Transcriptome analysis reveals the GRAS family in *Salvia miltiorrhiza* hairy roots in response to gibberellin

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Research article

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Abstract

Background: *Salvia miltiorrhiza* is one of the most important traditional Chinese medicinal plants with high medicinal value. Gibberellins are growth-promoting phytohormones that regulate numerous growth and developmental processes. However, their role in regulating secondary metabolism has not been investigated.

Results: In this study, we found that GA can promote hairy roots growth and increase the content of tanshinones and phenolic acids. Transcriptome sequencing revealed that secondary metabolism pathway genes were enriched in the GA-responded. After further analysis of the changes of GA signaling pathway genes, it was found that the GRAS transcription factors family had a significant response to GA. We identified 35 SmGRAS genes in *S. miltiorrhiza*. SmGRAS genes can be divided into ten subfamilies in which members of the same subfamily showed similar conserved motifs and gene structures, suggesting the possible conserved functions.

Conclusions: Most SmGRAS genes are significantly responsive to GA, indicating that they may play an important role in the GA signaling pathway and participate in GA regulation of root growth and secondary metabolism in *S. miltiorrhiza*.

Background

Salvia miltiorrhiza Bunge (Danshen) is a well-known traditional Chinese medicine with high medicinal and economic value. It is mainly used to treat cardiovascular and cerebrovascular diseases [1]. The Chinese pharmacopeia stipulates that the medicinal part of S. miltiorrhiza is its dried root. There are two main bioactive components of S. miltiorrhiza, lipophilic tanshinones, and hydrophilic phenolic acids [2]. More than 40 tanshinones and 20 hydrophilic phenolic acids have been isolated and identified from S. miltiorrhiza [3]. Tanshinones including dihydrotanshinone I (DT-I), cryptotanshinone (CT), tanshinone I (T-I) and tanshinone IIA (T-IIA) in S. miltiorrhiza are biosynthesized through the mevalonic acid (MVA) and 2-C-methyl-D-erythritol-4-phosphate (MEP) pathways [4, 5]. Phenolic acids including salvianolic acid B (Sal B) and rosmarinic acid (RA) in S. miltiorrhiza are biosynthesized via the phenylpropanoid and the tyrosine-derived pathways [6, 7]. Most of the key biosynthetic enzyme genes of those pathways have been identified [8, 9]. But these limited levels of bioactive compounds do not meet the ever-increasing market demand. Methods of improving the secondary metabolites have been tried, such as adversity stress, addition elicitor, overexpressing or suppressing key enzyme genes or transcription factors involved in the biosynthetic pathways of these secondary metabolites. However, the regulation of gibberellin on secondary metabolites biosynthesis remains unknown.

Gibberellins (GAs) are growth-promoting phytohormones that regulate numerous growth and developmental processes throughout the whole life cycle of the plant, including seed germination, root and stem elongation, and flower development [10]. Since the 1950s, more than 130 GAs have been identified in various plants (http://www.plant-hormones.info/gibberellin_nomenclature.htm) [11]. Only a

few of them, such as G1, G3, G4 and G7, are bioactive [10]. GAs biosynthesis and catabolism pathways in plants have been well characterized. GAs are biosynthesized from the common precursor trans-geranylgeranyl diphosphate (GGPP), formed via the methylerythritol phosphate pathway, through the sequential action of two terpene cyclases CPS and KS, followed by oxidation by cytochrome P450 monooxygenases and then by 2-oxoglutarate-dependent dioxygenases [12] Subsequently, GA functions in plants through its signaling pathways [13]. Binding of GA to the nuclear receptor, GID1, causes a conformational change in the N-Ex of protein that promotes its association with GRAS domain of DELLA protein. This stable complex enables efficient SCF^{SLY1} recognition and subsequent degradation of DELLA by the proteasome [14].

The plant-specific GAI-RGA-and-SCR (GRAS) family proteins function as transcriptional regulators and play critical roles in GA signaling [15]. Most GRAS proteins comprise an N-terminal less-conserved variable region and a C-terminal conserved GRAS domain. Typical GRAS domains comprise 5 conserved sequence motifs: leucine heptad repeats I (LHRI), VHIID, leucine heptad repeats II (LHRII), PFYRE and SAW. Flanked by the two leucine-rich regions, the VHIID motif is present in all GRAS family members [16]. Based on amino acid sequences, the GRAS family is divided into 10 distinct subfamilies: DELLA, SCL3, LAS, SCL4/7, SCR, SHR, SCL9 (LISCL), HAM, PAT1, and DLT [16]. Different subfamily protein sequences have different characteristics and perform different functions. For example, DELLA proteins possess a conserved DELLA sequence motif in the N-terminal region. They function as repressors of GA and act as key regulatory targets in the GA signaling pathway in regulating plant growth [17, 18]. SCL3 functions as a repressor of DELLA, which can positively regulate the GA signaling pathway and control GA homeostasis in Arabidopsis thaliana root development [19, 20]. The SCL subfamily participates in root cell elongation, GA/DELLA signaling and stress response [15]. The VHIID and PFYRE motifs in the GRAS domain of SHR are essential for the interaction between SCR and SHR [16]. They could form a complex in order to participate in regulating root-related developmental processes in Arabidopsis [21, 22]. The PAT1 subfamily has been shown to mediate phytochrome and defense signaling pathways [23]. And LISCL has two conserved subfamily-restricted acidic motifs in the N-domain and has been reported to be involved in stress response and adventitious root formation in response to auxin [16].

Although GA plays an important role in many aspects of plant growth and development, little is known about its role in regulating secondary metabolism. As diterpenoids, GA and tanshinones have common precursors GGPP [9]. There might be some correlation between GA and the biosynthesis of tanshinones. In addition, GRAS has crucial roles in the GA signaling pathway. Therefore, we speculated that SmGRASs might be involved in the GA regulating secondary metabolism in S. miltiorrhiza. To address these questions, we treated the hairy roots of wild-type S. miltiorrhiza with GA and determined the changes in biomass, tanshinones, phenolic acids, total phenolics, and total flavonoids. At the same time, we also measured the transcriptome changes and specifically analyzed the transcriptional level changes of the secondary metabolic pathway and GA signaling pathway genes. Finally, the bioinformatics of all SmGRAS family genes in S. miltiorrhiza and their responses to GA were analyzed. Our results revealed the possible pathways of GA regulating secondary metabolism and the response of SmGRASs to GA in

regulating the secondary metabolism in S. miltiorrhiza, which provided a reference for the GA signaling pathway to regulate secondary metabolism.

