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SmGRAS3 negatively responds to GA signaling while promotes tanshinones biosynthesis in *Salvia miltiorrhiza*

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ABSTRACT

Salvia miltiorrhiza Bunge is the origin plant of traditional Chinese medicine, Danshen. Demand of high-quality *S. miltiorrhiza* is increasing because of its excellent pharmaceutical value. Tanshinones and salvianolic acids (SAs) are the main active ingredients of *S. miltiorrhiza*. GA was reported to induce tanshinones and SAs accumulation, however, its mechanism was unclear. Here, we got a GA responsive protein, SmGRAS3. *SmGRAS3* expressed highest in the periderm where tanshinones produced and accumulated. Results of *SmGRAS3* over and antisense expression indicated that SmGRAS3 is a positive regulator of tanshinones biosynthesis in *S. miltiorrhiza* hairy roots. But overexpression of *SmGRAS3* depressed SAs and GA biosynthesis as well as hairy roots growth, and application of GA could partially recover these depressions. Pathway enzyme genes expressions of tanshinones, SAs and GA biosynthesis were comprehensively changed in *SmGRAS3* transgenic hairy roots. SmGRAS3 acted as a transcription factor that located in nucleus and has the transcription activation activity. Yeast one-hybrid (Y1H) and electrophoretic mobility shift assay (EMSA) showed that SmGRAS3 directly bound to GARE-motif in promoter of *KSL1* and activated *KSL1* transcription. Our study suggested that the SmGRAS3 may be a good potential target for further metabolic engineering of bioactive components biosynthesis in *S. miltiorrhiza*.

1. Introduction

Salvia miltiorrhiza Bunge is the source of a widely used traditional Chinese medicine, 'Danshen'. It has good curative effect on cardiovascular and cerebrovascular diseases (Feng et al., 2019). As cardiovascular and cerebrovascular diseases are the major killer of the human world, demand of *S. miltiorrhiza* has increased (Benjamin et al., 2018; Timmis et al., 2018). Tanshinones and salvianolic acids (SAs) are the two groups of its active substances (Liu et al., 2006; Shi et al., 2005). And their contents are the main evaluation indexes of *S. miltiorrhiza* quality. Many reports, from genetic engineering to metabolic engineering, have focused on regulation of tanshinones or SAs biosynthesis (Xing et al., 2018).

Biosynthetic pathways of tanshinones and SAs are nearly cleared in *S. miltiorrhiza*. Tanshinones are derived from mevalonate (MVA) pathway and methylerythritol phosphate (MEP) pathway (Gao et al., 2014) (Fig. S1). Most of the key enzyme genes involved in tanshinones biosynthesis have been cloned and functionally analyzed (Cui et al., 2015; Li et al., 2017; Ma et al., 2015, 2016; Shi et al., 2016). And SAs are derived via the phenylpropanoid pathway and the tyrosine pathway (Di et al., 2013). Parts of the key enzymes were identified as the limiting velocity enzymes for SAs biosynthesis (Ma et al., 2015; Song and Li, 2015; Zhang et al., 2015; Zhou et al., 2018).

Gibberellic acid (GA) is a plant hormone which plays important roles in many aspects of plant growth and stress responses to abiotic stress, such as seed germination, stem elongation, flower development, salinity drought or cold stress, and secondary metabolism regulation. GRAS proteins play essential roles in these processes. The GRAS proteins are a plant-specific protein family contains 13 subgroups, SCR, DELLA, PAT1, SHR, HAM, SCL3, SCL4/7, LISCL(SCL9), LAS, DLT, OS4, OS19 and OS43 (Sun et al., 2012). The first GRAS family member isolated was SCARECROW (AtSCR) which is essential to maintain the stem cell status of the surrounding initial cells (Laurenzio et al., 1996; Sabatini et al., 2003). VaPAT1 enhanced cold, drought and salt

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tolerance in transgenic *Arabidopsis* via modulation of the expressions of a series of stress-related genes. However, it was repressed by exogenous GA application (Yuan et al., 2016). OsGRAS23 positively modulates rice drought tolerance via the induction of stress-responsive genes (Xu et al., 2015a). GA inhibits flavanol biosynthesis via DELLA proteins in *Arabidopsis* (Tan et al., 2019). DELLA interacting with MYC2 integrates both the GA and the JA signals into the induction of sesquiterpene production in *Arabidopsis* flowers (Hong et al., 2012). GA treatment also could improve the tanshinones accumulation in *S. miltiorrhiza* wild-type hairy roots by inducing the expressions of *CPS* and *KSL* (Bai et al., 2017a). And GA improved SAs production through inducing PAL and TAT activities (Liang et al., 2013). But their mechanisms need to be further revealed.

Previously, we got a SmGRAS3 which belongs to SCL9 subfamily and is sensitive to GA response in *S. miltiorrhiza* wild- type hairy roots (Bai et al., 2017b). It was reported that SCL9 subfamily was involved in plant stress responses, adventitious root formation in response to auxin (Hakoshima, 2018). For instance, the family member AtSCL14 is essential for the activation of stress-inducible promoters (Fode et al., 2008). Taking into consideration that GA could regulate tanshinones and SAs biosynthesis, SmGRAS3 could be a potential factor used in *S. miltiorrhiza* genetic engineering.

To investigate the functions of SmGRAS3 on tanshinones and SAs biosynthesis, we generated its over and antisense expression hairy roots. Overexpression of *SmGRAS3* substantially increased the accumulation of tanshinones. However, SAs, GA contents and hairy roots growth simultaneously decreased in *SmGRAS3* overexpression lines. Exogenous GA treatment induced all these contents in both transgenic and control lines. Results of Y1H and EMSA assays showed that SmGRAS3 directly bound to the promoter of *KSL1*. These findings indicate that SmGRAS3 acts as a negative response factor in SAs, GA biosynthesis and roots growth and a positive regulator for tanshinones biosynthesis through directly regulating *KSL1*.

2. Material and methods

2.1. Plant materials, growth conditions and GA treatment

The *S. miltiorrhiza* hairy roots were derived from sterile leaves infected with *Agrobacterium rhizogenes* bacterium (ATCC15834 strain). The 0.3 g fresh hairy roots were cultured in 50 ml of liquid 6,7-V medium on an orbital shaker and sub-cultured every 30 days. *Nicotiana benthamiana* was grown in a greenhouse (16 h: 8 h, light: dark) at 25 °C for 30 days and used for the subcellular localization experiments.

To analyse tissue-specific *SmGRAS3* gene expression, phloem tissue, xylem tissue and periderm tissue were collected at the flowering stage of 2-year-old *S. miltiorrhiza* growth in the botanical garden of Northwest A&F University.

A GA_3 (Sigma, USA) stock solution was added to the 21-day-old hairy roots to obtain a final concentration of 100 μM. The hairy roots were treated for 2, 24 h and 6 days. Hairy roots without GA treatment were used as controls. The control and treated roots were collected at 2, 24 h after treatment and used for real-time quantitative PCR analysis (qRT-PCR) analysis. And hairy roots treated 6 days were used to detect GA effects on the contents of tanshinones, SAs, and GA and root biomass.

