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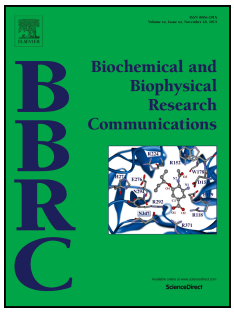

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RNA interference targeting *CYP76AH1* in hairy roots of *Salvia miltiorrhiza* reveals its key role in the biosynthetic pathway of tanshinones

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Abstract

Plant cytochrome P450s (CYPs) are well known as the largest family of enzymes that contribute to both primary metabolism and the chemical diversity of plant secondary metabolites. It is important to elucidate the *in vivo* role of CYPs in secondary metabolism, in order to apply them in the production of valuable metabolites in medicinal plants via metabolic engineering. CYP76AH1 has been suggested to catalyze the conversion of the carbon skeleton miltiradiene into the intermediate ferruginol, which is involved in the biosynthesis of tanshinones, the chief bioactive ingredients of *Salvia miltiorrhiza*. However, its role *in planta* remains to be elucidated. In this work, we constructed a *CYP76AH1* RNAi system for hairy roots. Metabolic analysis of RNAi-AH1 hairy root lines showed a significantly increased accumulation of miltiradiene compared to the control lines. At the same time, the concentration of ferruginol decreased revealing the *in vivo* catalytic activity of

CYP76AH1. The content of tanshinones decreased significantly after silencing of *CYP76AH1*, which verified its key role in the biosynthesis of tanshinones, and indicated that it could be used as a target for metabolic engineering.

Keywords: tanshinones; *Salvia miltiorrhiza*; biosynthetic pathway; RNAi; cytochrome P450

Introduction

Plant cytochrome P450s (CYPs) are well known as the largest family of enzymes that contribute to both the primary metabolism and the chemical diversity of plant secondary metabolites, and they fulfill essential functions in the production of various therapeutically valuable compounds [1]. Usually, they are characterized by a complex catalytic activity with high regio- and stereospecificity [2]. However, increasing numbers of studies also report on the promiscuity of CYPs involved in biosynthesis of secondary metabolites [3; 4; 5]. The complex catalytic functions of CYPs are thought to account for a large share of the structural diversity and complex metabolite networks in plant secondary metabolism. Thus, it is important to functionally characterize the many different CYPs both *in vitro* and *in vivo*, in order to make them accessible as targets for further genetic improvement of organisms by metabolic engineering.

The dry root and rhizome of *Salvia miltiorrhiza* Bunge, also known as Danshen, , has been considered to have a wealth of medicinal properties throughout Chinese history, and it is commonly used for the treatment of cardiovascular diseases. More than 40 tanshinones and their analogues have been isolated from Danshen, and these are considered to be the most bioactive constituents. They possess significant antioxidant, anti-inflammatory, antineoplastic and anticancer activities [6; 7]. Considering the remarkable medicinal value of tanshinones, characterization of the biosynthetic pathway of tanshinones and using metabolic engineering and/or synthetic biology methods to produce tanshinones seems to be a reasonable strategy for sustainable utilization of this traditional Chinese medicinal resource. Tanshinones are produced starting from a terpene skeleton by the action of terpene synthases SmCPSs and SmKSL [8], and further modified by CYPs, dehydrogenases, demethylases, and other enzymes to produce structurally varied compounds (fig.1)[9]. Four SmCPSs and two SmKSLs were identified and functionally characterized via expression of the