Results

GA treatment effects root growth and secondary metabolism

After 6-day cultivation, the GA-treated S. miltiorrhiza hairy roots showed bigger and redder than the control (Fig. 1A). The fresh and dry weights of the GA-treated hairy roots were all significantly higher than the controls (Fig. 1B, C). These results showed that the GA application promoted the growth of hairy roots. In order to investigate the changes of secondary metabolites affected by GA application, the contents of phenolic acids and tanshinones were determined after GA treatment. The two phenolic acids and four tanshinones contents in the hairy roots were all significantly increased after treated with GA (Fig. 1D, E). In addition, the contents of total phenolics and total flavonoids were also determined. However, the contents of total phenolics and total flavonoids were all decreased after GA treatment (Fig. 1F, G). Collectively, the data indicated that the GA treatment promoted the root growth, increased the accumulation of phenolic acids and tanshinones, while decreased the total phenolics and total flavonoids contents in the S. miltiorrhiza hairy roots.

Transcriptome-scale Analysis Of Ga-responsive Genes

To gain a comprehensive overview of the GA-responsive genes, we performed a transcriptome analysis of CK and GA-treated hairy roots. There were 10321 differentially expressed genes (DEGs) that were annotated in the volcano plot. The comparison of CK and GA-treated hairy roots revealed 4945 were GA-induced genes and 5376 were GA-repressed genes (Fig. 2A). To verify the results from RNA-seq, 10 genes were selected for Quantitative Reverse-Transcription PCR (qRT-PCR) analysis. The results showed the expression patterns consistent with the RNA-seq, indicating that the RNA-seq data was reliable (Fig. S1). The global functional analysis of the DEGs was revealed that the “biological processes”, “metabolic processes” and “cellular processes” were the top three categories in the most enriched gene ontology (GO) terms (Fig. 2B). And these DEGs identified were next assessed using the Kyoto Encyclopedia of Genes and Genomes (KEGG). The most significantly enriched term was biosynthesis of secondary metabolites, following by ribosome, plant-pathogen interaction and some primary and secondary metabolic pathways (Fig. 2C).

Secondary metabolism pathway genes in response to GA treatment

In order to further explore the effect of GA on secondary metabolism, we used the Mapman program to analyze the transcriptome data (Fig. 3). The result showed that most DEGs of secondary metabolism were GA induced, especially the shikimate pathway, MVA pathway, simple phenols, betaines, wax, and anthocyanins. Diterpenoids such as GA and tanshinones are biosynthesized by MVA and MEP pathways. Most of the DEGs in the MVA pathway were GA induced at the transcriptional level, which might be related to the fact that GA induced tanshinones biosynthesis. Phenolic acids are mainly biosynthesized

by shikimate and phenylpropane pathways. The results showed that most of the shikimate pathway DEGs and some of the phenylpropane pathway DEGs were induced by GA. In addition, GA also affected the biosynthesis pathways of flavonoids and alkaloids-like, wax and glucosinolates. In summary, GA regulated the transcription of many secondary metabolite synthesis pathway genes.

GA biosynthetic and signaling pathways genes in response to GA treatment

To investigate the effect of GA treatment on GA biosynthesis signaling pathway, we further analyzed and summarized the DEGs of GA biosynthesis and signaling pathway (Fig. 4). The results showed that the changes in transcriptional levels of GA biosynthesis pathway genes were diverse, the expressions of some DEGs were increased and others were decreased. In the downstream GA signaling pathway, the expression of GA receptor GID1 was increased. And the expression levels of most GRAS family genes, which were the key regulators of the GA signaling pathway, were increased. The expressions of F-box proteins SCF in the GA signaling pathway were different under GA treatment. In conclusion, GA could regulate the expressions of biosynthetic pathway genes and downstream signaling pathway genes, and further regulating many downstream physiological processes, such as cell growth, secondary metabolism, and plant resistance.

Identification and phylogenetic analysis of GRAS proteins in S. miltiorrhiza

In order to study the roles of SmGRAS in GA regulation of root growth and secondary metabolism of S. miltiorrhiza, we conducted a comprehensive analysis of the GRAS family genes in S. miltiorrhiza. We used HMMER to screen the protein sequences based on the HMM profiles from the S. miltiorrhiza genome database to identify putative GRAS proteins. We identified 35 SmGRAS proteins from S. miltiorrhiza, named GRAS1 ~ 35. The putative amino acid sequences of SmGRAS1 ~ 35 contained the conserved GRAS domain for the GRAS protein [15]. To study the evolutionary relationships of SmGRAS genes, we constructed a phylogenetic tree analysis with dicotyledons of Arabidopsis and monocotyledons of rice. We revealed that the SmGRAS proteins were divided into 10 subfamilies (Fig. 5). Among these GRAS family proteins, there were 9 proteins from the PAT1 subfamily, 6 proteins from the LISCL subfamily, 5 proteins from the SHR subfamily, 4 proteins from the SCL and DELLA subfamilies, 2 proteins from the DLT and SCR subfamilies, and the other 3 subfamilies only have 1 protein. Therefore, we speculated that these SmGRAS genes might be similar to Arabidopsis GRAS genes of the same subfamily. They might be involved in the GA signaling pathway, root and stem cortex development, light morphogenesis and resistance to stress.

SmGRAS proteins sequence alignments and conserved motifs

In order to further confirm that these 35 genes belong to the GRAS family, we used DNAMAN and online MEME to perform multiple sequences alignment and conservative domain analysis on them. The multiple sequences alignment result showed that the amino acid sequences of all these 35 proteins have high identities (Fig. 6A). Almost all SmGRAS proteins contained conserved GRAS domain consisting of LHRI, VHIID, LHRII, PFYRE and SAW. We also used online MEME to identify the conserved motifs of full-length

SmGRAS proteins (Fig. 6B, C). We showed the 10 most conserved motifs located in the GRAS domain in Fig. 6B. In Fig. 6C, 20 conserved motifs were identified in the 35 SmGRAS proteins. The VHIID and SAW motifs which were the most conserved motifs in the GRAS domain, were found in all SmGRAS proteins. The LHRI, LHRII, and PFYRE motifs were found in most of SmGRAS proteins. And other 15 motifs were found in some SmGRAS proteins. The results suggested that all these 35 SmGRAS proteins have the conserved GRAS domain, but SmGRAS proteins from different subfamilies that have different motifs to perform different functions.

Structural Analysis Of Smgras Genes

To further analyze the structural components and physicochemical properties of 35 SmGRAS genes, we did an in-depth analysis with ExPASy (Table 1). The result showed that the open reading frame length of most GRAS family genes was over 1000 bp. Of the 35 SmGRAS proteins, SmGRAS34 was the shortest gene with 453 bp, and the longest was SmGRAS3 with 2247 bp. Protein length ranged from 150 amino acids (SmGRAS34) to 748 amino acids (SmGRAS3), and the molecular weight ranged from 16.8 to 84.0 kDa. The protein isoelectric points ranged from 4.8 (SmGRAS20) to 9.0 (SmGRAS35), with most of them ranging from 5 to 6. Most of GRAS proteins contained 1 exon, and few contained 2 exons. Only SmGRAS28 has 4 exons and SmGRAS35 has 5 exons.

Table 1

The information of 35 SmGRAS transcription factors identified in S.miltiorrhiza genome.