2.2. Vector construction, genetic transformation and molecular detection of transgenic hairy roots

The full-length sequence of *SmGRAS3* was amplified and cloned into the restriction sites *NocI* and *SpeI* of the *pCAMBIA1304* binary vector in sense and antisense orientations under the control of the CaMV35S promoter. The positive clones were confirmed by PCR and restriction enzyme digestion. Afterwards, the plasmids were transformed into ATCC15834 which were then used for infect *S. miltiorrhiza* leaves. The transformants were screened with a combination of cefotaxime (Sigma, USA) and hygromycin B (MP Bio, USA). Genomic DNA was isolated from hairy roots by using the cetyl trimethylammonium bromide (CTAB) method. Four primer pairs, *rolB*, *rolC*, *hptII*, 35S forward primer (35S F) and *SmGRAS3* reverse primer (GRAS3 R), were used for the positive transgenic lines screening. The positive transgenic and control hairy roots lines being cultured 30 days were used for the qRT-PCR and HPLC analyses. All the primers used in this experiment are listed in Supplemental Table S1.

2.3. RNA extraction and qRT-PCR assays

Total RNA was extracted by using the RNAprep pure plant kit (TIANGEN, China), and then being reversely transcribed to cDNA by using the PrimeScript™ RT reagent kit (TaKaRa, Japan). No reverse transcriptase control (NRT) involves carrying out the reverse transcription step as a negative control. qRT-PCR was performed on a real-time PCR system (Bio-Rad CFX96, USA) using the SYBR Premix Ex Taq II Kit (Takara, Japan). ddH_2O replaced cDNA in the reaction was used as no template control (NTC). The *SmActin* was used as the endogenous control (Yang et al., 2010). The relative expression levels of the genes were calculated by the $2^{-\triangle\triangle ct}$ method. All the primers used for the qRT-PCR analysis are listed in Supplemental Table S2. The data were obtained from three independent biological replicates and three technical replicates.

2.4. HPLC analysis of SAs and tanshinones contents

The contents of tanshinones and SAs in the hairy roots were determined by HPLC, according to the general method in our laboratory that was described previously (Liu et al., 2016). In brief, 0.04 g of dried hairy roots was extracted by soaking the sample overnight in 8 mL of 70 % methanol and then sonicating the sample for 45 min. The mixture was centrifuged at 8000 *g* for 10 min, and the supernatant was filtered through a 0.2 μm filter and analysed by HPLC.

2.5. Total phenolics and total flavonoids contents analyses

Total phenolics and total flavonoids were detected as previously described (Pei et al., 2018; Xin et al., 2011) with some modifications. The absorbance of the samples for the total phenolics and total flavonoids analyses was measured at 765 nm and 506 nm, respectively. Gallic acid and rutin (Solarbio, China) were used to construct a calibration curve to determine the total phenolics and total flavonoids contents, respectively.

2.6. Subcellular localization

The *pA7*0390-*SmGRAS3-GFP* and *pA7*0390-*GFP* plasmids were transformed into the *Agrobacterium strain* GV3101. The GV3101 suspension cultures were infiltrated into leaves of 4-week-old *N. benthamiana*, following the previously described method (Bai et al., 2018). After 48 h of co-culture, the protoplasts were prepared as previously described (Li, 2011). The protoplasts were stained with DAPI for 15 min and later observed under a confocal laser scanning microscope. The primers used for the subcellular localization analysis are listed in Supplemental Table S1.

2.7. Transcriptional activity analysis

The *pDEST-GBKT7-SmGRAS3* and *pDEST-GBKT7* plasmids were transformed into the yeast strain AH109. The *pGBKT7-53 + pGADT7-T* plasmid was constructed as a positive control. The transformed AH109 were first screened on synthetic dropout (SD) medium lacking tryptophan (SD/-Trp) and then selected on SD medium without tryptophan, histidine and adenine (SD/-Trp/-His/-Ade). The transcriptional activity

was evaluated according to the growth status of the yeast.

2.8. Y1H assay

The coding sequence of full-length *SmGRAS3* was inserted into the *pGADT7* vector. The primers for the *pGADT7-SmGRAS3* vector are listed in Table S1. The 649-bp *KSL1* promoter sequences were cloned into the *pBait-AbAi* vector and then transformed to yeast as Bai et al. described (Bai et al., 2018). The primers used for the bait vectors construction are listed in Table S1. *pGADT7-SmGRAS3* was verified by interactions with yeast strains which recombined the *KSL1* promoter in SD/-Leu/AbA. The following step and X-α-gal staining were described in the yeast protocol handbook (Clontech, PT3024-1).

2.9. EMSA analysis

The full-length coding sequence of *SmGRAS3* was cloned into the *pMAL-2A* vector (Novagen) by using specific primers (Supplemental Table S1). Protein induction and purification were performed as previously described (Bai et al., 2018). The SDS-PAGE analyses of MBP (malE) and SmGRAS3-MBP purified proteins were conducted and showed major bands with an approximate molecular mass of 42.5 and 124.9, respectively (Fig. S2). Subsequently, the purified proteins were verified using Western blot as previously described (Ru et al., 2017).

EMSA assays were performed as Bai et al. (2018) described. The oligonucleotide probes used in this experiment were listed in Table S1

2.10. Statistical analysis

All experiments were performed with three biological replicates, and the results are presented as the means ± standard errors. The significance analyses of gene expression levels and metabolites contents were performed by one-way ANOVA followed by a Student-Newman-Keuls (S-N-K) test or Student's *t*-test (indicated in the figure legends) in Statistical Package for Social Science (SPSS 23.0, USA). For all analyses, $0.01 < p < 0.05$ and $p < 0.01$ were significant and highly significant, respectively. The figures were generated using GraphPad Prism 7.

3. Results

3.1. Expression pattern of SmGRAS3

SmGRAS3 expression was performed in different root parts (phloem, xylem and periderm) of 2-year-old *S. miltiorrhiza*. Results showed that *SmGRAS3* presented in all three root tissues (Fig. 1). And it was expressed highest in the periderm, hundredfold more than that in phloem and xylem. These results indicated that SmGRAS3 might have functional role in the periderm.

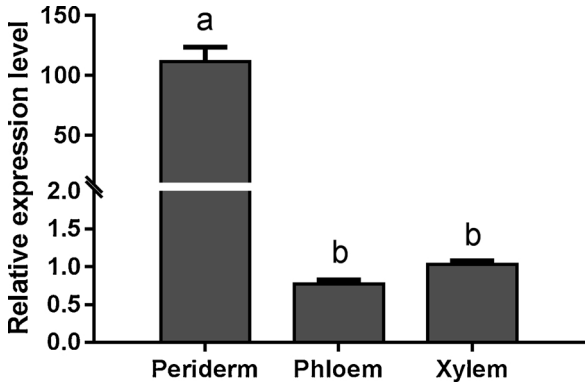

Fig. 1. Expression pattern of SmGRAS3 in different root tissues of *S. miltiorrhiza*.