proteins in *Escherichia coli*, which corroborated the roles of SmCPS1, SmCPS2, and SmKSL1 in tanshinone biosynthesis leading to the miltiradiene skeleton [8; 10]. RNAi analysis revealed that SmCPS1 was the class II terpene synthase responsible for tanshinone biosynthesis in the root and rhizome of Danshen [11]. Comparative transcriptome analysis of induced hairy roots was used to identify candidate CYP genes co-regulated with the diterpenoid synthase genes, and *CYP76AH1* was identified as the enzyme that catalyzes the conversion of miltiradiene into ferruginol [9]. Based on co-expression analysis, two new CYPs, *CYP76AH3* and *CYP76AK1*, were found and functionally characterized. They might work sequentially to catalyze the conversion of ferruginol into the metabolic intermediate of tanshinones[12]. RNA-Seq data of YE & Ag^{+}-induced hairy roots of Danshen led to the detection of a total of 125 expressed CYPs belonging to 8 clans and 31 families, of which 40 were up-regulated during the accumulation of tanshinones [13]. This offers a strong indication that downstream pathways of tanshinone biosynthesis may be closely associated with CYPs. Therefore, the characterization of both *in vivo* and *in vitro* functions of CYPs could clarify the secondary metabolite network, and this knowledge is essential for any future genetic improvement of Danshen using metabolic engineering.

RNA interference (RNAi) was first discovered as a natural response to double-stranded RNA, which results in gene-specific silencing [14; 15]. It was subsequently developed into a commonly used genetic expression and regulation technology for the functional identification of candidate genes [16]. RNAi is considered to be a potent reverse genetic tool for the identification of gene functions in plants. It was successfully implemented in the opium poppy [17], maize [18], and cotton [19], and was used to regulate the biosynthesis of target compounds and exert signal control in different plant tissues including leaves [20], seeds [21], fruits [22] and hairy roots [23]. Silencing of SmCPS1 with RNAi resulted in decreased accumulation of tanshinones in Danshen hairy roots, which verified the key role of SmCPS1 in tanshinone biosynthesis [11]. Down-regulation of SmCPS1 by RNAi resulted in substantial reduction of tanshinones, and metabolomics analysis revealed 21 potentially affected intermediates, indicating a complex network for tanshinone metabolism defined by certain key biosynthetic steps [10] (Fig.1). RNAi results indicated that *CYP76AK1* plays a key role in the production of tanshinones[12]. On the other hand, the reduction of the contents of tanshinones was not statistically

significant in *CYP76AH3* RNAi lines, implying that the reactions catalyzed by *CYP76AH3* most likely is not rate limiting for tanshinone biosynthesis. This indicates that the downstream pathway of tanshinone biosynthesis might contain multifunctional enzymes and substrates that interact with multiple binding sites involving different enzymes. These complex factors form the biosynthesis pathway of tanshinones into a network, and due to the high level of sequence similarity between *CYP76AH1* and *CYP76AH3*, CYP76AH1 may also constitute a node in the complex catalytic network of tanshinone biosynthesis.

The CYP76AH1-catalyzed turnover of miltiradiene into ferruginol was presumed to be the key step involved in tanshinone biosynthesis. However, there are more than 40 tanshinone analogs in Danshen, and the role of CYP76AH1 in tanshinone biosynthesis remains to be characterized in detail. In this study, in order to investigate the contributing role of CYP76AH1 in tanshinone biosynthesis *in vivo*, we employed RNAi to validate the effect of expression of CYP76AH1 on the accumulation of tanshinones and its analogues in the hairy roots of Danshen.

Materials and methods

Plant materials and culture conditions

Plants were grown in MS solid medium in a growth chamber maintained at 25℃ with a 16 h light/8 h dark cycle and transformed hairy roots were collected after culture in 1/2 MS solid medium at 25 °C in the dark for 40 days.

Construction of RNAi vector and hairy root transformation

The cDNA segment derived from *CYP76AH1* (Table. 1) was cloned into the entry vector of the pENTR Directional TOPO Cloning Kit (Invitrogen). The entry vector fragment was then subcloned into the pK7GWIWG2D(II) binary transformation vector using Gateway LR Clonase Enzyme Mix (Invitrogen) to generate the RNAi expression clones. The resulting recombinant constructs were introduced into *Agrobacterium rhizogenes* C58C1 using the freeze-thaw method. The strain C58C1 harboring the pK7GWIWG2D(II) vector was used as a negative control [24]. The constructs were subsequently used to transform injured leaves of *Salvia miltiorrhiza* using strain C58C1 as described previously [11; 25].