Gene	Gene ID	ORF length (bp)	Protein length (aa)	Mw (Da)	pI	Exon no.
SmGRAS1	SMil_00008598	1470	489	54680.21	5.813	1
SmGRAS2	SMil_00025700	1380	459	51215.14	6.184	1
SmGRAS3	SMil_00006393	2247	748	83952.38	4.953	1
SmGRAS4	SMil_00023920	1581	526	58112.72	5.427	1
SmGRAS5	SMil_00019115	1041	335	37542.27	5.774	1
SmGRAS6	SMil_00022108	1626	541	59758.38	5.462	1
SmGRAS7	SMil_00020530	1563	520	57762.17	6.031	1
SmGRAS8	SMil_00027641	1662	553	62006.62	5.661	2
SmGRAS9	SMil_00022663	1653	550	60292.19	5.429	2
SmGRAS10	SMil_00013932	1872	623	70517.82	6.435	1
SmGRAS11	SMil_00010266	1575	524	58338.81	5.321	1
SmGRAS12	SMil_00005963	1584	527	58587.25	5.121	1
SmGRAS13	SMil_00014572	1308	435	48756.82	6.053	1
SmGRAS14	SMil_00014379	1692	563	61920.17	6.916	1
SmGRAS15	SMil_00013931	1905	634	71762.76	6.203	1
SmGRAS16	SMil_00019995	1326	441	48523.73	5.853	1
SmGRAS17	SMil_00012335	1269	422	47557.06	6.496	1
SmGRAS18	SMil_00023609	1944	647	70502.62	5.986	2
SmGRAS19	SMil_00027306	1617	538	59384.69	5.694	1
SmGRAS20	SMil_00025750	1628	545	59449.16	4.832	1
SmGRAS21	SMil_00015621	1548	515	56501.40	5.806	1
SmGRAS22	SMil_00016132	1695	564	61825.58	5.768	2
SmGRAS23	SMil_00019107	1911	636	70786.90	6.863	1
SmGRAS24	SMil_00011905	1419	472	52047.14	6.533	1
SmGRAS25	SMil_00024909	1326	441	48314.18	5.097	1

Gene	Gene ID	ORF length (bp)	Protein length (aa)	Mw (Da)	pI	Exon no.
SmGRAS26	SMil_00017304	1266	421	47024.52	5.397	1
SmGRAS27	SMil_00018880	1206	401	44337.81	8.832	2
SmGRAS28	SMil_00018052	2205	734	81680.17	6.038	4
SmGRAS29	SMil_00012105	1248	415	44237.29	9.695	2
SmGRAS30	SMil_00000751	1440	479	53599.74	6.116	1
SmGRAS31	SMil_00012472	1218	405	44533.13	5.761	1
SmGRAS32	SMil_00017280	2235	744	83995.86	5.793	1
SmGRAS33	SMil_00000430	1998	665	71197.52	5.928	2
SmGRAS34	SMil_00006319	453	150	16797.00	4.990	1
SmGRAS35	SMil_00004080	1326	441	49830.65	9.001	5

Expression analysis of SmGRAS genes in response to GA treatment

As the key regulator of the GA signaling pathway, the transcriptional levels of GRASs were significantly affected by GA. We comprehensively analyzed the transcription level changes of these 35 SmGRAS family genes after 100 μm GA treatment for 2 hours. The expression levels of these SmGRAS genes changed a lot. The heatmap showed that 15 SmGRAS genes were GA induced, and 11 SmGRAS genes were GA repressed, while the other 9 genes did not change significantly under GA treatment (Fig. 7). The most significantly increased of these was SmGRAS5, which increased 4-fold expression levels and was followed with SmGRAS20 (2.3-fold) and SmGRAS14 (2.1-fold). The most significantly reduced of these genes was SmGRAS28, which reduced about 90% of the control. The expressions of SmGRAS31, SmGRAS8, SmGRAS11, and SmGRAS12 were all fell by more than half.

Discussion

It is well known that bioactive GAs are diterpene phytohormones that regulate growth and development throughout the whole life cycle [14]. There are many reports on the important roles of GA in plant growth, development and stress [10–13], but few reports on the relationship between GA and secondary metabolism. In addition, GA shares the same biosynthetic pathway and precursor substances with diterpenoid metabolites tanshinones, which were the main secondary metabolites of S. miltiorrhiza [9]. We speculated that there might have some correlation between GA and tanshinones. Therefore, we treated the hairy roots of S. miltiorrhiza with GA and found that it not only promoted the root growth but also increased the accumulation of tanshinones and phenolic acids. These results make us begin to pay attention to the specific mechanism of GA regulating secondary metabolism.

GA regulates many growth and development processes such as seed germination, root and shoot elongation, metabolism, stress, flowering and fruit patterning [10, 12]. The GA signaling pathway includes the biosynthesis of the active GA, perception, signal transduction and inactivation [12]. It is now clear that GAs accumulate in the elongating endodermal cells of Arabidopsis root, and play central roles in growth regulation through key transcription factor DELLA (GRAS family member) [10, 13]. Several genes of the GRAS transcription factor family in the GA signaling pathway have been reported to regulate the development of the cortex in the root. For instance, GRAS family genes form a complex SHR-SCR-SCL3 to regulate middle cortex formation in the Arabidopsis root [24]. SCL28 also plays a role in the root growth response to stress-induced microtubule organization in Arabidopsis [25]. Moreover, there is an interaction between energy metabolism and the GA-mediated control of growth that coordinates cell wall extension, lipid metabolism and secondary metabolism in Arabidopsis [26]. Similarly, the overexpression of HaGRASL reduces the metabolic flow of GAs in Arabidopsis, and this modification could be relevant in axillary meristem development [27]. After treating the wild-type hairy roots of S. miltiorrhiza with GA, we found that GA promoted the root growth, promoted the accumulation of tanshinones and phenolic acids, but inhibited the contents of total phenolics and total flavonoids. In order to explore the possible molecular mechanism, we performed transcriptome analysis. The results showed that the gene response of the secondary metabolic pathway was significant after GA treatment. We speculated that GA regulated the accumulation of secondary metabolites by regulating genes in the secondary metabolites biosynthetic pathway. After further analysis of the DEGs in secondary metabolic pathways, we found that most of the DEGs in MVA, shikimate and phenylpropane pathways are GA induced at the transcriptional level. This result also explains a series of composition content changes in the hairy root of S. miltiorrhiza at the transcriptional level after GA treatment, such as increased tanshinones and phenolic acids contents. However, there are too many components in the content of total phenolics and total flavonoids, so it is difficult to find the corresponding law through the changes of these DEGs. In addition, we also deeply analyzed the expressions of DEGs in GA signaling pathway. The results showed that the gene expression levels of most key regulatory factors GRAS family genes and GA receptor GID1 in the pathway were significantly increased. Therefore, it is speculated that GA induction affects the GA signaling pathway and causes the response of key regulatory factors GRASs in the GA signaling pathway, and further regulating the downstream physiological processes such as growth, secondary metabolism, and stress response.