3.2. SmGRAS3 localized in the nucleus

Subcellular localization of the protein could help reveal its potential function. We used the GFP transient expression in tobacco protoplasts to examine the subcellular localization of SmGRAS3. Results showed that GFP fluorescence of the control existed in the nucleus and cytoplasm. The GFP fluorescence of SmGRAS3 was only dispersive in the nucleus (Fig. 2). Like many other transcription factors, SmGRAS3 may play a role in the transcriptional regulation system.

3.3. Transcriptional activation analysis of SmGRAS3

The AH109 with pGBKT7-53 (positive control), pGBKT7 (negative control), or SmGRAS3-pGBKT7 grown well in SD/-Trp medium, respectively. In the SD/-Trp/-His/-Ade medium, positive control grown well and the negative control could not grow. AH109 with SmGRAS3-pGBKT7 can grow in SD/-Trp/-His/-Ade medium (Fig. 3). These results demonstrated that SmGRAS3 could activate transcription in the AH109 yeast cells.

3.4. SmGRAS3 up-regulated tanshinones biosynthesis, while down-regulated SAs, GA biosynthesis and hairy roots growth

To investigate the function of SmGRAS3 on active ingredients biosynthesis in *S. miltiorrhiza*. Transgenic hairy roots of *SmGRAS3* overexpression and antisense expression were obtained. After molecular identification and *SmGRAS3* expression analyzed by qRT-PCR, three overexpression lines (G3O8, G3O16, G3O17) and three antisense expression lines (G3A7, G3A10, G3A14) were chosen for further study (Figs. S3, 4 **A**). Hairy roots developed using ATCC15834 without plasmids were the control (ATCC). Compared with the control, OE lines obviously grown slower while AE lines grown faster (Figs. S3A, 4 **B**). Antisense of *SmGRAS3* significantly inhibited flavanol content in hairy roots (Fig. S4A, B). And the content of the growth promoter - GA was also decreased in *SmGRAS3* overexpression lines (Fig. 4**C**).

Four tanshinones contents, tanshinone I (T-I), cryptotanshinone (CT), dihydrotanshinone I (DT-I), and tanshinone IIA (T-IIA) were detected in transgenic and control hairy roots lines. Results showed that contents of DT-I, CT, T-I were suppressed in *SmGRAS3* antisense expression lines when compared with control (Fig. 4**E**). DT- I contents were decreased as much as 35 % in G3A7 and G3A10. CT content was decreased nearly half of the control in G3A10. And T-I content was present about 70 % of the control in G3A7, G3A10 and G3A14, respectively. In the three overexpression lines, contents of DT-I, CT, T-I were present significant increase in G3O17. On the other hand, effect of SmGRAS3 on SAs accumulations were opposed to that on tanshinones (Fig. 4**D**). Contents of two SAs, rosemarinic acid (RA), and salvianolic acid B (Sal B) were decreased in G3O8, G3O16, G3O17. And RA accumulation was improved in G3A10, G3A14. These results indicated that SmGRAS3 could promote tanshinones biosynthesis while inhibited SAs biosynthesis at the same time.

3.5. SmGRAS3 effects on expressions of pathway genes involved in SAs, tanshinones and GA biosynthesis

To further illustrate SmGRAS3 regulation mechanism on tanshinones and SAs biosynthesis, expressions of key pathways genes which contains GA-response element were detected in transgenic hairy roots. Expressions of *4CL2*, *TAT1* and *CYP98A14* in SAs biosynthesis pathways were depressed by *SmGRAS3* overexpression, and activated by antisense expression (Fig. 5).

For tanshinones biosynthesis, most of the key genes' transcription was activated by SmGRAS3. Includes *AACT2*, *HMGS*, *HMGR2* involved in MVA pathway, *DXS2*, *DXR*, *CMK*, *HDS* involved in MEP pathway and

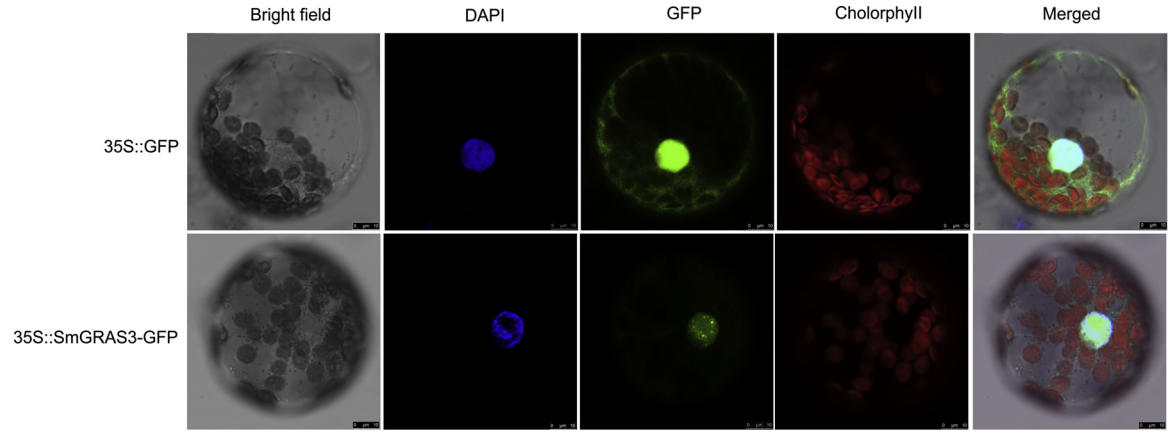

Fig. 2. Subcellular localization of SmGRAS3 protein in *N. benthamiana* protoplasts. The pictures show bright field, green fluorescent field (GFP), DAPI, Cholorphyll and overlay of four fields (Merge). The numerical reading of red ruler is 10 μm.

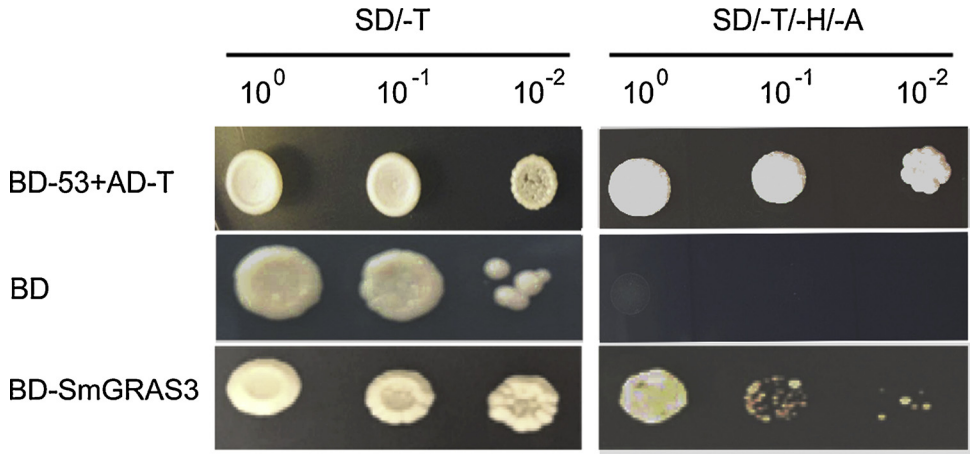

Fig. 3. Transactivation activity of SmGRAS3 in yeast. Yeast cells carrying *pGBKT7-53* (positive control), *GRAS3-pGBKT7*, or *pGBKT7* (negative control) were spotted onto SD/-Trp and SD/-Trp-His/-Ade plates.