Sequence	Forward primer (5'-3')	Reverse primer (5'-3')	Insert size (bp)
(a)AH1	CACCGCCCAACTTCGCCGACTACTTC	GCATTGCCGACTCATCCACGAT	395
(b)q-AH1	TCGTGGATGAGTCGGCAAT	TGAGTATCTGAGTTCCCT	78

Table. 1 Primers used for (a) the assembly of RNAi fragment and (b) qRT-PCR analysis

Quantitative real-time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, USA) and first-strand cDNA was synthesized using the PrimeScript® RT reagent Kit with gDNA Eraser (Takara, Tokyo, Japan). Relative transcript abundance was determined by qRT-PCR using SYBR Premix Ex Taq II (Takara, Tokyo, Japan) on an ABI 7500 apparatus (Applied Biosystems, Foster City, CA, USA). The primers used for qRT-PCR analysis are listed in Table 1. All experiments were done in triplicate.

GC-MS and UPLC-QTOF/MS analysis

Authentic ferruginol and sugiol samples were purchased from BioBioPha (Yunnan, China). Tanshinone I, cryptotanshinone, tanshinone IIA and 11-hydroxy-sugiol were purchased from Chengdu Must Bio-Technology Co., Ltd (Sichuan, China). Tanshinones were extracted from lyophilized hairy roots using acetonitrile in an ultrasonic water bath. The content of tanshinones and relative transcript abundance data were generated from 5 individually cultured hairy root lines. UPLC-QTOF/MS was carried out using an Acquity UPLCTM system (Waters Corp., Milford, MA, USA) with an Acquity UPLC BEH C18 column (50×2.1mm,1.7μm). GC-MS was carried out using a Trace 1310 series GC system with detection via a TSQ8000 MS (Thermo Fisher Scientific Co. Ltd.). Chromatographic separation was performed on a TR-5ms capillary column (30m × 0.25mm ID DF = 0.25μm; Thermo Fisher Scientific Co. Ltd.).

Results

RNAi-mediated silencing of *CYP76AH1* in hairy roots

CYPs are a diverse group comprising a large number of members with low sequence similarity. 437 CYPs were identified in the genome sequence of Danshen [26]. Six fragments from four scaffolds were found to be highly similar and homologous to *CYP76AH1* in the Danshen genomic data, with 79% to 99% similarity. These six fragments were then used in a BLAST analysis of the transcriptome of the hairy roots of Danshen. It was revealed that only three of them were transcribed in hairy root cells, namely CYP76AH1, CYP76AH3, and a fragment with the designation isotig22502 with an FPKM value of less than 10. In order to avoid co-suppression, a 395 bp fragment (the region from 635 bp to 1,029 bp of the CDS of *CYP76AH1*), which was less conserved, was introduced into Danshen hairy roots via a Gateway vector. The targeted fragments from *CYP76AH1* were cloned into the plant expression vector pK7GWIWG2D, which expresses a self-complementary 'hairpin' RNA fragments that induces silencing. The constructs pK7GWIWG2D-AH1 (Fig.S1) and pK7GWIWG2D-control were transfected into Danshen leaf explants using *A. rhizogenes* C58C1, in order to produce recombinant hairy root cultures.

The induced hairy roots were screened with kanamycin (50 mg/L), whereby the leaves started to lose their green coloration and callus tissue grew from the wound after prolonged culture in the dark. Hairy roots began to form within 15 days after infection (Fig. S2A) and started to elongate rapidly after 20 days (Fig. S2B). Each single twig of hairy root was cut off and cultured as a separate hairy root line (Fig. S2C). Hairy root lines were harvested after 40 days after being cut off (Fig. S2D). The root and hairy root of Danshen, which accumulates tanshinones is always red, as most tanshinone compounds have distinct red and dark orange colors. In accordance with this here was a readily perceivable color difference between the control (Fig. S2E) and RNAi-AH1 lines (Fig. S2F), which indicated that the accumulation of tanshinones might indeed be suppressed.