In order to study the roles of GRAS in GA regulation of growth and secondary metabolism, we used HMMER to search for all SmGRAS protein sequences based on the HMM profiles from the S. miltiorrhiza genome database. SmGRAS was found to be a large family of transcription factors with 35 genes. These SmGRAS proteins were systematically analyzed in relation to the GRAS family proteins of monocotyledons rice and dicotyledons Arabidopsis. Phylogenetic tree revealed that these SmGRASs had gathered into 10 subfamilies, PAT1 (9 SmGRASs), LISCL (6), SHR (5), DELLA (4), SCL3 (4), SCR (2), DLT (2), HAM (1), SCL4/7 (1), LAS (1). Orthologs generally retained similar functions. The PAT1 subfamily has been reported to mediate phytochrome, light and defense signaling pathways [15]. The LISCL subfamily was involved in stress response and response to auxin. SHR, SCR and SCL3 formed a complex to

regulate middle cortex formation. DELLA has a central role in suppressing GA signaling [28]. DLT was involved in the brassinosteroid signaling, SCL4/7 was involved in the response to environmental stresses and LAS participated in the formation of lateral shoots [15]. Therefore, we speculated that SmGRASs might also be involved in the regulation of the above processes in S. miltiorrhiza. In addition, we further analyzed the conservative sequence. Most GRAS proteins clustered into similar subfamilies sharing similar motifs and had family-specific functions. The conservative sequence is slightly different in different subfamilies. For example, motif 15 was only present in subfamily PAT1; motifs 10 and 16 were only present in DELLA subfamily; motif 17 was only found in subfamily LISCI, indicating their specific functions to other subfamily members. The distributions of conserved motifs also reflect the relations between different subfamilies. For example, motifs 13 and 20 were found and motifs 8, 15 and 17 were completely lost in subfamilies SCL3 and DELLA. This result indicated the close evolutionary relationships between subfamilies SCL3 and DELLA. Therefore, structural analysis also provided a clue to locate which sub-family of GRAS genes is the ancient origin.

Finally, we analyzed the responses of all SmGRAS family genes to GA induction. The expression analysis indicated that most SmGRAS genes were significantly induced or inhibited in GA induction. In Arabidopsis, DELLA proteins act as repressors of GA-responsive plant growth. Three DELLA genes (SmGRAS16, 19 and 20) were induced by GA treatment. SCL3 functioned as a repressor of DELLA, which could positively regulate the GA signaling pathway and control GA homeostasis in Arabidopsis root development [20]. SmGRAS13, 17 and 30 is an orthologous SCL3 gene in S. miltiorrhiza, and it was evidently downregulated under GA treatment. Therefore, we speculated that SmGRAS proteins of different subfamilies played different regulatory roles through the GA signaling pathway, and ultimately regulated many physiological processes in S. miltiorrhiza. Finally, we also proposed a model diagram of the regulation in the GA signaling pathway. The results showed that GA could regulate the expressions of biosynthetic pathway genes and downstream signaling pathway genes, especially SmGRAS transcription factors, and further regulating many downstream physiological processes, such as cell growth, secondary metabolism, and plant resistance.

Conclusions

We found that GA could promote the growth of S. miltiorrhiza hairy roots and increased the accumulation of tanshinones and phenolic acids. In order to study the possible pathway of GA regulating root growth and secondary metabolites accumulation, we treated the hairy roots of S. miltiorrhiza during the growing period with GA, and determined the component contents and transcriptome sequencing. The results showed that GA treatment could significantly increase the accumulation of tanshinones and phenolic acids. Moreover, many DEGs of metabolic processes, especially the secondary metabolic processes, could significantly be induced under GA treatment. At the same time, we also deeply analyzed the gene expression changes in the process of the GA biosynthesis and signaling pathway, and comprehensively analyzed the biological information and expressions of the key regulatory factors SmGRAS family. The results revealed the important roles of SmGRASs and the possible pathways in the GA-regulated root

growth and secondary metabolism in medicinal plants, which providing references for further studies. Of course, there are still many studies to further explain the specific molecular mechanism.

Methods

Plant materials and treatment

The leaves of S. miltiorrhiza were from the sterile seedling line preserved in our laboratory at Northwest A&F University in Yangling, Shaanxi Province, China. The establishment of S. miltiorrhiza hairy roots was derived from aseptic leaves of S. miltiorrhiza infected with Agrobacterium rhizogenes (ATCC15834), as previously reported [29]. Samples of the hairy roots (0.3 g fresh weight) were cultured in 100 ml beaker flasks contains 50 ml of the 6,7-V liquid medium on an orbital shaker 120 rpm·min^{-1}, 25℃ in the dark and sub-cultured every 30 days.

For GA treatment, GA_3 (Sigma, CA, USA) stock solution was filter sterilized through 0.22 μm filters and added to cultures to the 21-day-old hairy roots to a desired final concentration of 100 μM. After 2 h and 6 days of treatment with GA, hairy roots were collected for the determination of qRT-PCR and HPLC analysis. Hairy roots without GA treatment were used as controls. All treatments were performed in three independent biological replicates.
HPLC Analysis

The 21-day-old hairy roots were treated with 100 μM GA_3 for 6 days and collected in three biological replicates. 0.04 g of the dried hairy roots was powdered and extracted by 8 mL of 70% methanol overnight 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 analyzed by HPLC, according to the general method in our laboratory that was described previously [30]. The GA_3 concentration analysis was measured by HPLC as previously described [31].

Determination of total phenols and total flavonoids

Total phenolics and total flavonoids were detected as previously described with some modifications [5, 32]. 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.

RNA-seq Library Construction And Sequencing

At 2 h after GA treatment, GA-treated hairy roots (GA) and controls (CK) were collected from three biological replicates and analyzed by transcriptome technology. Total RNA was extracted using the RNA extraction kit following the manufacturer's instruction (Tiangen Biotech, Beijing, China). After measuring the quality, the strand-specific RNA-Seq libraries were constructed and sequenced on the Illumina PE150

platform (Novogene, Tianjin, China). The high-quality clean data were calculated and used for downstream analysis.

Identification Of DEGs And Functional Enrichment

The reference genome and gene model annotation files were downloaded from genome websites [33]. The fragments per kilobase of transcript per million mapped reads (FPKM) were used to determine the relative expression levels of each gene. DEGs were determined using the DESeq R package [34]. The expression levels of DEGs were considered significantly differentially expressed genes with an adjusted P-value < 0.05 and |fold-change|≥2. The GO term enrichment of DEGs was evaluated using the GOseq R package [35]. The statistical enrichment of DEGs in the KEGG was identified with KOBAS [36]. Mapman program was used to analyze the transcriptome data of metabolic and signal pathways.

qRT-PCR Validation

A total of 10 DEGs were randomly chosen to verify the RNA-seq data (Fig. S1). The 10 DEGs specific primers were designed by primer 5 (Table S1). qRT-PCR was performed on a real-time PCR system (Bio-Rad CFX96, USA) using the SYBR Premix Ex Taq II Kit (Takara, China) by heating at 95℃ for 30 s, followed by 39 cycles of 95℃for 5 s, and 60℃ for 30 s. The SmActin gene was used as the endogenous control [37]. The relative expression levels of the genes were calculated by the $2^{-\triangle\triangle ct}$ method. All assays for each gene were performed in triplicate under identical conditions.