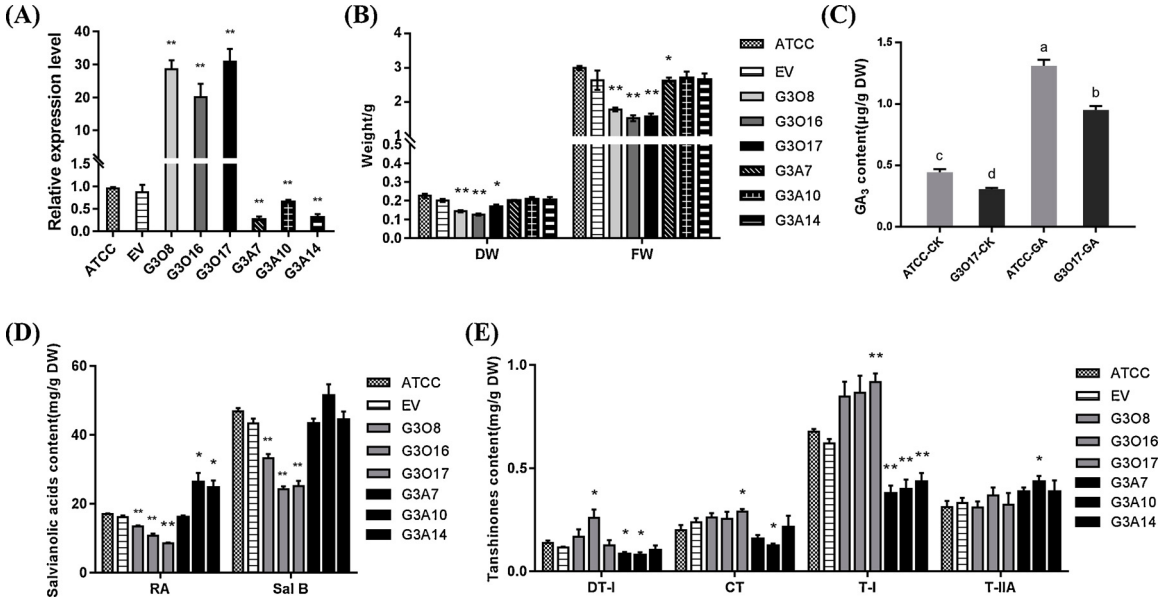

Fig. 4. (A) Relative quantitative analysis of *SmGRAS3* expression in transgenic and control of *S. miltiorrhiza* hairy roots lines. **(B)** Biomass of *SmGRAS3* transgenic and control hairy root lines. **(C)** GA content in *SmGRAS3* overexpression and control lines after 6 days of GA treatment. **(D)** Content of RA, Sal B in transgenic and the control hairy roots lines of *S. miltiorrhiza*. **(E)** Content of T-I, T-IIA, CT, DT-I in transgenic and the control hairy roots lines of *S. miltiorrhiza*. The hairy roots used in A, B, D and E were obtained after cultured for 30 days.

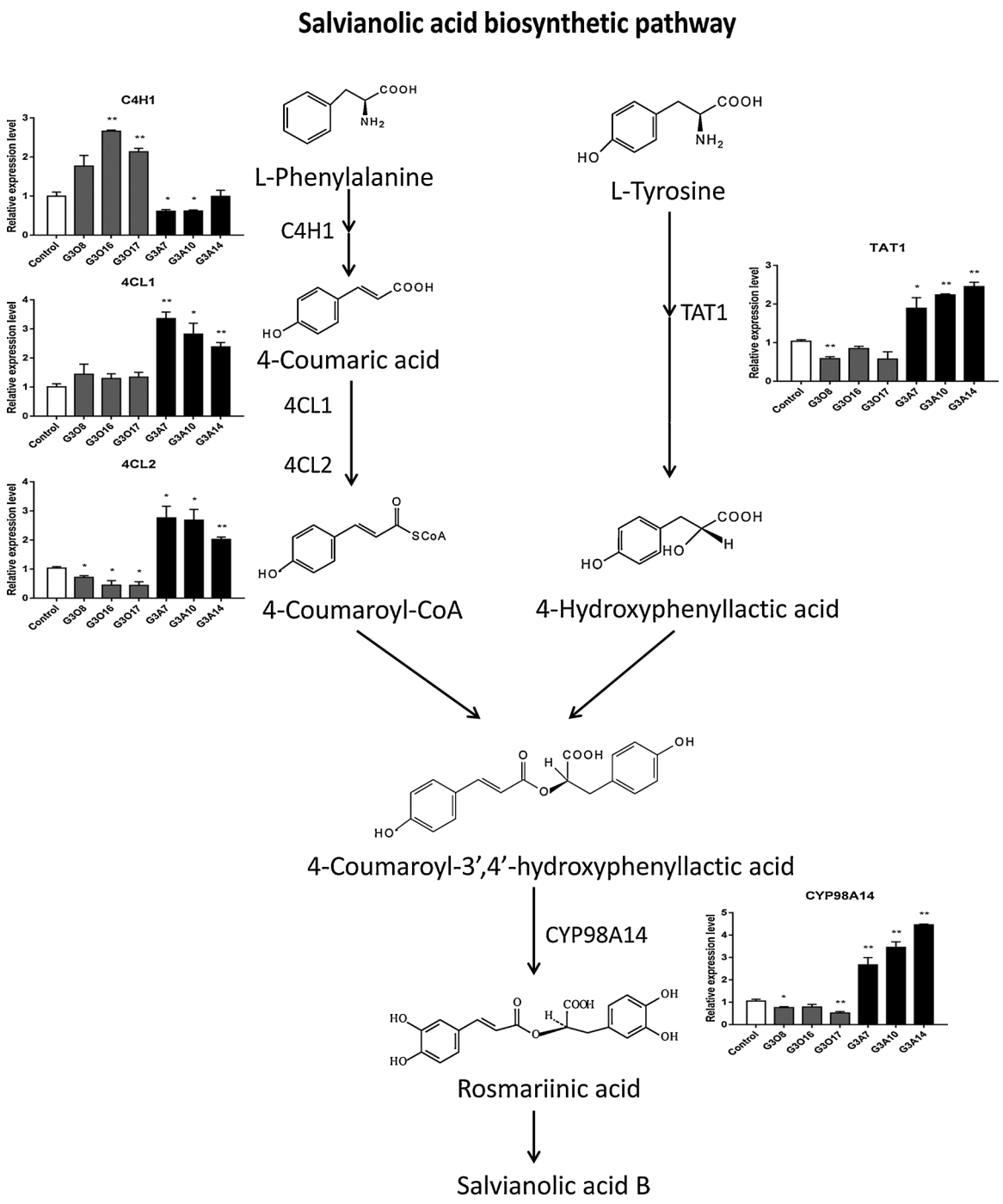

Fig. 5. Relative expression level of SAs biosynthetic pathways genes in transgenic and the control hairy roots lines.

