# SmGRAS5 acts as a positive regulator in GA-induced biosynthesis of tanshinones in Salvia miltiorrhiza hairy roots

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**Abstract**

GA is an important phytohormone that regulates root growth and secondary metabolism. GRAS family transcription factors (TFs) are the key regulators of GA signaling. Here, we found that SmGRAS5 was co-expressed in the root periderm with tanshinones in Salvia miltiorrhiza. Overexpression (OE) of SmGRAS5 increased tanshinones accumulation and upregulated the biosynthetic genes. Antisense expression (AE) of SmGRAS5 reduced tanshinones accumulation and downregulated the biosynthetic genes. Yeast one-hybrid (Y1H), dual-luciferase (Dual-LUC) and electrophoretic mobility shift assays (EMSA) showed that SmGRAS5 promoted tanshinones biosynthesis by directly binding to the GARE motif in the promoter of SmKSL1 to induce its expression. However, overexpressing SmGRAS5 reduced GA content through downregulating biosynthetic genes. GA treatment further increased tanshinones accumulation and restored the accumulation of GA inhibited by overexpressing SmGRAS5. SmGRAS5 could not directly bind to the GA biosynthetic genes. Transcriptome analysis revealed the potential functions of SmGRAS5 in regulating secondary metabolism. Taken together, SmGRAS5 regulated GA-promoted tanshinones biosynthesis by directly activating the expression of SmKSL1, which suggested that SmGRAS5 may be a potential target for further metabolic engineering of tanshinones biosynthesis in S. miltiorrhiza.

## INTRODUCTION

The dried roots of *Salvia miltiorrhiza* Bunge (Danshen) are widely used in treating inflammation and heart diseases (Dong *et al* ., 2011; Liu *et al* ., 2018). *S. miltiorrhiza* has been considered as a model medicinal plant due to its important medicinal value, small genome (˜600 Mb), short life cycle, established transgenic system, and ease of tissue culture (Ma *et al* ., 2012; Xu *et al* ., 2015). The bioactive compounds of*S. miltiorrhiza* form two main groups: hydrophilic components (SAs), such as salvianolic acid B (Sal B) and rosmarinic acid (RA), and lipophilic components (tanshinones, which are a group of diterpenoids), such as dihydrotanshinone I (DT-I), cryptotanshinone (CT), tanshinone I (T-I) and tanshinone IIA (T-IIA) (Wang *et al* ., 2007; Pei *et al* ., 2018). The Chinese Pharmacopoeia stipulates that the main quality markers of Danshen are tanshinones and SAs (The State Pharmacopoeia Commission of China, 2015). The biosynthetic pathways of SAs include phenylpropanoid and tyrosine-derived pathways (Petersen, 2013; Sun *et al.* , 2018). Due to the great medicinal value of tanshinones, the biosynthetic pathways have been well studied (Ma *et al* ., 2015; Song *et al.* , 2015; Zhou*et al.,* 2017). The tanshinones are synthesized from diterpenoids universal precursor GGPP, which is produced by the mevalonic acid (MVA) and 2-C-methyl-D-erythritol-4-phosphate (MEP) pathways (Guo *et al.* , 2016; Pei *et al.* , 2018). The key enzyme genes involved in

tanshinones biosyntheses, such as *AACT* , *DXS* , *CMK* ,*GGPPS* , *CPS* , *KSL1* , and *CYP76AH1* have been characterized in *S. miltiorrhiza* (Ma *et al* ., 2012; Xu*et al.* , 2015). There have been many reports of overexpressing or RNA interference these biosynthetic genes could regulate the accumulation of tanshinones (Kai *et al* ., 2011; Ma *et al* ., 2016; Shi *et al* ., 2016). Overall, the supply of these bioactive compounds is limited because of their low concentrations in the roots of *S. miltiorrhiza* . And the yields and quality of *S. miltiorrhiza* are often affected by unfavorable environmental conditions. Therefore, research on regulating secondary metabolites has become a hot topic. The application of TFs is a promising approach to improve the efficiency of metabolic engineering in the plant (Fu *et al.* , 2018).

TFs play an important role in secondary metabolic engineering. TF can coordinately regulate the expressions of several genes encoding these enzymes, therefore guide the metabolic flux towards certain pathways (Ying *et al* ., 2019). It is an effective methodology using TFs to improve the production of secondary metabolites in plants. Several MYBs have been reported to regulate tanshinones biosynthesis of *S. miltiorrhiza.* Overexpression of *SmMYB36* promoted tanshinones accumulation by binding to the promoters of *DXR* , *MCT* and*GGPPS1* (Ding *et al* ., 2017). Overexpression of*SmMYB9b* also increases tanshinones concentration by stimulating the MEP pathway (Zhang *et al.* , 2017). And overexpression of*SmbHLH10* enhanced the tanshinones biosynthesis by binding with the G-box of *DXS2* and *CPS5* in *S. miltiorrhiza* (Xing*et al* ., 2018). Moreover, SmWRKY1 could positively regulate tanshinones biosynthesis via target gene *DXR* in *S. miltiorrhiza* (Cao *et al.* , 2018). But ethylene response factor (ERF), SmERF115 decreased tanshinones content in *S. miltiorrhiza*(Sun *et al.* , 2018). However, the regulatory function of GA signaling key regulator SmGRASs in tanshinones biosynthesis of *S. miltiorrhiza* remains unknown.

GRAS TF family has been reported playing diverse roles in GA signaling, root development, light signaling and stress responses (Livne *et al.* , 2015; Xu *et al.* , 2015; Heck *et al.* , 2016). GRAS has been found in many plants, such as *Arabidopsis* , tomato, rice, grapevine, cotton and Danshen (Tian *et al.* , 2004; Huang *et al.* , 2015; Grimplet *et al.* , 2016; Bai *et al.* , 2017; Zhang*et al.* , 2018). The GRAS family is divided into 13 distinct subfamilies based on amino acid sequences: DELLA, SCL3, LAS, SCL28, SCL4/7, SCR, SHR, SCL9 (LISCL), HAM, PAT1, OS4, DLT and OS19 (Zhang*et al.* , 2018). Among them, the SCL3 subfamily has been shown to participate in root cell elongation, GA/DELLA signaling and stress responses (Hakoshima, 2018). Furthermore, some evidence has shown that GRAS proteins participated in the GA-dependent regulatory network and root periderm formation. For instance, DELLA protein is the repressor of GA and acted as key regulatory targets in the GA signaling pathway in regulating plant growth (Murase *et al.* , 2008; Yoshida *et al.* , 2014). SCL3 has been reported as a repressor of DELLA. It could positively regulate the GA signaling pathway and control GA homeostasis in*Arabidopsis* root development (Heo *et al.* , 2011; Zhang*et al.* , 2011).

GA is an important phytohormone that plays vital roles in many processes of plant growth and metabolism (Brian, 2010; Du *et al.* , 2015; Davière and Achard, 2016). For instance, GA could regulate the flavonol biosynthesis through DELLA to further promote root growth in *Arabidopsis* (Tan *et al.* , 2019). And the GA-mediated control of growth has an interaction with energy metabolism to coordinates cell wall extension, lipid and secondary metabolism in *Arabidopsis* (Ribeiro *et al.* , 2012). Moreover, the biosynthesis pathways of GA and tanshinones are different branches of the diterpenoid biosynthesis pathway, since they have a universal precursor GGPP (Xu *et al* ., 2015). The biosynthesis of GA from GGPP involves many synthase genes, such as *CPS5* ,*KS* , *KAO* , *GA20ox* , *GA3ox* and *GA2ox* (Ma*et al.* , 2012; Cui *et al.* , 2015; Su *et al.* , 2016). Thus, crosstalk may occur between GA and tanshinones biosynthesis in*S. miltiorrhiza* . In our previous report, GA treatment was able to increase tanshinones accumulation and induce the expressions of*SmGRAS1˜5* genes in wild-type hairy roots of*S. miltiorrhiza* (Bai *et al.* , 2018). Therefore, we speculated that SmGRASs might be involved in the regulation of GA to tanshinones biosynthesis in *S. miltiorrhiza* . Although five GRAS family genes have been cloned in *S. miltiorrhiza* , however, we found the *SmGRAS5* was the most sensitive gene of them responding to GA treatment (Bai *et al.* , 2017). But the roles of SmGRAS5 in regulating tanshinones biosynthesis through GA signaling are still unknown.

