, and these genes exhibit different patterns of expression in time and space, underlining the complexity of terpenoid biosynthesis in dānshēn. Different isoenzymes of one or all of these enzymes may be involved in the biosynthesis of specific terpenoids.[25] The later steps in tanshinone biosynthesis remain particularly obscure, so a current research priority is to identify which enzymes catalyse these steps. As yet, the identity of the transcription factors involved in the regulation of tanshinone biosynthesis has not been ascertained, although it is known that such factors represent a critical element in the biosynthesis of terpenoids in other species. For example, the *Arabidopsis thaliana* MYC2 protein, which belongs to the family of basic helix-loop-helix transcription factors, binds to the promoters of the sesquiterpene synthase genes *TPS21* and *TPS11*.[82] Similarly in tobacco the binding of an AP/ERF transcription factor to the promoter of a putrescine *N*-methyltransferase gene regulates its MJ-induced transcription and thereby influences the accumulation of nicotine and total alkaloids.[83] A key future line of research in dānshēn will therefore be the exploration of the identity and role of transcription factors in tanshinone biosynthesis. A combination of large-scale transcriptome sequencing and co-expression analysis, as demonstrated in *Catharanthus roseus* by Góngora-Castillo *et al.*[84], provides a model strategy for exploring the regulation of tanshinone biosynthesis in dānshēn. The combined heterologous expression of the flavonoid activator transcription factor *AtMYB12* and the legume isoflavone synthase gene *IFS* has been shown to promote the biosynthesis of isoflavone in tobacco leaves, even though the quantity of substrate

present is unlikely to be sufficient.[85] Therefore the over-expression of both structural and regulatory genes, together with the suppression of side-branch enzyme genes, could represent a viable means of improving tanshinone production in dānshēn. Given that the tanshinone precursors miltiradiene and ferruginol have both been synthesized heterologously in yeast,[29,44] it should also be possible to produce tanshinone heterologously in well-developed, rapidly growing, high biomass-producing crop species such as tobacco and tomato. All the elicitors and treatments in this paper have positive effect on tanshinone biosynthesis. Combining any of yeast extract, fungal elicitor, sucrose, AIB, BABA, sorbitol, Co^{2+}, Cu^{2+}, Zn^{2+}, Ag^{+}, PEG, MJ, ABA, IAA, GA, NAA and 6-BA with one another has a larger enhancing effect than would be predicted from the effect each induces on its own.[49,50,52−54,60,61,67,68,72] Cu^{2+}, Zn^{2+}, MJ, PEG and ABA all trigger a burst of ROS, which serves to raise the level of tanshinone production.[63,65,70] Yang *et al*.[71] have shown that the crosstalk between PEG and ABA signalling pathways also has a regulatory effect on tanshinone biosynthesis. The variable effect on tanshinone accumulation and biomass growth of different elicitors and of the culture medium composition indicates that an effort needs to make to clarify the regulatory mechanisms underlying tanshinone biosynthesis.[86] A combination of metabolic engineering and elicitor treatments has the potential to support the sustainable production of tanshinone in the future.[26,43]

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Figure legends

Fig. 1 Chemical structure of tanshinone I, tanshinone IIA, tanshinone IIB, cryptotanshinone and dihydrotanshinone I.

Fig. 2 A proposed pathway for tanshinone biosynthesis in dānshēn (modified from Yang *et al.*[16], Ma *et al.*[19], Gao *et al.*[22] and Guo *et al.*[23]). HMG-CoA: 3-hydroxy-3-methylglutary-CoA; MVA: mevalonic acid; MVAP: mevalonate-5-phosphate; MVAPP: mevalonate diphosphate; GA-3P: glyceraldehyde 3-phosphate; DXP: 1-deoxy-D-xylulose 5-phosphate; MEP: 2-C-methyl-D-erythritol 4-phosphate; CDP-ME: 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; CDP-MEP: 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol 2-phosphate; cMEPP: 2-C-Methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP: 1-hydroxy-2-methyl-2-(E)-buteny 4-diphosphate; IPP: isopentencyl diphosphate; DMAPP: dimethylallyl diphosphate; GPP: geranyl pyrophosphate; FPP: farnesyl diphosphate; GGPP: geranylgeranyl diphosphate; CPP: copalyl diphosphate; AACT: acetyl-CoA C-acetyltransferase; HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase; MK: mevalonate kinase; PMK: 5-phosphomevalonate kinase; MDC: mevalonate 5-diphosphate decarboxylase; DXS: 1-deoxy-D-xylulose 5-phosphate synthase; DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MCT: 2-C-methyl-D-erythritol 4-phosphate cytidyl transferase; CMK: 4-(cytidine 5-diphospho)-2-C-methyl-Derythritol kinase; MDS: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS: 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase; HDR: 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase; IPPI: isopentenyl-diphosphate deltaisomerase; GPPS: geranyl diphosphate synthase; FPPS: farnesyl diphosphate synthase; GGPPS: geranylgeranyl diphosphate synthase; CPS: copalyl diphosphate synthase; KSL: *ent*-kaurene synthase-like.

Table 1 Tanshinone biosynthesis-related genes in dānshēn

Name	Accession no.	Gene length (bp)	Protein length (aa)	Reference
SmDXS1	EU670744	2519	714	[25]
SmDXS2	FJ643618	2522	724	[25]
SmDXS3	JN831116	2142	713	[25]
SmDXS4	JN831117	2142	713	[25]
SmDXS5	JN831118	2112	703	[25]
SmDXR	FJ476255	1425	474	[30]
	DQ991431	1665	474	[31]
SmMCT	JN831096	915	304	[25]
SmCMK	EF534309	1477	396	[32]
SmMDS	JN831097	705	234	[25]
	JX233816	988	234	[33]
SmHDS	JN831098	2229	742	[25]
SmHDR1	JN831099	1392	463	[25]
	JX233817	1647	463	[34]
SmHDR2	JN831100	1389	462	[25]
SmAACT1	EF635969	1569	399	[35]
SmAACT2	JN831101	1212	403	[25]
SmHMGS	FJ785326	1655	460	[36]
SmHMGR1	EU680958	2115	565	[37]
SmHMGR2	FJ747636	1653	550	[38]
SmHMGR3	JN831102	1689	562	[39]
SmHMGR4	JN831103	1653	550	[25]
SmMK	JN831104	1164	387	[25]
SmPMK	JN831095	1530	509	[25]
SmMDC	JN831105	1269	422	[25]
SmIPPI1	EF635967	1234	305	[23]
SmIPPI2	JN831106	810	269	[25]
SmGPPS	JN831107	1275	424	[25]
SmFPPS	EF635968	1494	349	[23]
	HQ687768	1319	349	[40]
SmGGPPS1	FJ643617	1563	364	[41]
	FJ178784	1563	364	[42]
SmGGPPS2	JN831112	1041	346	[25]
SmGGPPS3	JN831113	1140	379	[25]
SmCPS1	EU003997	2613	793	[28]
SmCPS2	JN831114	2274	757	[25]
SmCPS3	JN831115	2106	701	[25]
SmCPS4	JN831120	1983	660	[25]
SmCPS5	JN831121	1338	445	[25]
SmKSL1	EF635966	2110	595	[28]
SmKSL2	JN831119	2289	762	[23]
SmCYP76AH1	JX422213	1488	495	[29]

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