CPS1, *KSL1*, *CYP76AH1* of the downstream pathway (Fig. 6**A**, **B**). GA biosynthesis shares the same precursor substance, GGPP, with tanshinones in plants (Su et al., 2016) (Fig. S1). The qRT-PCR results showed that SmGRAS3 depressed the expressions of GA specific biosynthetic genes. Transcriptions of *CPS5*, *KS*, *GA20ox2*, *GA3ox1*, *GA2ox9* were all inhibited by *SmGRAS3* overexpression, while induced by *SmGRAS3* antisense expression (Fig. 6**C**). The results indicated that the SmGRAS3 regulated the biosynthesis of tanshinones, GA and SA through adjusting their biosynthetic genes.

3.6. Exogenous GA induced SAs, tanshinones accumulation and hairy roots growth in both control and transgenic hairy roots

To further illustrate the roles that SmGRAS3 takes in GA signaling, exogenous GA was used to treat *SmGRAS3*-OEand control lines. After GA treatment, yield of both *SmGRAS3*-OE and control hairy roots was all improved (Fig. 7**A**, **B**). Two SAs contents were also induced by applications of GA in both *SmGRAS3*-OE and control lines (Fig. 7**C**). And tanshinones contents of the *SmGRAS3*-OE and control lines were significantly increased under GA treatments (Fig. 7**D**). The increase level in *SmGRAS3*-OE lines was greater than that in the control lines. However, the total phenolics and flavonoids were substantially reduced after GA treatment (Fig. S4C, D). GA content in hairy roots was also

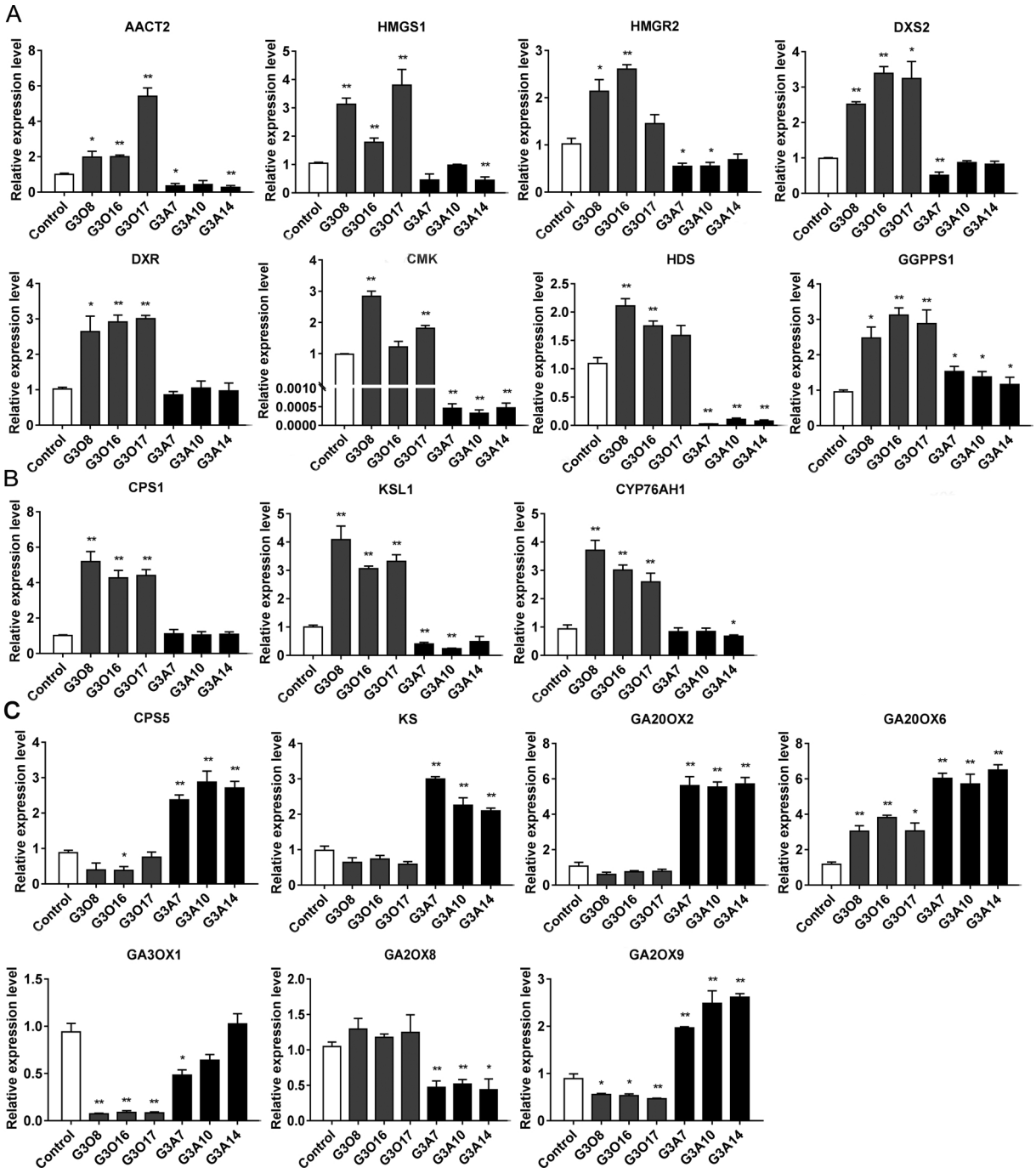

Fig. 6. Relative expression level of upstream genes **(A)**, downstream genes of tanshonones biosynthesis **(B)** and downstream genes of GA biosynthesis **(C)** in transgenic and the control hairy roots lines.

improved after treatment (Fig. 4**C**). However, increase degree of GA content in G3O17 line was lower than that in control lines. These results indicated that SmGRAS3 takes negative regulation on GA-mediated the SAs, GA biosynthesis and hairy roots growth. However, SmGRAS3 positively regulated tanshinones biosynthesis whether with GA application or not.

The expressions of most tanshinones biosynthetic genes were quickly induced by GA application (Fig. 8). Among the tanshinones biosynthetic genes, the expressions of *GGPP1, CPS1* and *KSL1* had the most significant increase. Expression changes of these genes were greater in *SmGRAS3* OE lines than that in control lines. Most of the SAs biosynthetic genes, except for *C4H1*, were also upregulated by GA. The expression of SAs biosynthetic downstream key enzyme gene *CYP98A14* was significantly increased. However, expressions of these genes induced by GA were present higher in control than that in *SmGRAS3* OE lines. *SmGRAS3* expression was induced by exogenous GA in control while suppressed in *SmGRAS3*-OE hairy roots (Fig. 7**E**). The expression patterns of the key genes in GA biosynthesis were different. For instance, *KS*, *GA20xo6* were mostly activated and *GA30xo1* was the most significantly downregulated gene among them. These findings showed that GA was an effective abiotic elicitor that promoted the accumulation of tanshinones and SAs in *S. miltiorrhiza* hairy roots and pointed to the possible co-regulation of these two pathways. And regulation of SmGRAS3 in SAs biosynthesis was likely related to GA, while regulate tanshinones biosynthesis was not.