Identification of GRAS family members in S. miltiorrhiza and phylogenetic analysis

A Hidden Markov Model (HMM) of the GRAS domain (PF03514) was downloaded from the Pfam database (http://pfam.xfam.org). HMM algorithm (HMMER) was used (http://www.hmmer.org/) to search for the GRAS domain in the S. miltiorrhiza genome database [38], with an E-value < $1e^{-6}$. The protein motifs obtained via the HMMER were queried against the Pfam database. Multiple sequence alignments of GRAS protein sequences from S. miltiorrhiza, A. thaliana and O. sativa (https://phytozome.jgi.doe.gov/pz/portal.html) were performed using the Clustal X program. A phylogenetic tree based on the alignment was constructed with MEGA 6.0 by the neighbor-joining method with the bootstrap test (n = 500 replications) [39].

Analysis of conserved motifs and gene structures

The Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) was used to obtain the gene structure of introns and exons based on the CDS and corresponding to genic sequences. The GRAS domains were aligned and the conserved sites were checked manually for their corresponding amino acid residues, which were shaded using DNAMAN software. The conserved motif analysis of SmGRASs was performed by online MEME tools with 20 motif numbers. The theoretical isoelectric point (pI) and molecular weight (Mw) were predicted by the ExPASy server (http://web.expasy.org/computepi/).

Abbreviations

GRAS:GA:gibberellin; DT-I:dihydrotanshinone I; CT:cryptotanshinone; T-I:tanshinone I; T-IIA:tanshinone IIA; MVA:mevalonic acid; MEP:2-C-methyl-D-erythritol-4-phosphate; SAs:salvianolic acids; Sal B:salvianolic acid B; RA:rosmarinic acid; GGPP:trans-geranylgeranyl diphosphate; LHR:leucine heptad repeats; DEGs:Differentially expressed genes; qRT-PCR:Quantitative Reverse-Transcription PCR; GO:gene ontology; KEGG:Kyoto Encyclopedia of Genes and Genomes; FPKM:The fragments per kilobase of transcript per million mapped reads; HMM:Hidden Markov Model.

Declarations

Ethics approcal and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data setssupporting the conclusions of this article are included with in the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authers' contributions

WL and ZL conceived and designed the experiment; WL, CL, JL, and ZB performed the experiments; WL analyzed the data and wrote the article with input from other authors. All authors read and approved the final manuscript.

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Supplementary Information

Additional file 1: Figure S1. The classification of raw reads

Additional file 2: Figure S2. Pearson correlation between sample replicates

Additional file 3: Figure S3. Validating the different expression levels of the identified genes from RNA-seq data by qRT-PCR

Additional file 4: Table S1. Data output quality list

Additional file 5: Table S2. Reads and reference genome comparison list

Additional file 6: Table S3. Go enrichment of DEGs between CK and GA-treated hairy roots

Additional file 7: Table S4. KEGG Pathway enrichment of DEGs between CK and GA-treated hairy roots

Additional file 8: Table S5. Primers used for qRT-PCR

Figures

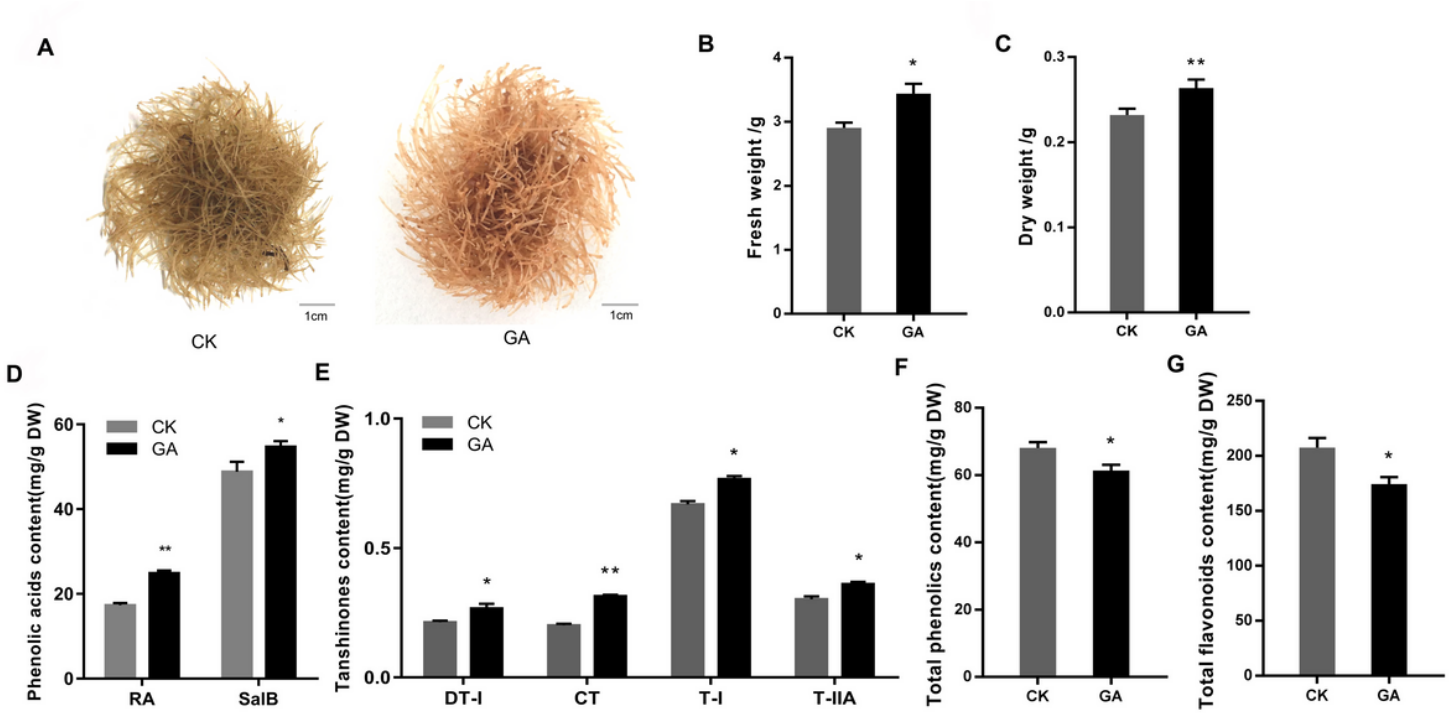

Figure 1

Phenotype, root biomass and secondary metabolites contents in S. miltiorrhiza hairy roots under CK and GA treatment. a Phenotype of hairy roots under CK and GA treatment for 6 days. b Fresh weight of hairy roots under CK and GA treatment for 6 days. c Dry weight of hairy roots under CK and GA treatment for 6 days. d Phenolic acids content of hairy roots under CK and GA treatment for 6 days. e Tanshinones content of hairy roots under CK and GA treatment for 6 days. f Total phenolics of hairy roots under CK and GA treatment for 6 days. g Total flavonoids of hairy roots under CK and GA treatment for 6 days.