Transcript levels in RNAi -AH1 hairy roots

Five independently transformed hairy root lines were selected to assess the effects of silencing CYP76AH1 on the biosynthesis of tanshinones. Normalized real-time quantitative PCR showed significant decreases of *CYP76AH1*, corresponding to a 37.6% expression level relative to control. The effect of cross-reactivity and co-suppression with other CYPs in the biosynthesis of tanshinones was also tested. For *CYP76AH3*, which shows more than 80% sequence similarity to *CYP76AH1*, the transcript level was relatively lower than control, though without significant decrease in the RNAi lines. There was no significant effect on the transcription of *CYP76AK1*, which showed less than 59 % sequence similarity to *CYP76AH1* (Fig.2). This suggested that RNA interference targeting CYP76AH1 did not inhibit the expression of other CYPs by RNAi or co-suppression, at least among the known genes involved in tanshinone biosynthesis.

Tanshinone accumulation in RNAi-AH1 hairy roots

UPLC-QTOF/MS and GC-MS were used to determine the relative contents of tanshinones and their biosynthesis intermediates. The profiles revealed an alteration in the accumulation of tanshinones in hairy root cultures that reflected the modulation in transcript levels. Suppressing the expression of *CYP76AH1* resulted in a significant reduction of the three main active compounds, the content of tanshinone IIA, cryptotanshinone, and tanshinone I in RNAi-AH1 hairy root lines were 56.8%, 61.7%, and 50.0% of the control lines, respectively (Fig. 3A). This confirmed the key role of CYP76AH1 in the biosynthesis of tanshinones.

The normalized results showed that the accumulation of miltiradiene was raised 12.7 times after the silencing of *CYP76AH1*(Fig. 3B), and the content of ferruginol decreased to 37.4 % of that in the control lines (Fig. 3B). Moreover, the concentrations of verified compounds involved in the downstream biosynthetic pathway of tanshinones, namely sugiol, 11-hydroxy sugiol, 11-hydroxy ferruginol, 11,20-dihydroxy ferruginol, 11,20-dihydroxy sugiol, 10-hydroxymethyl tetrahydromiltirone and miltirone, also decreased in the RNAi-AH1 hairy root lines. The contents of these intermediate products decreased to 42.8%, 17.5%, 12.5%, 5.2%, 7.2%, 10.5% and 21.6%, respectively (Fig.3C). This illustrated that interference targeting the expression of *CYP76AH1* impeded the biosynthesis of ferruginol, which proved the *in vivo* catalytic function of CYP76AH1.

Candidate intermediates in the downstream pathway of tanshinone biosynthesis

OPLS-DA analysis was done with the chromatography results of RNAi-AH1 hairy root lines and control lines (Fig. 4A). The data clearly clustered into two categories which showed significant differences in the metabolic components of the two transcript lines. Further analysis of the different compounds between the two hairy root lines provided more evidence for the derivation of downstream compounds, and some compounds were found to be significantly decreased in abundance after silencing of *CYP76AH1* (Fig.4B).

The ions that showed significant differences in abundance between the RNAi-AH1 lines and control lines were contributed to the observed separation and selected from the respective S-plots and VIP-plots as potential markers in negative modes. We also found peaks of 8 ions that were reduced significantly in the RNAi-AH1 lines compared with control. Their parent ion masses were 299.2034(-), 301.1684(-), 315.1825(-), 317.2058(-), 311.1896(-) and 325.2103(-), respectively (Fig.S3). These compounds seem to be situated downstream of ferruginol in the biosynthesis pathway of tanshinones, and may serve as new targets in screening for functional genes in the future. Finally, no additional peak with related molecular weight was found to accumulate in the RNAi-AH1 line, which indicated that miltiradiene might be the only substrate for CYP76AH1 involved in the biosynthesis of tanshinones in Danshen.