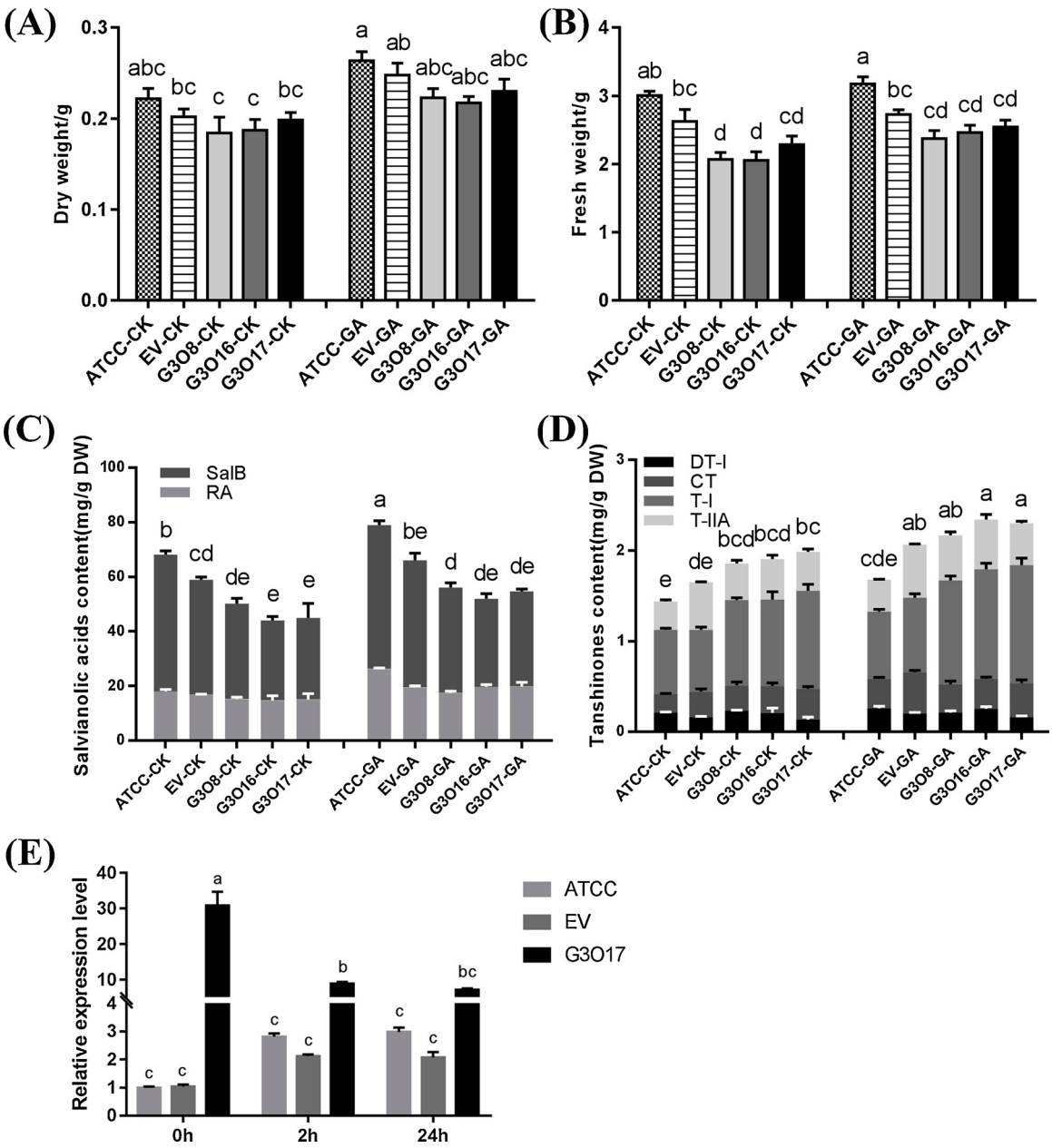

Fig. 7. Dry weight **(A)**, fresh weight **(B),** content of Sal B, RA **(C)**, four tanshinones components **(D)** of transgenic and the control hairy roots after 6 days of GA treatment. *SmGRAS3* relative expression in transgenic and the control hairy roots after 2 and 24 h of GA treatment (E).

3.7. SmGRAS3 binds to the promoter of tanshinones biosynthesis key gene KSL1

To investigate whether SmGRAS3 could binds the promoter of one or more genes involved in tanshinones biosynthesis, we analyzed the key genes that induced or repressed by SmGRAS3, and found GARE-motif in *KSL1* promoter. We constructed the *pGADT7-SmGRAS3* plasmid, and then co-transformed with *proKSL1* to yeast. The Y1H result showed that yeast with *proKSL1*and *pGADT7* could not grow in SD/-Leu/AbA (700 ng/ml)/X-α-gal, while yeast with proKSL1and *pGADT7-SmGRAS3* grow normally and appeared blue (Fig. 9**A**). These indicated that SmGRAS3 could bind the promoter of *KSL1*. EMSA assay was also performed to verify result of Y1H. Firstly, we got the SmGRAS3 protein from the *E. coli* which contained *pMALL-SmGRAS3* plasmid. Then probe and protein were mixed and tested in gels. The result showed probes for *KSL1* promoter could interact with SmGRAS3 and the control probes could not (Fig. 9**B**). These results showed that SmGRAS3 could directly regulate tanshinones biosynthesis by interacting with *KSL1*.

4. Discussion

4.1. SmGRAS3 expression pattern accords with tanshinones accumulation

As a member of gene family which specifically respond to GA signaling, transcription of *SmGRAS3* could be induced by exogenous GA. GA elicited accumulation of tanshinones as well as key genes expressions (*CPS1* and *KSL1*) in *S. miltiorrhiza* wild-type hairy roots (Bai et al., 2018, 2017b). Roots and rhizome of *S. miltiorrhiza,* where most of its pharmacodynamic ingredients mainly distribute, are used for traditional Chinese medicine. We analyzed *SmGRAS3* expression pattern in phloem, xylem and periderm tissue of roots. Results showed that *SmGRAS3* presented in all three tissues with the highest expression in the periderm (Fig. 1). It was reported that tanshinones produced and