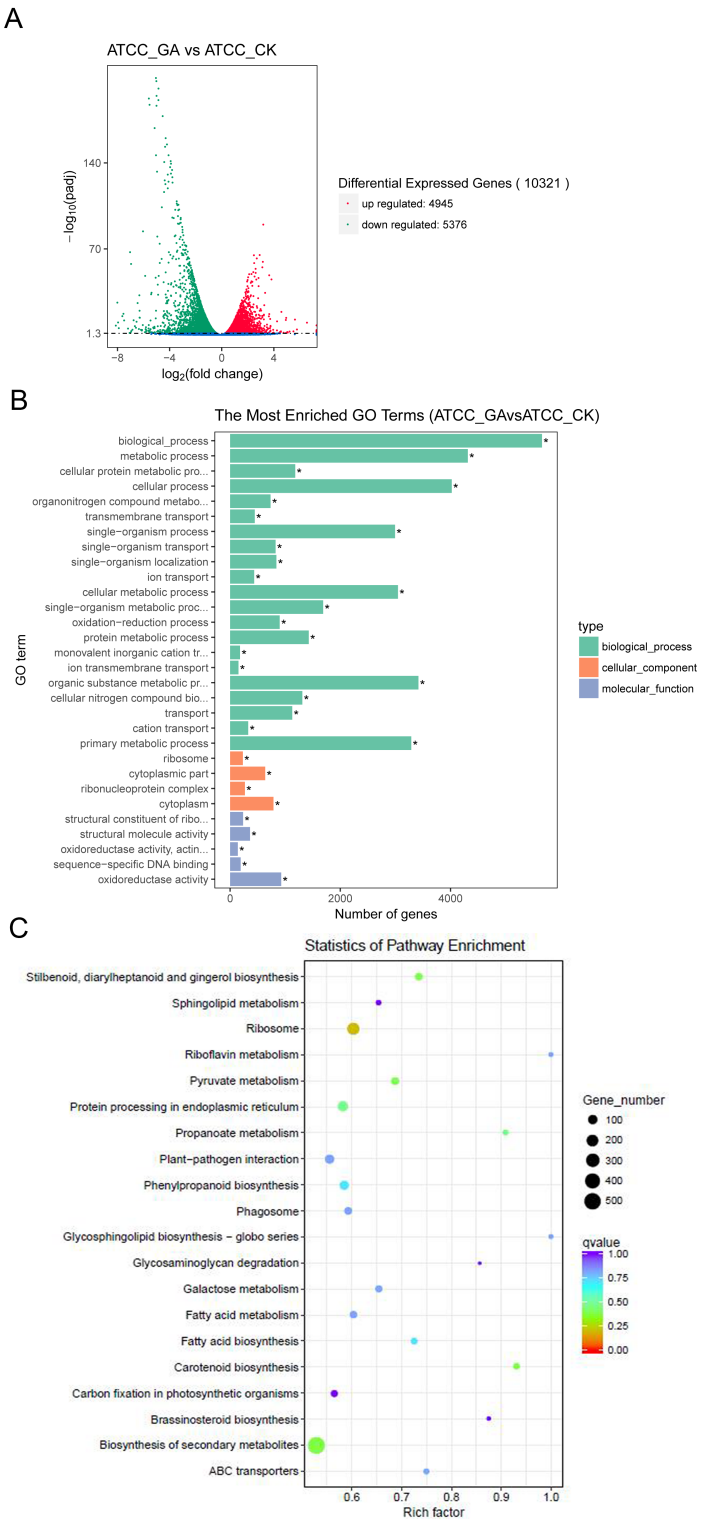

Figure 2

Transcriptome profiling analysis under CK and GA treatment in hairy roots. a Volcano plots of the differentially expressed genes (DEGs) in the comparison of GA (ATCC-GA) and CK (ATCC-CK) hairy roots. b Functional gene ontology (GO) term classifications of DEGs from comparisons of GA and CK hairy roots. c Kyoto Encyclopedia of Genes and Genomes (KEGG) classification of DEGs in the comparisons of GA and CK hairy roots.