In this study, we found that overexpressing *SmGRAS5* significantly increased the tanshinones' content and

decreased GA content in *S. miltiorrhiza* hairy roots. Y1H, Dual-LUC and EMSA assays indicated that SmGRAS5 could directly bind to the promoter of *SmKSL1* to induce its expression. And GA treatment improved the tanshinones and GA accumulation in the *SmGRAS5* OE lines. Subsequently, transcriptome analysis revealed the potential functions of SmGRAS5 in regulating secondary metabolism. Finally, the molecular mechanism of SmGRAS5 regulated the GA-induced tanshinones biosynthesis was analyzed and discussed. Our findings revealed a link between SmGRAS and secondary metabolism and provided important information on GA-mediated secondary metabolite biosynthesis in *S. miltiorrhiza* .

## MATERIALS AND METHODS

### Plant materials and GA treatment

*Agrobacterium rhizogenes* (*ATCC15834* ) was used to infect the terile plantlets to obtain the *S. miltiorrhiza* hairy roots, as previously reported (Ru *et al.* , 2016). (0.3 g fresh weight hairy roots were cultured in the liquid 6,7-V medium and sub-cultured every 30 days. The 21-day-old hairy roots were treated with $GA_3$ (100 μM final concentration) for 2 h, 24 h or 6 days. Control hairy roots were not treated without $GA_3$. Three independent biological replicates were performed in all treatments.

The phloem tissue, xylem tissue and periderm tissue of the roots were collected from the 2-year-old *S. miltiorrhiza* to analyze the tissue-specific expressions of *SmGRAS5* and the tanshinones accumulation.

### Bioinformatics analysis of SmGRAS5

ClustalX program was used to perform multiple sequence alignments of SmGRAS5 (GenBank accession number KY435890) protein sequences and AtGRAS protein sequences (http://www.arabidopsis.org). MEGA6 software was used to construct a phylogenetic tree by the maximum-likelihood method with the bootstrap test (n = 500 replications) (Tamura *et al.* , 2013).

### RNA extraction and qRT-PCR assays

RNAprep pure plant kit (TIANGEN, China) was used to extracted total RNA and then using the PrimeScript RT reagent kit (TaKaRa, China) to reverse-transcribed RNA to cDNA. SYBR Premix Ex Taq II Kit (Takara, China) was used to performed a real-time PCR system (Bio-Rad CFX96, USA). The *SmActin* gene was selected as endogenous control (Yang*et al.* , 2010). $2^{-t}$ method was used to calculate the relative expression levels. All the qRT-PCR analysis primers are listed in Supplemental Table S1. Three independent biological replicates and three technical replicates were used to obtain the data.

### High-performance liquid chromatography (HPLC) analysis of tanshinones and $GA_3$ contents

The contents of tanshinones and $GA_3$ were determined by HPLC, according to the general method in our laboratory that was described previously (Wang *et al.* , 2008; Liu *et al.* , 2016). 0.04 g dried hairy roots power were extracted by 8 mL of 70% methanol overnight. After centrifugation, the supernatant was filtered through a 0.2-μm filter for HPLC analysis.

### Subcellular localization

The full-length open reading frame (ORF) of *SmGRAS5* was fused with GFP in the *pA7-GFP* vector. Gene gun (Bio-Rad, USA) was used to transform the *pA7 -SmGRAS5-GFP* and control plasmids transiently into onion epidermis. The onion epidermis was stained with 4,6-diamidino-2-phenylindole dihydrochloride (Solarbio, China) for 20 min after 1 day of incubation. And then washed the epidermis twice with PBS buffer (pH 7.2) and observed under a confocal laser scanning microscope (Nikon A1R, Japan).

The *pA7* 0390-*SmGRAS5-GFP* and control plasmids were transformed into *Agrobacterium GV3101* . 4-week-old *N. benthamiana* leaves were infiltrated with the *GV3101* suspension cultures, following the previously described method (Bai *et al.* , 2018). The protoplasts were prepared after 2 days of co-culture, as previously described (Li, 2011). The subcellular localization analysis primers are listed in Supplemental Table S2.

### Analysis of transcriptional activity

The *pDEST-GBKT7-SmGRAS5* and negative control plasmids were transformed into the yeast AH109. The positive control plasmid was*pGBKT7-53+pGADT7-T* . First, screened the transformed AH109 on synthetic dropout (SD) medium lacking tryptophan (SD/-Trp) and then on SD medium without tryptophan, histidine and adenine (SD/-Trp/-His/-Ade).

### Plasmid construction and genetic transformation

The ORF of *SmGRAS5* was amplified and cloned into the *Noc* I and *Spe* I restriction sites of the *pCAMBIA1304* in sense and antisense orientations with the CaMV35S promoter. PCR and restriction enzyme digestion were used to confirm the positive clones. And then the plasmids were transformed into *ATCC15834* . The combination of cefotaxime (Sigma, USA) and hygromycin B (MP Bio, USA) was used to screen the transformants. PCR identification and the positive transgenic lines screening use four primer pairs, *rolB* , *rolC* , *hptII* , 35S forward primer (35S-F) and *GRAS5* reverse primer (GRAS5-R). All the expression vector construction and the PCR identification primers are listed in Supplementary Table S2.

### Y1H verification

The ORF of *SmGRAS5* was inserted into the *pGADT7* vector. The 649-bp *SmKSL1* and 826-bp *SmCPS1* promoter sequences were cloned into the *pBait-AbAi* vector. The primers for vectors (*pBait-AbAi-SmKSL1-649* and *pBait-AbAi-SmCPS1-826* ) are listed in Table S2. Aureobasidin A suppressed the basal expression of the *Y1H-pAbAi-SmKSL1-649* (*PYK649* ) and*Y1H-pAbAi-SmCPS1-649* (*PYC826)* yeast strain (Bai *et al.* , 2018). *pGADT7-SmGRAS5* was verified by interactions with*PYK649* and *PYC826* yeast strains, which recombined the*SmKSL1* and *SmCPS1* promoter in SD/-Leu/AbA.

### Protein extraction and western blot

The ORF of *SmGRAS5* was cloned into the *pMAL-c2X* vector. The primers for vectors are listed in Table S2. The plasmids were transformed and proteins were expressed in*Escherichia coli* (*Rosetta* strain). Protein induction and purification were performed as previously described (Bai *et al.* , 2018). The MBP (malE) and SmGRAS5-MBP purified proteins were conducted and SDS-PAGE analyses showed major bands with approximate 80.7 kDa molecular mass (Fig. S7). The western blot was performed as previously described (Ru*et al.* , 2017).

### Dual-luciferase assay

The 649-bp promoter of *SmKSL1* was cloned and inserted into*pGREEN* . The vector *pCAMBIA1304-SmGRAS5* was transferred into *Agrobacterium* strain GV3101. *pCAMBIA1304* empty vector was used as a negative control and The 35S promoter-driven*Renilla* luciferase as an internal control. The two GV3101 strains were co-infiltrated into tobacco leaves. Infiltrated leaves were incubated in darkness for 8h and then in light for 40h. Three biological replicates of each sample were assayed using the Dual-Luciferase Reporter Assay System (Promega, USA).