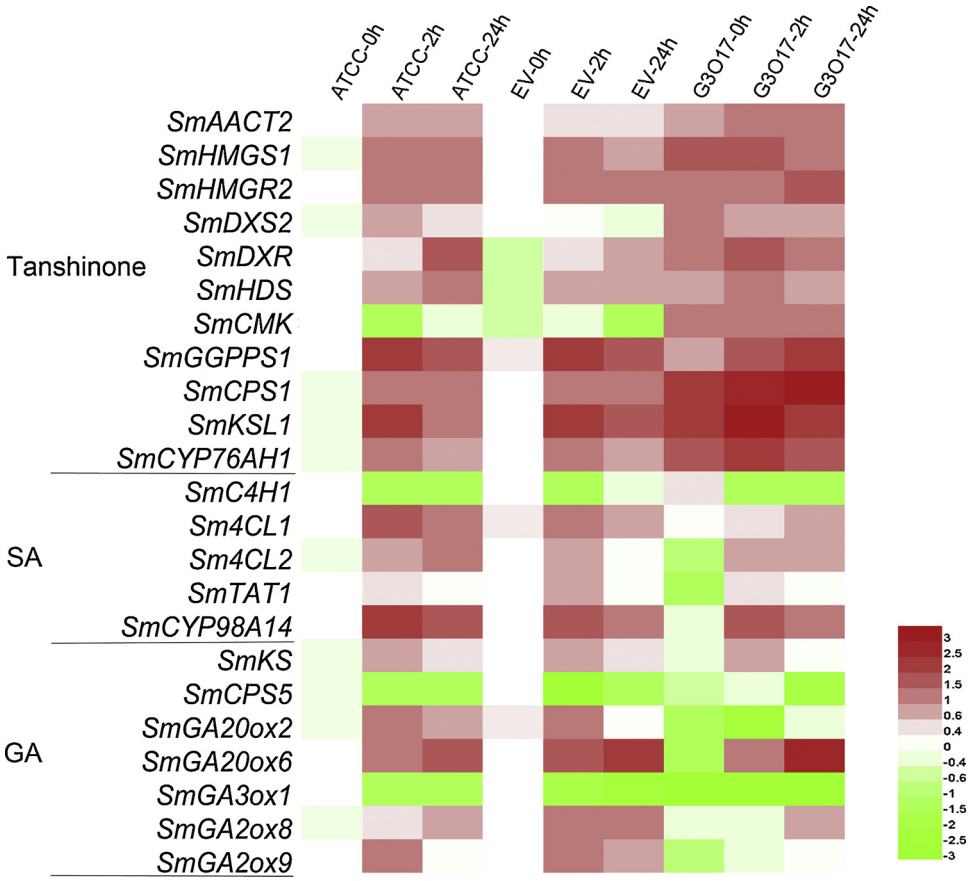

Fig. 8. Relative expressions of pathway genes involved in tanshinones, SAs and GA in transgenic hairy roots and the control after 2 and 24 h of GA treatment.

accumulated in the root periderm (Xu et al., 2015b). GRAS functions on root growth, lateral root elongation and adventitious root, middle cortex formation (Sun et al., 2012). PrSCL1 and CsSCL1 predominantly expressed in roots and played a role at early stages of adventitious root formation (Sanchez et al., 2007). SHR-SCR-SCL3 regulate middle cortex formation in the *Arabidopsis* root (Gong et al., 2016). Taken together, these findings indicated that SmGRAS3 could be a key factor in the GA signaling process regulating tanshinones biosynthesis in the periderm of *S. miltiorrhiza*.

4.2. SmGRAS3 down-regulate hairy roots growth, SAs and GA biosynthesis via GA signaling dependent way

GA regulates numerous developmental processes throughout the plant life cycle, including seed germination, stem elongation, flowering, root growth and stress tolerances (Colebrook et al., 2014). As a key regulator in GA signaling, overexpression of *SmGRAS3* inhibited hairy roots biomass and SAs biosynthesis while induced flavanol content (Figs. 4**B**, **D**, S2A, S2B). And GA content was decreased in *SmGRAS3* OE line (Fig. 4**C**). Antisense of *SmGRAS3* significantly inhibited flavanol, content in hairy roots, and improved GA and SAs biosynthesis. In *Arabidopsis*, GA promotes root growth via the inhibition of DELLA on flavanol biosynthesis (Tan et al., 2019). Transcription of *SmGRAS3* was induced by exogenous GA in control while suppressed in *SmGRAS3* OE lines (Fig. 7**E**). Exogenous GA almost recovered the biomass and SAs reduction caused by overexpressing *SmGRAS3* (Figs. 7**C**, S2A, S2B). In contrast, the total phenolics and flavonoids contents were reduced after GA application (Fig. S3C, D). These results indicated that SmGRAS3 played a negative role in GA-induced root growth and accumulation of SAs in *S. miltiorrhiza* hairy roots.

Expressions of key pathways genes changing in transgenic hairy roots showed that *4CL2*, *TAT1* and *CYP98A14* in SAs biosynthetic pathways were negatively regulated by SmGRAS3 (Fig. 5). *4CL2* was reported playing an important role in the biosynthesis of water-soluble phenolic compounds rather than *4CL1* (Jin et al., 2011). And overexpression of *TAT* could improve the productions of RA and Sal B (Xiao et al., 2011). The accumulation of RA and Sal B obviously decreased in *CYP98A14* antisense transgenic *S. miltiorrhiza* hairy root lines (Di et al., 2013). These three genes expressions were significantly activated by GA treatment and *SmGRAS3* antisense expression. The qRT-PCR results showed that SmGRAS3 also negatively regulated expressions of GA specific biosynthetic pathway genes (*CPS5*, *KS*, *GA20ox2*, *GA3ox1*, *GA2ox9*) (Fig. 6**C**). These genes take crucial roles in GA biosynthesis, and overexpression of these genes in transgenic plants in some cases alters the concentrations of bioactive GA (Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000). These finding indicated that SmGRAS3 took part in GA signaling, negatively regulated GA biosynthesis, and thus repressed GA-induced accumulation of SAs by supressing the expressions of key enzyme genes.

4.3. SmGRAS3 directly regulates tanshinones biosynthesis through binding to the KSL1 promoter

Effect of SmGRAS3 on tanshinones biosynthesis was opposite to that on SAs or GA biosynthesis. GA could induce the *SmGRAS3* response and promote root growth and the accumulation of tanshinones and SAs through signaling pathway. However, *SmGRAS3* expression was inhibited by GA in *SmGRAS3* OE lines. And exogenous GA further enhanced tanshinones accumulation in *SmGRAS3* OE lines. Transcription of the key genes in tanshinones biosynthesis were activated by SmGRAS3, including *AACT2*, *HMGS*, *HMGR2* involved in MVA pathway, *DXS2*, *DXR*, *CMK*, *HDS* involved in MEP pathway and *CPS1*, *KSL1*, *CYP76AH1* of the downstream pathway (Fig. 6**A**, **B**). Participation of GA also induced these genes' expressions in both *SmGRAS3*-OE