Discussion

CYPs were suggested to play an essential role in the structural diversity and complex metabolic network of secondary metabolites in plants. It is indispensable to verify their functions *in vivo*, in order to provide a basis for the improvement of production strains by metabolic engineering. CYP76AH1, which catalyzes the conversion of the miltiradiene skeleton into ferruginol *in vitro*, was the first CYP characterized in Danshen. RNA interference analysis and metabolite profile analysis further confirmed that CYP76AH1 was responsible for tanshinone production in this study. The inhibition of transcript accumulation of CYP76AH1 effectively suppressed the conversion process, which resulted in the accumulation of its substrate miltiradiene and a decrease of its product ferruginol [9]. OPLS-DA analysis detected

8 related peaks that were reduced significantly in abundance compared with control. However, there were no peaks with related molecular weights detected to accumulate in addition to miltiradiene. This was in accordance with the fact that CYP76AH1 uses miltiradiene as the optimum substrate, with high catalytic efficiency and a low Michaelis-Menten constant [9]. Suppression of the expression of *CYP76AH1* resulted in a significant reduction of active metabolic end-products, such as tanshinone IIA, cryptotanshinone, and tanshinone I, and this confirmed the key role of CYP76AH1 in the biosynthesis of tanshinones. This indicated that CYP76AH1 was a chief CYP in the main route of the tanshinone biosynthetic pathway, and that it could be considered as a target for regulation in metabolic engineering.

Tanshinones are a series of extensively aromatized abietane-type ortho-quinone and furan ring-containing nor-diterpenoids, which are formed from the miltiradiene skeleton via a multistep oxidation pathway. In addition to the functionally characterized CYPs (CYP76AH1, CYP76AH3, and CYP76AK1), other CYPs likely play essential roles in the decoration process in the downstream pathways of tanshinones biosynthesis. Upon BLAST analysis using the KEGG database, 10 CYPs were found to be associated with diterpenoid biosynthesis. Among them, SmCYP76AK2 and SmCYP76AK3, which belong to the CYP76 family, have undergone significant expansion after speciation [27]. Unfortunately, their functions remain to be elucidated.

Long metabolic pathways and low contents of metabolic intermediates in plants make it very challenging to screen the functional genes in downstream biosynthetic pathways of pharmaceutical ingredients, as it is difficult to obtain sufficient quantities of the intermediate products used as substrates for enzymatic reactions or structural identification. *In vivo* experiments demonstrated their advantages in screening for the candidate structure-modifying enzymes. Empowered by a sophisticated genetic transformation system, *in vivo* analysis will be employed as a faster and more efficient way to identify candidate genes in diterpene biosynthetic pathways and become a powerful method for the thorough study of complex functions of CYPs.

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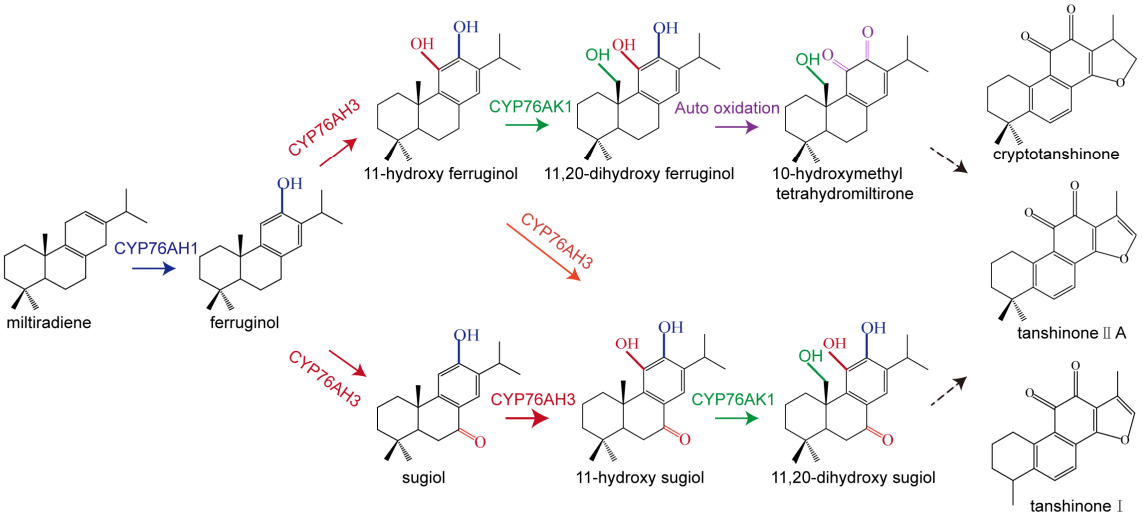
Figure Legends