### EMSA analysis

EMSA kit (Invitrogen, E33075) was used to perform EMSA analysis. The oligonucleotide probes were synthesized (listed in Table S2) and annealed. The protein-free sample was used as blank control. The mass ratios of the probe and protein were 1:5/1:15/1:50 in each reaction mixture (10 μL). ChemiDoc XRS+ system (Bio-Rad, USA) was used to observe the gels on a 490 nm SYBR photographic filter.

### Construction of RNA-seq libraries and transcriptome data analysis

21-day-old transgenic hairy roots (G5O14) and control roots (ATCC) were collected from three biological replicates and used for total RNA extraction. 3 μg RNA of each sample was used to construct Illumina sequencing libraries using the Illumina PE150 platform (Novogene, China.). Three biological replicates were used for each sample.

The reference genome and gene model annotation files were downloaded from genome websites (Xu *et al.* , 2016). Genes with an adjusted$P$ -value<0.05 and |fold-change|[?]2 were considered significantly differentially expressed. GO Slim enrichment was evaluated using the GOseq R package (Young *et al.* , 2010). Significantly enriched KEGG pathways were identified with KOBAS 2.0 (Mao*et al.* , 2005).

## RESULTS

### SmGRAS5 and tanshinones are co-located in the periderm of*S. miltiorrhiza* roots

To study the functions of *SmGRAS5* gene, the coding regions were amplified by using root-derived cDNAs from *S. miltiorrhiza* .*SmGRAS5* contains an open reading frame (ORF) encoding 335 amino acids. The amino acid sequences of *SmGRAS5* contained the conserved GRAS domain. The phylogenetic analysis indicated that SmGRAS5 belongs to SCL3 subfamily (Fig. S1).

Since the medicinal part of *S. miltiorrhiza* is the roots and the tanshinones were mainly accumulated in the roots, the potential functions of *SmGRAS5* in tanshinones biosynthesis were detected by analyzing its expression patterns in the periderm, phloem and xylem tissues of*S. miltiorrhiza* roots. The expression levels of *SmGRAS5*were the highest in the periderm, suggesting the important roles of*SmGRAS5* in the periderm (Fig. 1A). In addition, the four contents of tanshinones (DT-I, CT, T-I and T-IIA) were also detected in the three tissues of the roots. Tanshinones were mainly concentrated in the periderm (Fig. 1B). The results showed *SmGRAS5* to be co-located with tanshinones in the periderm (Fig. 1C). Therefore, we speculated that SmGRAS5 might be involved in the regulation of tanshinones biosynthesis.

### Subcellular localization and transcriptional activity analysis of SmGRAS5

To determine the subcellular localization of SmGRAS5, SmGRAS5 protein was fused with a green fluorescent protein (GFP) label. The protoplasts of tobacco leaves and onion epidermal cells were used to analyze the SmGRA5-GFP signal (Fig. 2). The results showed that the GFP fluorescence of SmGRAS5 was enriched in the nucleus, indicating that SmGRAS5 might function as a TF.

To further identify the characteristics of SmGRAS5, the transcriptional activity of SmGRAS5 was analyzed. The results showed that the yeast with*SmGRAS5-pGBKT7* and control constructs were able to grow on the SD/-Trp medium. However, the yeast with *SmGRAS5-pGBKT7* and negative control constructs could not survive on the SD/-Trp-His-Ade medium (Fig. S2). The results demonstrated that SmGRAS5 had no transcriptional activity.

### SmGRAS5 promotes tanshinones biosynthesis in *S. miltiorrhiza*

To further explore the roles of SmGRAS5 in the biosynthesis of tanshinones, the transgenic hairy roots lines were generated through overexpression and antisense expression approaches. The positive transgenic hairy roots were identified by PCR (Fig. S3). Control hairy roots were developed using *ATCC15834* without plasmids. Three independent OE and three AE lines were randomly selected for functional analysis (G5O14, G5O17, G5O20 and G5A11, G5A16, G5A21). Collectively, the gene expression levels of *SmGRAS5* were 18- to 50-fold higher in the OE lines than in the control but decreased by 70%–85% in the AE lines (Fig. 3A).

The phenotypes of control and *SmGRAS5* transgenic hairy roots are shown in Figure S4. The *SmGRAS5* OE lines showed redder and smaller compared with the control. The HPLC analysis showed that the contents of four tanshinones (DT-I, CT, T-I, T-IIA) were all significantly increased in the *SmGRAS5* OE lines (Fig. 3B). Tanshinones' contents were the highest in the G5O14 lines, reaching 5-fold (DT-I), 10.85-fold (CT), 2.94-fold (T-I), and 3.77-fold (T-IIA) of the control lines, respectively. In contrast, the contents of four tanshinones were reduced in the *SmGRAS5* AE lines, especially T-I (Fig. 3C). The results showed that SmGRAS5 promoted the accumulation of tanshinones.

To further confirm these results, we then analyzed the expression levels of key genes in the biosynthetic pathways of tanshinones (Fig. 3D). As expected, the expressions of the genes whose promoters contained the GA response motif were consistent with HPLC results. Expressions of most of the key genes in the tanshinones biosynthetic pathway were upregulated in the *SmGRAS* 5 OE lines. The expressions of downstream genes

of the tanshinones biosynthetic pathway, such as *GGPPS1* ,*CPS1* and *KSL1* , were indeed significantly upregulated in the *SmGRAS* 5 OE lines. In contrast, expressions of most of the tanshinones biosynthetic pathway genes were decreased in the AE lines, especially *AACT2* , *HDS* , *CMK* and *KSL1* . These data showed that SmGRAS5 could regulate the biosynthesis of tanshinones through regulating the expressions of key biosynthesis genes. Collectively, our findings indicated that SmGRAS5 acted as a positive regulator of tanshinones biosynthesis in *S. miltiorrhiza* hairy roots.

**SmGRAS5 regulates GA-promoted biosynthesis of tanshinones**

Since *SmGRAS5* OE lines grow slower than the control, which was similar to the GA-deficient phenotypes (Fig. S4). Consistent with the phenotypes, overexpression of *SmGRAS5* indeed decreased the GA content and downregulated the expressions of most GA biosynthetic genes (Fig. 4A,C). To further investigate whether the regulatory function of SmGRAS5 on tanshinones biosynthesis is mediated by GA, we then used exogenous GA to treat the *SmGRAS5* OE and control lines. We found that there are some GA-responded motifs (GARE-motif and P-box) in the promoter of SmGRAS5. And we also found GA treatment could induce the expression of *SmGRAS5* (Fig. S5). The results showed that the GA content of control and *SmGRAS5* OE lines were all significantly increased after GA treated. However, the expressions of the downstream genes of GA biosynthesis were different in the *SmGRAS5* OE lines. GA biosynthetic enzymes *SmGA20ox2/6* were upregulated, while*SmGA3ox1* was downregulated in the *SmGRAS5* OE lines. And the expressions of the GA deactivating enzymes *SmGA2ox8/9* were different in the *SmGRAS5* OE lines. The results after GA treatment indicated that the increased GA content in the *SmGRAS5* OE lines maybe not have occurred by promoting GA biosynthesis but could have also been caused by exogenous GA entering the cell. Intriguingly, the four tanshinones total contents of *SmGRAS5* OE and control lines were all significantly increased under GA treatments (Fig. 4B). As expected, the expression of most tanshinones biosynthesis genes was quickly induced by GA application in all the lines (Fig. 4C). Among the tanshinones biosynthesis genes, the downstream genes *GGPPS1* and *KSL1* had the most significantly increased in the *SmGRAS5* OE and control lines. Collectively, these results indicated that GA could induce*SmGRAS5* response to further promote tanshinone biosynthesis.