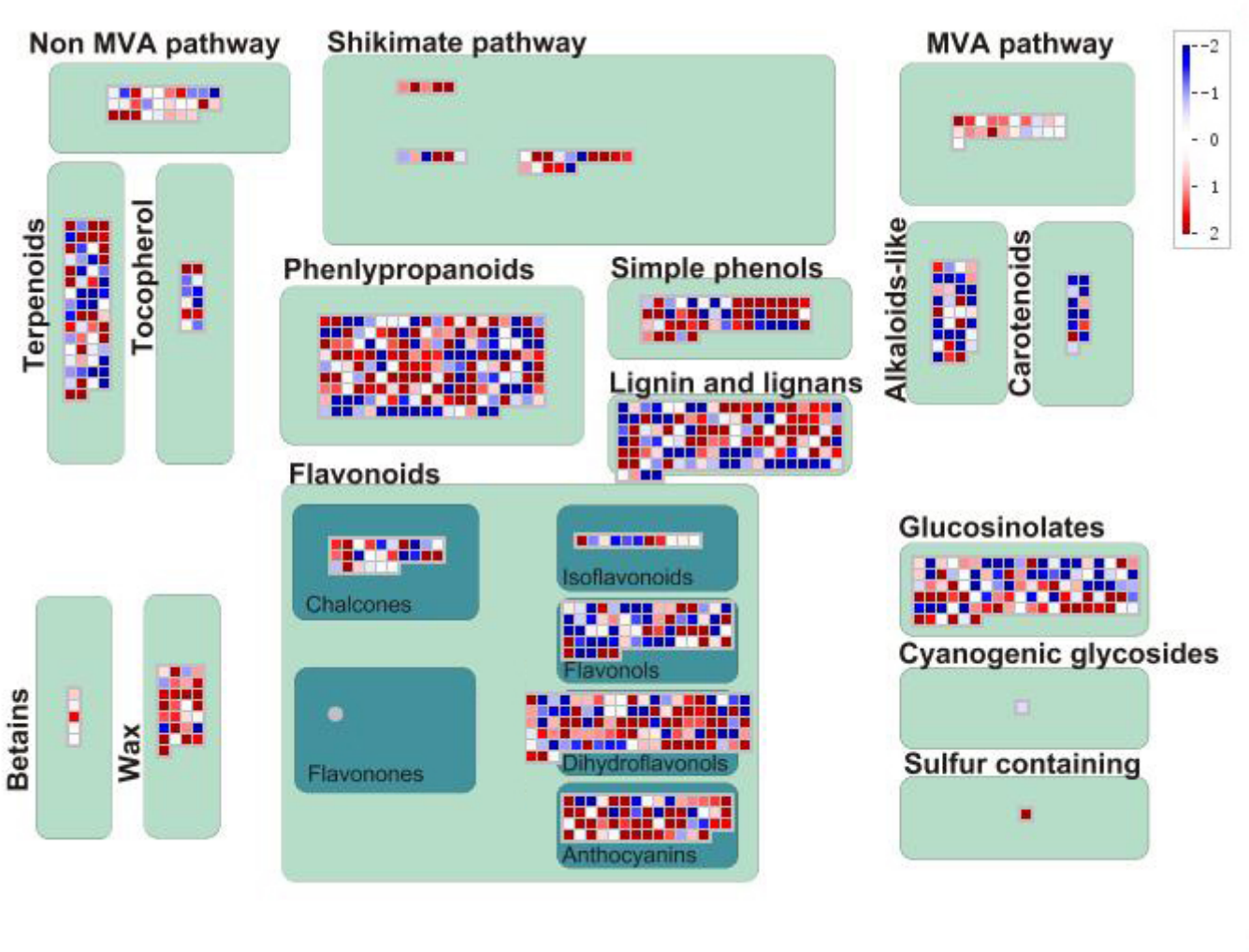

Figure 3

DEGs of secondary metabolism pathway between CK and GA treatment hairy roots. DEGs marked in red indicated they were GA induced, and blue ones indicated they were GA repressed.

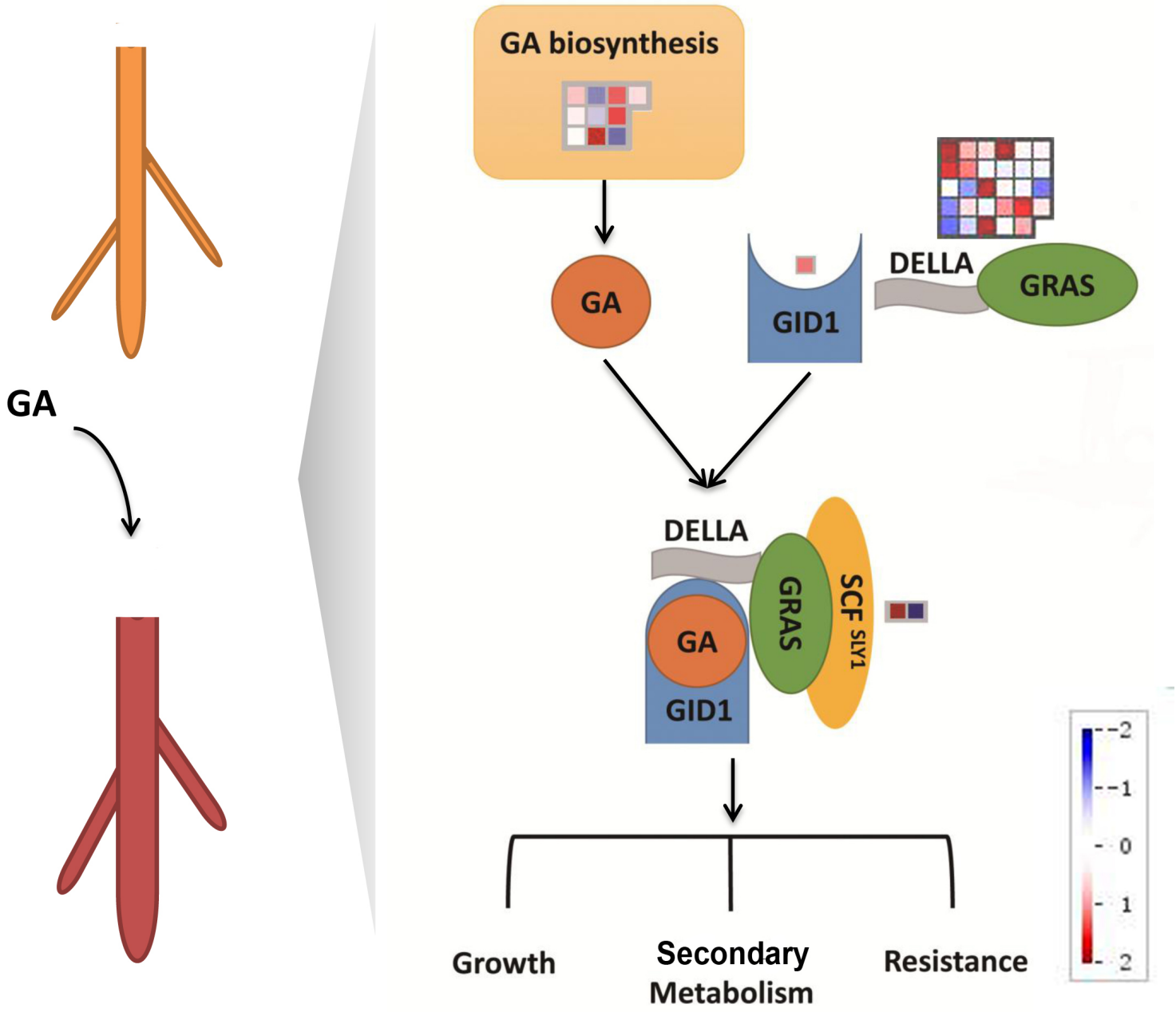

Figure 4

A model for a possible mechanism of the regulation of GA to root growth and secondary metabolism. DEGs marked in red indicated they were GA induced, and blue ones indicated they were GA repressed.