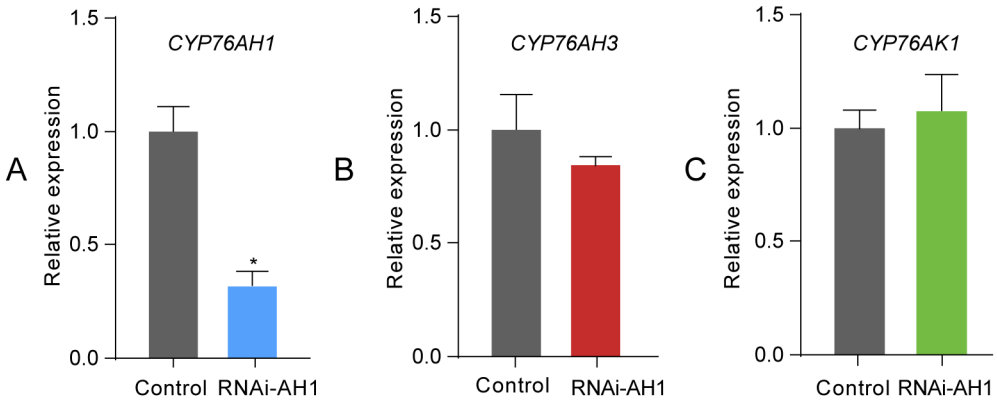
Fig.1 Reactions catalyzed by CYPs in the biosynthesis of tanshinones in Danshen.

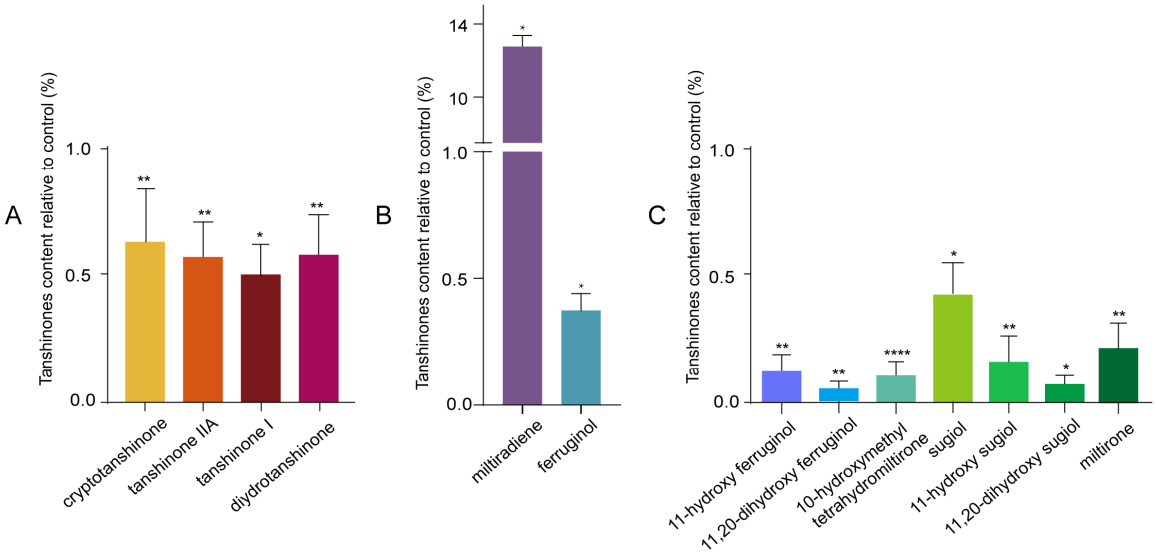
Fig.2 The relative expression levels of *CYP76AH1*、*AH3*、*AK1* in RNAi-AH1 hairy roots