**Interaction of SmGRAS5 with tanshinones and GA biosynthetic gene**

To further investigate how SmGRAS5 regulates tanshinones biosynthesis, we performed Y1H, Dual-LUC and EMS assays. According to our qRT-PCR results, the expressions of most genes in the tanshinones biosynthetic pathway were upregulated in the *SmGRAS5* OE lines. Among them,*SmCPS1* and *SmKSL1* , which are the key downstream genes in tanshinones biosynthesis, their expressions were remarkably upregulated. The Y1H Gold reporter strains that have the *SmKSL1* promoter transformed with SmGRAS5 prey plasmid and positive control could grow normally on SD/-Leu (700 ng/ml AbA) (Fig. 5A). However, the Y1H Gold reporter strains that have the *SmCPS1* promoter transformed with SmGRAS5 prey plasmid and negative control could not grow on SD/-Leu (700 ng/ml AbA) (Fig. S6). These results provided in vivo evidence that SmGRAS5 participates in tanshinones biosynthesis by directly binding to the *SmKSL1* promoter. We further confirmed this by dual-luciferase report system. The Dual-LUC assay showed that SmGRAS5 could directly activate the *SmKSL1* promoter (Fig. 5B).

Our promoter analysis indicated that the promoter sequence of*SmKSL1* contains a GARE motif at 774 bp upstream of the ATG codon (Fig. 5C). To further confirm binding between SmGRAS5 and the fragment containing the SmGRAS5 recognition element in vitro, purified SmGRAS5-MBP fusion proteins were combined with the fragment containing the GARE motif, and they were analyzed by EMSA. Subsequently, specific DNA–SmGRAS5 protein complexes were strongly detected (Fig. 5C). These results confirmed that SmGRAS5 regulated tanshinones biosynthesis by directly binding to the GARE motif of the *SmKSL1* promoter. All these data together indicated that SmGRAS5 could directly bind to the GRAE in the promoter of *SmKSL1* to induce its expression.

Furthermore, in order to further study whether SmGRAS5 directly regulates the expressions of GA biosynthesis genes. We analyzed some promoters of GA biosynthesis genes that were significantly downregulated

by SmGRAS5. We designed GA response element probes for some GA biosynthesis genes and conducted EMSA experiments (Fig. S8). Unfortunately, none of the promoters of these GA biosynthesis genes interacted with SmGRAS5. We speculated that SmGRAS5 might inhibit the expressions of GA biosynthesis genes by interacting with other negative regulatory factors or inhibit GA biosynthesis by other pathways.

### Transcriptome analysis of SmGRAS5-regulated genes

To gain a comprehensive overview of the changes regulated by SmGRAS5 at the transcript level, we performed a transcriptome analysis of G5O14 and ATCC hairy roots. A total of 3910 differentially expressed genes (DEGs) were designated as SmGRAS5-regulated genes (Fig. 6A). A functional gene ontology (GO) analysis indicated that the DEGs regulated by SmGRAS5 were mostly enriched in biological process, metabolic process and catalytic activity terms (Fig. 6B). The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that hundreds of genes for metabolic pathways and the biosynthesis of secondary metabolites were enriched. Moreover, there were many DEGs enriched in stilbenoid, diarylheptanoid and gingerol biosynthesis, phenylpropanoid biosynthesis, limonene and pinene degradation, flavonoid biosynthesis, diterpenoid biosynthesis and plant hormone signal transduction (Fig. 6C). Since many genes involved in secondary metabolites biosynthesis were identified as SmGRAS5 regulated genes in our transcriptomic results. We further analyzed the secondary metabolism pathway using Mapman software (Fig. 6D). We could see that most of the DEGs in MVA and non MVA pathways were upregulated. And there were more upregulated DEGs in the terpenoids, alkaloids-like, carotenoids, flavonoids, anthocyanins and glucosinolates pathways. However, most DEGs in wax and chalcones pathways were downregulated.

Furthermore, we further analyzed transcriptome data to determine the regulatory roles of SmGRAS5 in tanshinones, GA biosynthetic and GA signaling pathways. As shown in Figure 7, the expressions of most tanshinones biosynthetic pathway genes were upregulated while the expressions of most GA biosynthetic and signaling pathway genes were downregulated in the *SmGRAS5* OE lines. The qRT-PCR analysis verified the transcriptomic data (Fig. S9). These results were consistent with our above data and supported the function of SmGRAS5 in promoting tanshinones biosynthesis but inhibiting the GA biosynthesis and GA signaling pathway. Taken together, our results suggested that SmGRAS5 might reduce GA biosynthesis by promoting the universal precursor GGPP to synthesize more tanshinones and further to inhibit downstream GA signaling pathway.

## DISCUSSION

### GA signaling involved in the biosynthesis of tanshinones and SAs

GA is a diterpene phytohormone that modulates growth and development throughout the whole life cycle of the plant (Sun, 2011). A study has shown that GA could promote the accumulation of tanshinones in the wild-type hairy roots of *S. miltiorrhiza* (Bai *et al.* , 2017), but its regulatory mechanisms are poorly understood. We found the SmGRAS5 was the most sensitive genes to GA response of the five SmGRASs genes in our previous study (Bai *et al.* , 2017). We speculated that it might play a major role in SmGRASs in the GA signaling. As a key regulator of GA signaling, GRAS has been classified into 13 different subgroups in *Arabidopsis* (Zhang *et al* ., 2018). Our phylogenetic analysis showed SmGRAS5 belonged to SCL3 subfamily, which is involved in the regulation of *Arabidopsis* root cell elongation, GA homeostasis and signaling (Heo *et al.* , 2011). AtSCL3 inhibited GA biosynthesis and antagonized with DELLA in maintaining GA homeostasis by feedback regulating upstream GA biosynthesis genes, as well as in modulating downstream GA responses by direct protein-protein interaction (Zhang *et al.* , 2011). DELLA protein interacted with AtMYB12 activating expression of flavonol biosynthetic genes (Tan *et al.* , 2019). The tissue-specific expression of *GRAS* genes often indicates their functional roles in development. It has been reported that diterpenoid tanshinones not only accumulated but also biosynthesized in the roots periderm of*S. miltiorrhiza* (Xu *et al.* , 2015). We found that*SmGRAS5* highly expressed in the periderm and co-localized with tanshinones, which indicated that SmGRAS5 might be involved in the regulation of tanshinones biosynthesis. Overexpression of *SmGRAS5*significantly improved tanshinones accumulation through upregulating the expressions of biosynthetic genes, while in the *SmGRAS5* AE lines the tanshinones accumulation was decreased. Notably,

the promotion of SmGRAS5 to the tanshinones biosynthesis is the most significant in all of the SmGRASs, which we are currently studying. So we speculated that it might play a vital role in the regulation of tanshinones biosynthesis. Considering the correlation between tanshinones and GA biosynthetic pathways, we found that the expressions of the most of GA biosynthetic pathway genes were downregulated, which resulted in the decrease of GA content in the *SmGRAS5* OE lines. Similarly, overexpressing*HaGRASL* also reduced the metabolic flow of GAs in*Arabidopsis* (Fambrini *et al.* , 2015). Silencing*SlGRAS26* inhibited the GA biosynthetic pathway, promoted the GA inactivation pathway, and resulted in GA deficiency in tomato (Zhou*et al.* , 2018). *SlGRAS24* could downregulate GA biosynthetic genes, disrupt GA homeostasis and participate in a series of developmental processes through modulating GA and auxin signaling (Huang*et al.* , 2017). We speculated that SmGRAS5 might regulate the metabolic flow of diterpenoid GA and tanshinones biosynthesis. In addition, the GA application could promote the tanshinones biosynthesis in *SmGRAS5* OE lines as well as control lines. Therefore, these results implied that SmGRAS5 could be induced by GA and might catalyze the precursor GGPP to synthesize more tanshinones. Moreover, transcriptome data also revealed that SmGRAS5 could regulate many secondary metabolites biosynthesis. It could inhibit the GA biosynthetic and signaling pathway at the same time, and induce tanshinones biosynthetic pathway through regulating biosynthetic genes.