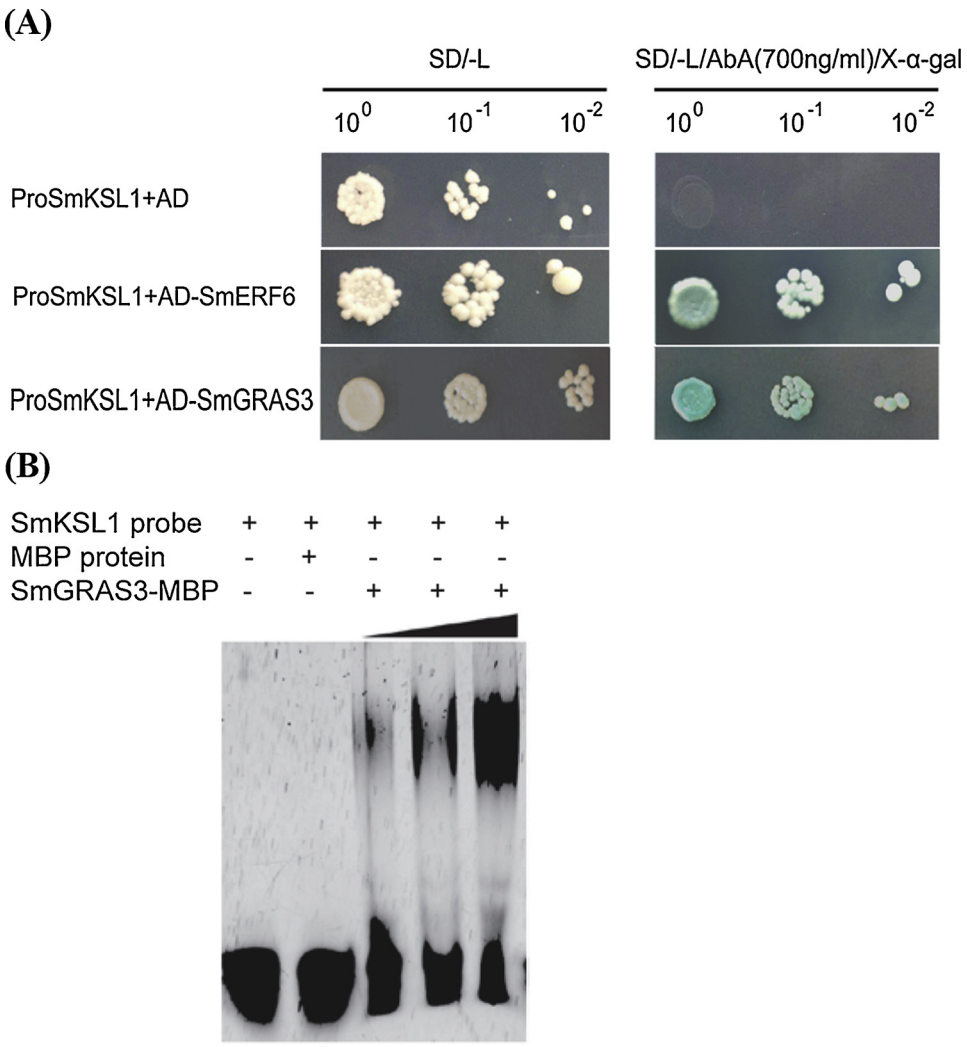

Fig. 9. Verification of the interaction between the SmGRAS3 protein and the *KSL1* promoter **(A)** SmGRAS3 can successfully binds the *KSL1* promoter in yeast cells under 700 ng/ml concentration of aureobasidin A (AbA). **(B)** EMSA result of SmGRAS3 binds GARE-motif in *KSL1* promoter.

and control lines, and with higher expression in *SmGRAS3* OE lines. These genes take important roles in tanshinones biosynthesis. AACT and HMGS are the first and second enzyme in MVA pathway of isoprenoid biosynthesis, and play significant roles in biosynthesis of tanshinones (Chang et al., 2015; Cui et al., 2010; Zhang et al., 2011). *DXS2* overexpression enhanced tanshinones accumulation, and overexpression of *HMGR* or *DXR* in hairy root lines can also significantly enhance the yield of tanshinones and with higher tanshinones contents in *HMGR* and *DXR* co-expressing lines (Kai et al., 2011; Shi et al., 2016, 2014). *CMK* and *HDS* also take part in the regulation of tanshinones biosynthesis by elicitors or transcription factors (Ding et al., 2017; Ma et al., 2015; Zhou et al., 2012). The overexpression of *CPS1* could enhance tanshinones accumulation, otherwise, silencing of *CPS1* caused a decrease in tanshinones levels. Silencing of *CYP76AH1* and *KSL1* also significantly decreased the tanshinones accumulation (Cheng et al., 2014; Cui et al., 2015; Ma et al., 2016). These results suggested that SmGRAS3 might directly regulate the expressions of tanshinones biosynthetic pathway genes. And the regulation of GA on tanshinones biosynthesis is not completely dependent on SmGRAS3.

GRAS protein always functions as transcription factor which activate or suppress gene expression by directly binds the cis-elements in gene' promoter. OsGRAS23 improved rice drought tolerance via directly modulating the expressions of a series of stress-related genes directly (Xu et al., 2015a). VaPAT1, a GRAS of *Vitis amurensis*, increased the cold, drought and high salinity tolerance in transgenic *Arabidopsis* via inducing stress-related genes such as *SIZ1*, *CBF1*, *ATR1/MYB34*, *MYC2*, *COR15A*, *RD29A* and *RD29B* expression levels (Yuan et al., 2016). In consideration that of SmGRAS3 located in the nucleus and has transcription activation activity, it could be functioned as a transcription factor. These results indicated that SmGRAS3 might binds the promoters of *AACT2*, *HMGS*, *HMGR2*, *DXS2*, *DXR*, *CMK*, *HDS*, *CPS1*, *KSL1*, *CYP76AH1*, directly regulated their transcription and then functioned on tanshinones or SAs biosynthesis.

The cis-elements which GRAS binds to was identified. AtSCL27 directly interacted with the G(A/G)(A/T)AA(A/T)GT cis-elements of the *PORC* promoter to regulated chlorophyll biosynthesis (Ma et al., 2014), whereas NSP1 induced nodulation signaling via interacting with the *ENOD11* promoter which had a shorter motif, AATTT (Hirsch et al., 2009). To investigate whether SmGRAS3 could binds one or more tanshinones biosynthetic genes' promoter, key genes that induced or repressed by SmGRAS3 were analyzed, and a GA response element (GARE motif) was found in *KSL1* promoter. We constructed the *pGADT7-SmGRAS3* plasmid, and then co-transformed with proKSL1 to yeast. The Y1H result indicated that SmGRAS3 could binds the promoter of *KSL1*. EMSA results verified the result of Y1H. Taken together, SmGRAS3 act as positive regulators in tanshinones biosynthesis by directly inducing the transcription of *KSL1*.

5. Conclusions

In the present study, the roles of SmGRAS3 in pharmaceutical components biosynthesis of *S. miltiorrhiza* hairy roots were investigated. SmGRAS3 could regulate both root growth and biosynthesis of tanshinones and SAs. Its regulation on root growth, SAs and tanshinones biosynthesis via different mechanisms. SmGRAS3 down-regulated root growth and SAs accumulation through inhibiting GA biosynthesis. It's a GA signaling related process. However, Y1H and EMSA assays results showed that SmGRAS3 could binds promoter of *KSL1*. These findings indicated SmGRAS3 regulated tanshinones biosynthesis by directly active transcription of key pathway gene *KSL1*. This study gave that SmGRAS3 has potential application value used for medical plants engineering, especially for roots medicine. Nevertheless, GA signaling has diversity functions, future study should be taken to identify other GRAS or transcription factors and their roles in the biosynthesis of tanshinones and SAs.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.112004.

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