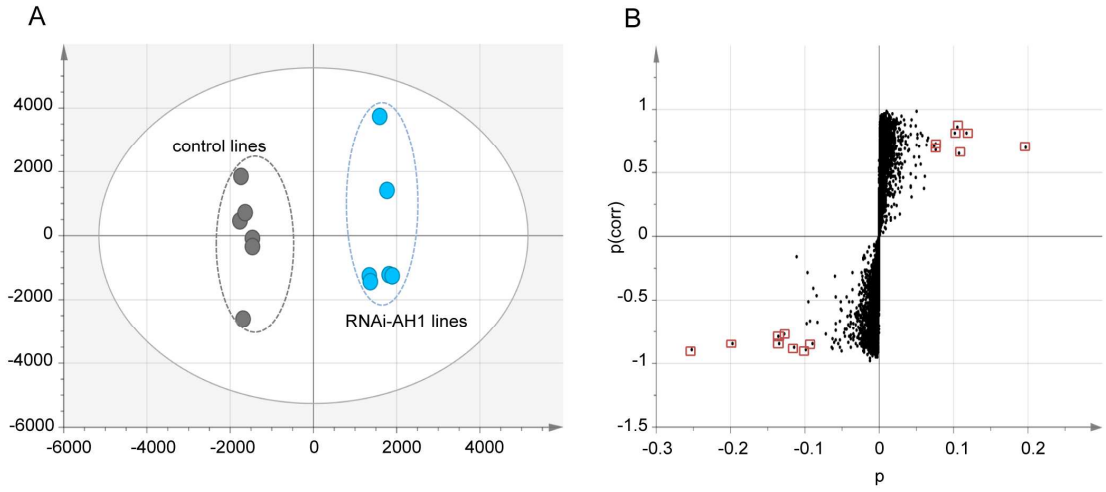
Fig.3 The relative contents of tanshinones in RNAi-AH1 hairy root lines. A: Contents of main tanshinones in RNAi-AH1 lines relative to control; B: Content of substrate and product of CYP76AH1 in RNAi-AH1 lines relative to control C: Content of verified intermediate products involved in the downstream biosynthetic pathway of tanshinones in RNAi-AH1 lines relative to control.

Fig.4 Metabolic component analysis of RNAi-AH1 hairy root lines. A: OPLS-DA analysis score scatter of RNAi-AH1 and control line; B: S Plot analysis of RNAi-AH1









RNA interference targeting *CYP76AH1* in hairy roots of *Salvia miltiorrhiza* reveals its key role in the biosynthetic pathway of tanshinones

Authors: Ying Ma [a,b], Xiao-Hui Ma [b,c], Fan-Yun Meng[a] ,Zhi-Lai Zhan[b], Juan Guo [b,*] and Lu-Qi Huang [b,*]

Highlights：▲A *CYP76AH1* RNAi system for Danshen hairy roots is constructed；▲The *in vivo* catalytic activity of CYP76AH1 is revealed by RNAi system；▲The RNAi system verified the key role of CYP76AH1 in the biosynthesis of tanshinones；▲CYP76AH1 could be used as a target for metabolic engineering.