**SmGRAS5 directly regulated tanshinones biosynthetic gene but not GA**

TFs usually regulate the transcription of target genes by directly binding to the elements in the promoter. Studies have shown that GRAS could interact with the promoter of downstream genes and regulate their expression (Smit *et al.* , 2005; Hirsch *et al.* , 2009). For instance, OsGRAS23 could bind to the potential target genes' promoters to further positively modulating rice drought tolerance (Xu *et al.* , 2015). AtSCL14 was shown to be essential for activating the stress-inducible promoters (Fode *et al.* , 2008). Moreover, SlGRAS2 also regulated the fruit development downstream genes (Li *et al.* , 2018). Therefore, we speculated that SmGRAS5 might directly regulate the expressions of the tanshinones and GA biosynthetic pathway genes. Previous work has indicated that tanshinones biosynthesis is initiated by cyclization of general diterpenoid precursor GGPP to copalyl diphosphate (CPP) by *SmCPS1* and subsequent further cyclization to the abietane miltiradiene by *SmKSL1* (Cui *et al.* , 2015).*SmCPS1 and KSL1* , as the downstream key enzyme genes in tanshinones biosynthetic pathway, were the most highly expressed in the periderm and played a vital role in tanshinones biosynthesis (Xu*et al.* , 2015). Considering the significant response of*SmCPS1 and SmKSL1* to SmGRAS5 and GA, Y1H, Dual-LUC and EMSA assays were performed and demonstrated that SmGRAS5 recognized the GARE motif of the *SmKSL1* promoter, leading to the activation of expression. However, we verified several GA biosynthetic genes and found that none of their promoters interacted with SmGRAS5. These results showed that SmGRAS5 could only directly regulate tanshinones biosynthesis by interacting with *SmKSL1* . However, *SmKSL1*was likely not the only target gene for SmGRAS5 regulation. GRAS could also interact with other TFs to mediate the regulation of the transcription activity of other target genes. For instance, GA inhibited flavonol biosynthesis via DELLA protein, which could interact with SG7 MYBs, to regulate the transcriptional levels of the biosynthesis pathway key genes (Tan *et al.* , 2019). In addition, the specific regulatory mechanism of SmGRAS5 on GA biosynthesis genes is also unclear. SmGRAS was likely to co-regulate GA biosynthesis by interacting with other negative regulatory factors and forming complexes. Therefore, identifying new interactive TFs or downstream targets of SmGRAS5 may provide further insight into the molecular mechanism of SmGRAS5-mediated regulation of secondary metabolite biosynthesis.

Finally, a potential mechanism for the SmGRAS5-mediated GA-promoted tanshinones biosynthesis was proposed (Fig. 8). GA could induce*SmGRAS5* response and promote the biosynthesis of tanshinones through directly activating the expression of *SmKSL1* . SmGRAS5 could inhibit the GA biosynthetic and signaling pathway at the same time and might promote metabolic flow to catalyze the precursor GGPP to synthesize more tanshinones.

**The importance of SmGRAS5 for metabolic engineering**

The biosynthesis of secondary metabolites usually involves multiple enzymes. We can promote the accumula-

tion of secondary metabolites by regulating the expressions of these enzymes. Overexpression of artemisinin biosynthesis gene *FPS* , *CYP71AV1* , *DXR* significantly increased the artemisinin content (Chen *et al.* , 2000; Shen*et al.* , 2012; Xiang *et al.* , 2012). Overexpressing the key enzyme gene *HMGR* in the MVA pathway increased the content of ajmalicine and catharanthine in *Catharanthus roseus* hairy roots (Ayora-Talavera *et al.* , 2002). However, in most biosynthetic pathways, the rate-limiting step is not limited to one or two enzymes. TFs often target the key steps in biosynthetic pathways. Many TFs, such as MYB, AP2/ERF, bHLH, and WRKY, have been reported to have global regulatory functions in pharmaceutical terpenoid biosynthesis (Xie*et al.* , 2012; Liu *et al.* , 2015; Lu *et al.* , 2016). CrWRKY1, TcWRKY1 and AaWRKY1 can significantly improve the content of serpentine, taxol and artemisinin biosynthesis in *Catharanthus roseus* , *Taxus chinensis* and *Artemisia annua* , respectively (Suttipanta *et al* ., 2011; Li *et al* ., 2012; Ma *et al.* , 2009). Overexpression of *AaORA* in *Artemisia annua*significantly increased the expressions of several biosynthetic genes including *ADS* , *CYP71AV1* , and *DBR2* , and led to a significant increase in artemisinin production (Lu *et al.* , 2013). SmGRAS5 as a positive regulator mediated GA-promoted diterpenoid compound tanshinines biosynthesis by regulating the expression of*SmKSL1* and induce the anthocyanin, diterpenoid compound, artemisinin, and triterpene saponins, cannabidiolic acid biosynthetic genes in transcriptome data (Solfanelli *et al.* , 2006; Han *et al.* , 2013; Weiblen *et al.* , 2015; Guo*et al.* , 2016; Xiang *et al.* , 2012). Therefore, make full use of TFs as a general tool to control metabolic pathways, is vital for successful metabolic engineering.


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## CONFLICT OF INTEREST

The authors have declared no conflict of interest.

## AUTHOR CONTRIBUTIONS

W.L., Z.B. and Z.L. conceived and designed the experiment; W.L., Z.B., T.P., R.M. and R.H. performed the experiments; W.L. analyzed data; W.L. and D.Y.wrote the article.

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**Figure legends**

Figure 1. Tissue-specific expression of *SmGRAS5* and determination of tanshinones. (A) Tissue-specific expression of *SmGRAS5* in xylem, phloem and periderm tissues of *S. miltiorrhiza* roots. The expression levels were normalized to values in the xylem. (B) Contents of tanshinones in different tissues of the roots. (C) A Sectional view of the *SmGRAS5* and tanshinones distribution in the root tissues. Standard errors were calculated from three sets of biological replicates. Significant differences using one-way ANOVA and S-N-K comparison tests, $P < 0.05$.

Figure 2. Subcellular localization of SmGRAS5 in protoplasts of tobacco leaves (A) and onion epidermal cells (B). Upper images represent the GFP control, while lower images represent the SmGRAS5-GFP fusion proteins. GFP: green fluorescence; DAPI: fluorescence of DAPI nuclear dye; Cholorophyll: chloroplast autofluorescence; Bright field: field observations; Merged: merge of bright field and relevant fluorescence.

Figure 3. SmGRAS5 regulates the biosynthesis of tanshinones in transgenic hairy roots. (A) Relative quantitative analysis of*SmGRAS5* expression in the transgenic lines and controls. (B) Analysis of tanshinones production from *SmGRAS5* OE hairy root lines. (C) Analysis of tanshinones production from *SmGRAS5* AE hairy root lines. (D) Relative expression levels of the genes involved in tanshinones biosyntheses in the *SmGRAS5* transgenic lines. Standard errors were calculated from three sets of biological replicates. Significant differences using Student's t-test, * $0.01 < P < 0.05$, ** $P < 0.01$.

Figure 4. GA affects the tanshinones and GA contents in *SmGRAS5*OE hairy roots. (A) Analysis of $GA_3$ production from*SmGRAS5* OE lines and controls with or without 100 μM GA treatment for 6 d. (B) Analysis of tanshinones production from *SmGRAS5* OE lines and controls with or without 100 μM GA treatment for 6 d. (C) Relative expression levels of genes involved in tanshinones and GA biosyntheses in the *SmGRAS5* OE lines with 100 μM GA treatment for 2/24 h. Standard errors were calculated from three sets of biological replicates. Significant differences using one-way ANOVA and S-N-K comparison tests, $P < 0.05$.

Figure 5. SmGRAS5 binds to the GARE motif of the *SmKSL1* promoter and activates its expression. (A) Y1H assay shows the interaction between SmGRAS5 and the *SmKSL1* promoter. *SmKSL1*promoter+pGADT7 as the negative control and *SmKSL1*promoter+pGADT7-SmERF6 as positive control. (B) Dual-LUC assay shows the effects of SmGRAS5 on *SmKSL1* promoter activation. (C) EMSA analysis of SmGRAS5 binding to the GARE-motif of the *SmKSL1*promoter. Schematic diagram showing the GARE motif in the *SmKSL1*promoter. Standard errors were calculated from three sets of biological replicates. Significant differences using Student's t-test, * $0.01 < P < 0.05$.

Figure 6. Differentially expressed genes (DEGs) regulated by SmGRAS5 from the RNA-seq data. (A) Volcano plot of the DEGs regulated by SmGRAS5 (G5O14 vs ATCC). (B) GO term classifications of the 3910 DEGs from G5O14 vs ATCC. (C) KEGG classification of the 3910 DEGs from G5O14 vs ATCC. (D) Expression levels of representative DEGs of secondary metabolism. The differentially expressed genes demonstrated significantly differential expression (FDR-adjusted *P*- value < 0.05, |fold-change| [?] 2). The average FPKM (log10 scale) of each gene is shown in the heat map.

Figure 7. The differentially expressed genes (DEGs) involved in tanshinones, GA biosynthetic pathway and signaling pathway. DEGs demonstrated significantly differential expression (FDR-adjusted*P*- value < 0.05, |fold-change| [?] 2). The average FPKM (log10 scale) of each gene is shown in the heat map.

Figure 8. Model for SmGRAS5 regulates the GA-mediated accumulation of tanshinones in *S. miltiorrhiza* . GA could induce *SmGRAS5*response and promote the accumulation of tanshinones. SmGRAS5 could inhibit GA biosynthetic, and catalyze the precursor GGPP to synthesize more tanshinones through directly binding to the promoter of*SmKSL1* .

**Supporting information**

Table S1. Primers used for qRT-PCR.

Table S2. Primers used for vector construction and positive hairy roots line selection.

Figure S1. Phylogenetic tree of SmGRAS5 transcription factor. The phylogenetic tree was constructed by the maximum-likelihood method of MEGA 6.0. Members in the same sub-branch were marked by a circle filled with the same color.

Figure S2. Transactivation activity of SmGRAS5. Yeast AH109 containing*pGBKT7-53+pGADT7-T* (positive control), *pGBKT7* (negative control) and *SmGRAS5-pGBKT7* were spotted onto SD/-Trp and SD/-Trp-His-Ade medium, respectively.

Figure S3. Verification of positive hairy roots lines by using PCR. PCR screening of *SmGRAS5* overexpressing or antisense expressing lines.

Figure S4. Growth phenotypes of *SmGRAS5* transgenic hairy roots lines.

Figure S5. GA induces SmGRAS5 responses.

(A) Relative quantitative analyzes the expression of SmGRAS5 with 100 μM GA treatment for 2/24 h. (B) Promoter analysis of the GA response element of SmGRAS5.

Figure S6. Y1H assay of SmGRAS5 and *SmCPS1* promoter.*SmCPS1* promoter+*pGADT7* as the negative control and*SmCPS1* promoter+*pGADT7-SmERF6* as the positive control.

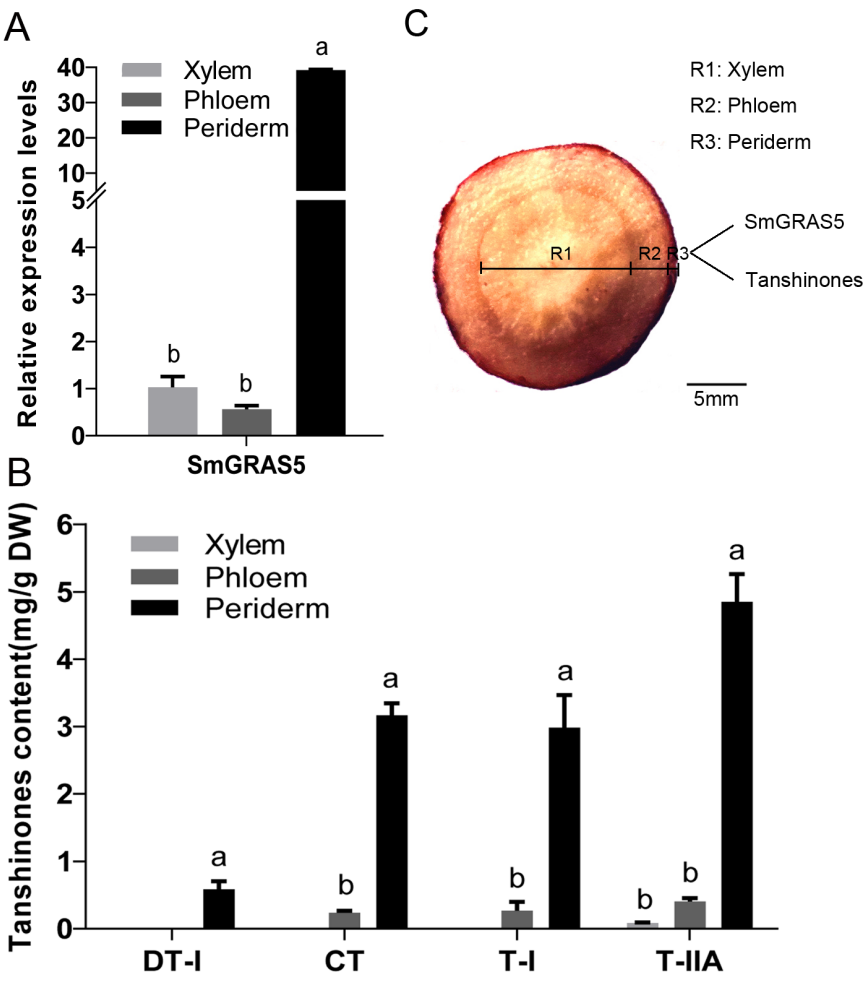
Figure S7. Purified MBP protein and SmGRAS5 protein with MBP label were verified via Western blot. M: protein marker.

Figure S8. EMSA analysis of the interaction of SmGRAS5 with the promoter of GA biosynthesis genes.

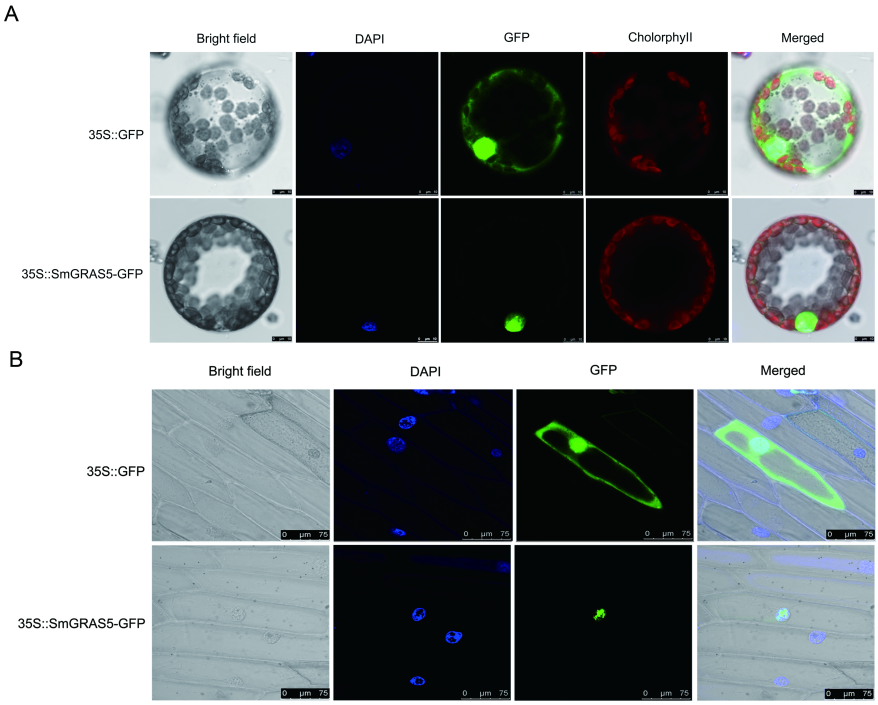
(A) EMSA analysis of the interaction of SmGRAS5 with CPS5 promoter. (B) EMSA analysis of the interaction of SmGRAS5 with KS promoter. (C) EMSA analysis of the interaction of SmGRAS5 with GA3ox1 promoter.

Figure S9. Validating the different expression levels of the identified genes from RNA-seq data by qRT-PCR. Expression of the representative GA-responsive genes in the RNA-seq experiments of G5O14. The expression of genes in the untreated ATCC hairy roots lines was set to 1. Standard errors were calculated from three sets of biological replicates. Significant differences using one-way ANOVA and S-N-K comparison tested, $P < 0.05$.

Figure 1



# Figure 2



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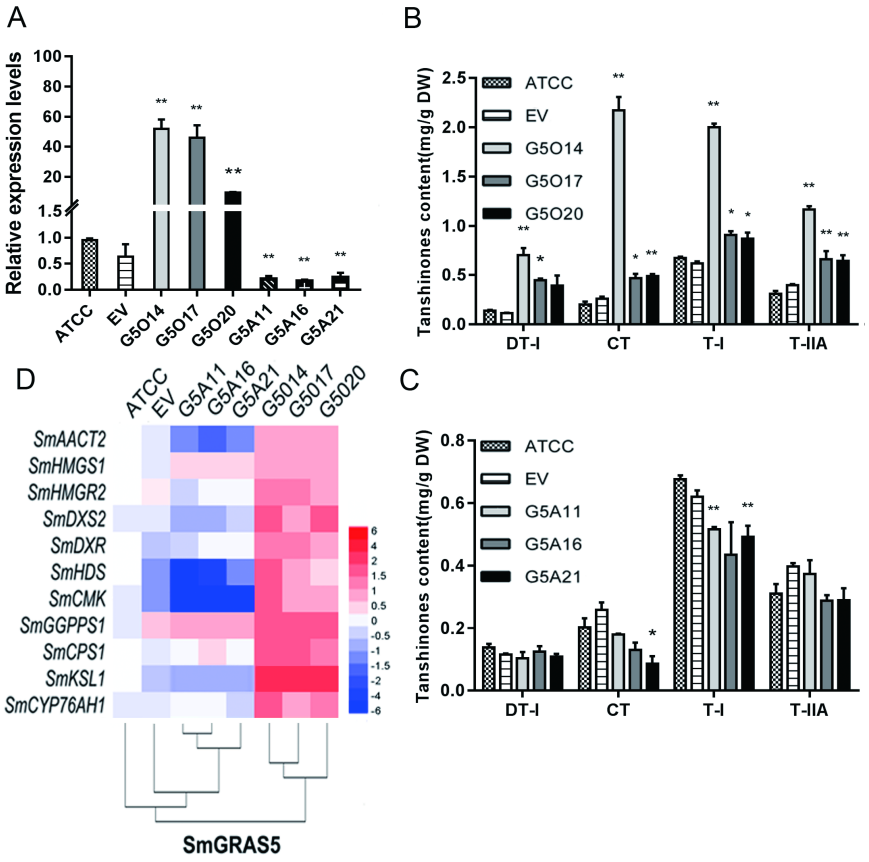



# Figure 4

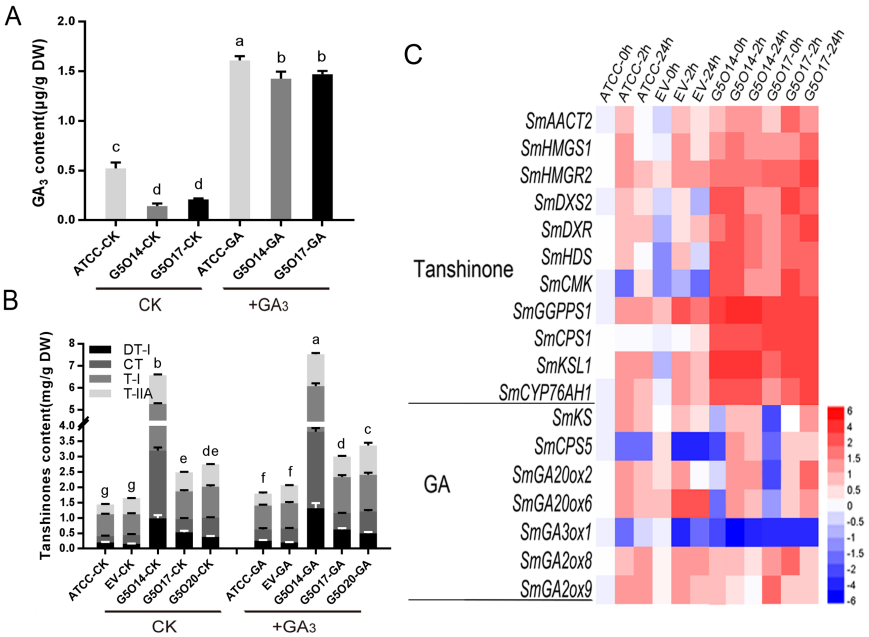
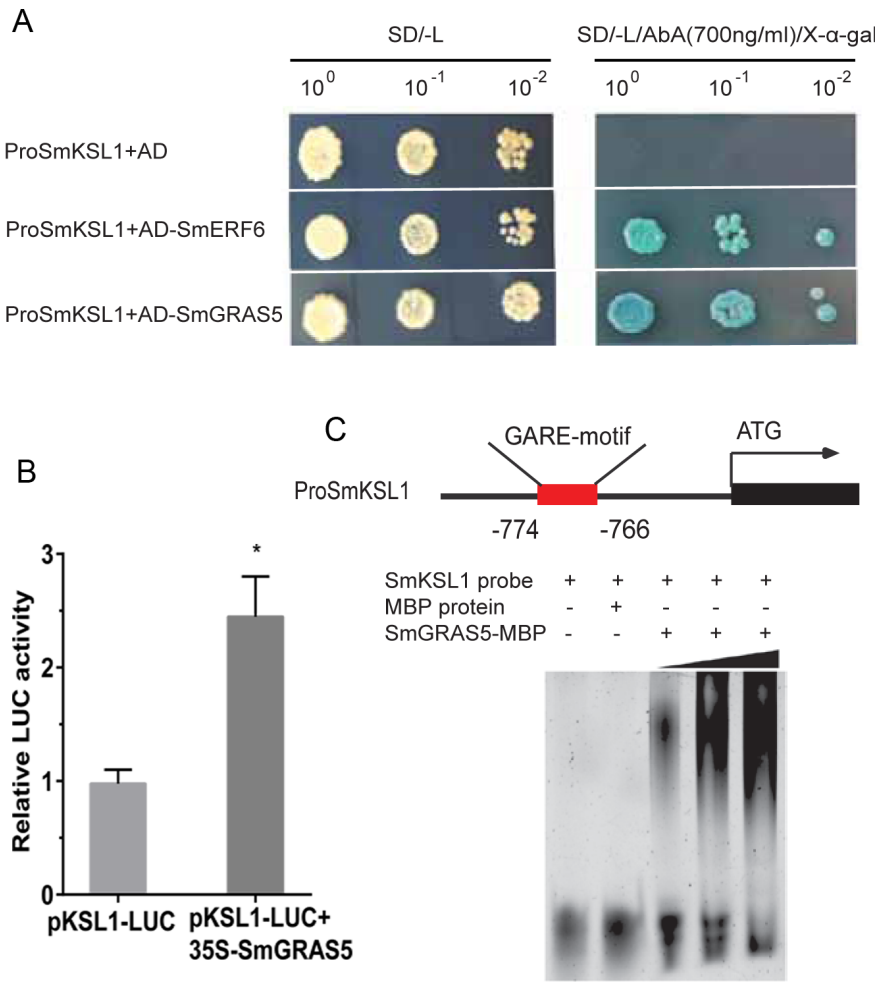
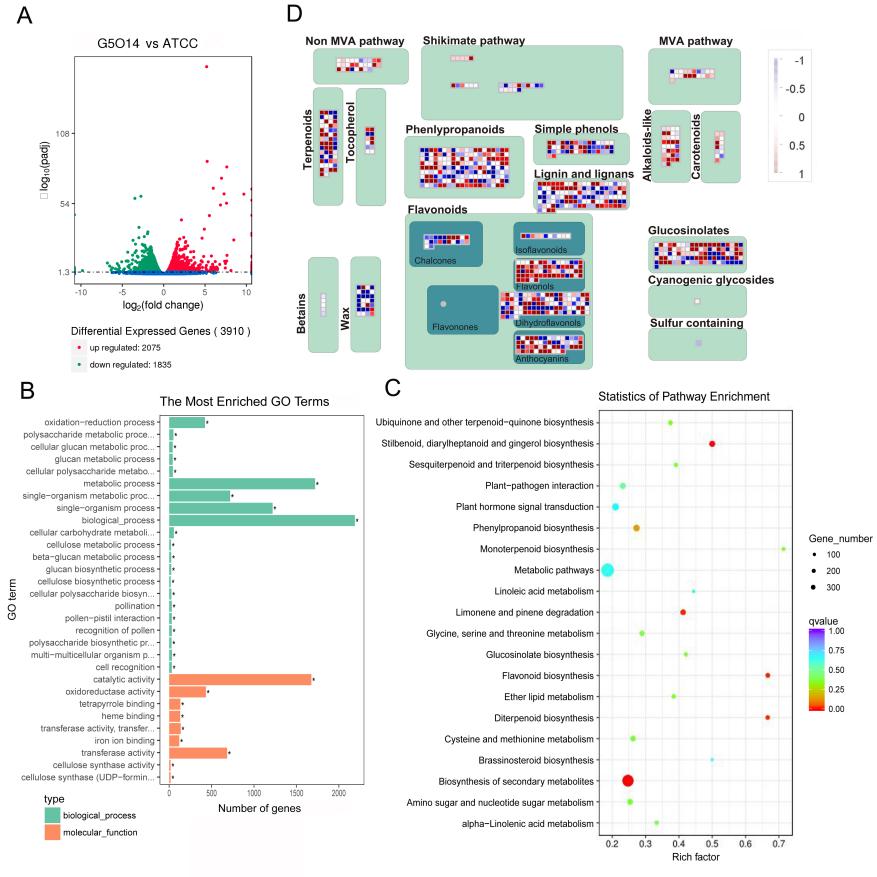


Figure 5



# Figure 6



# Figure 7

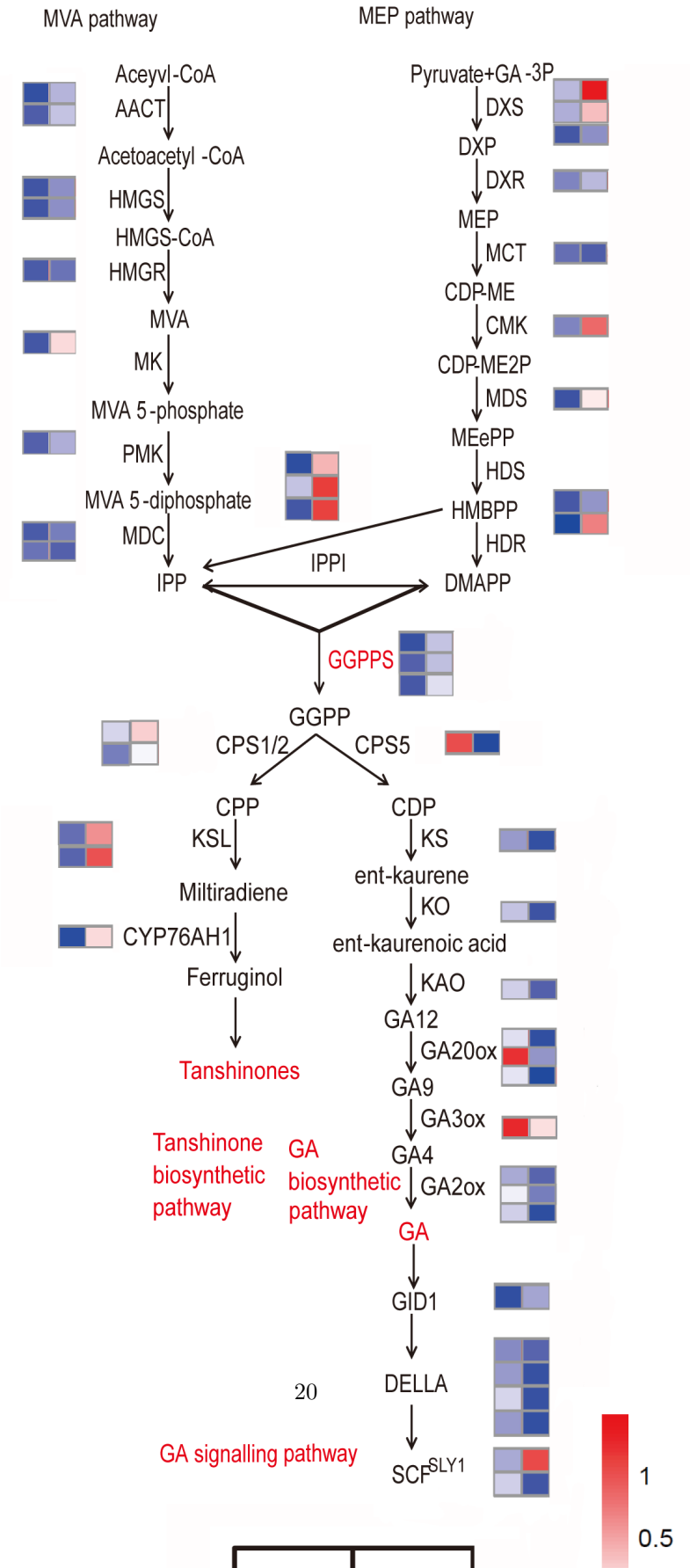


Figure 8