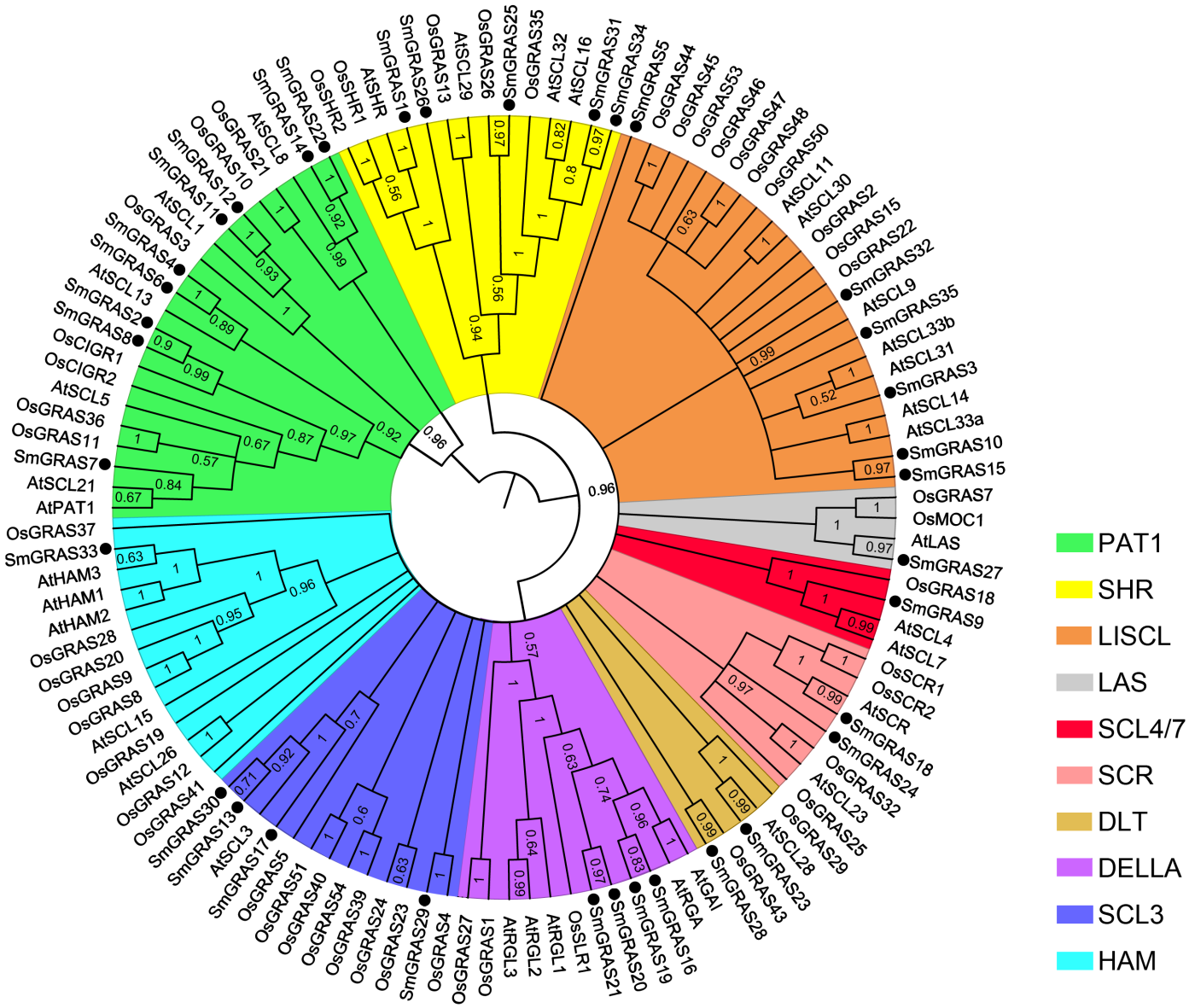

Figure 5

The phylogenic tree of GRAS transcription factors used the Neighbor-Joining (NJ) method from S. miltiorrhiza, Arabidopsis, and rice. Different subfamilies were marked with different background colors.

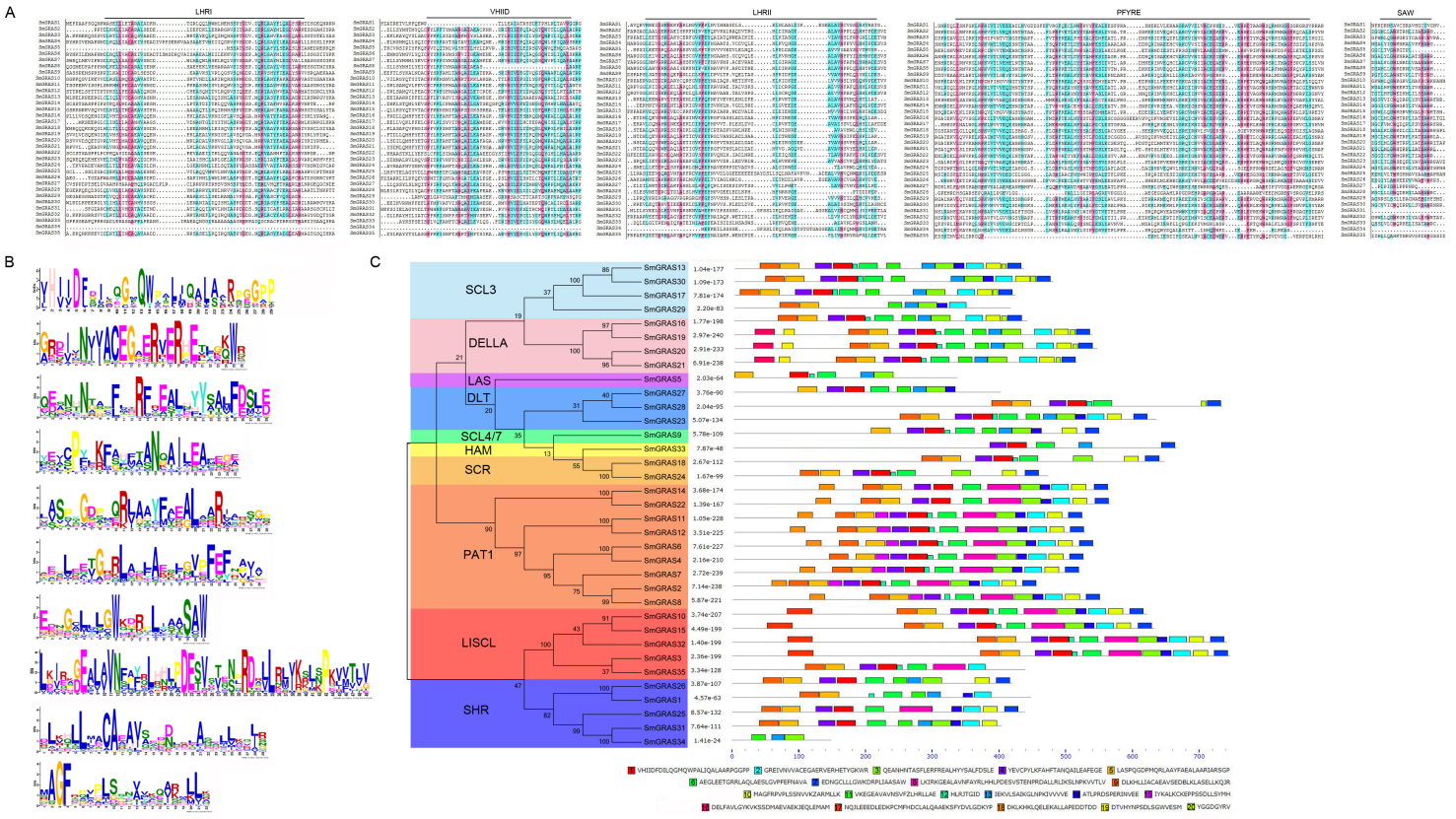

Figure 6

Conserved motifs and gene structures of SmGRAS transcription factors in S. miltiorrhiza. a Multiple sequence alignment of the GRAS domain from SmGRAS proteins. b Subfamily-specific motifs of SmGRAS. c Gene structures of SmGRAS proteins.

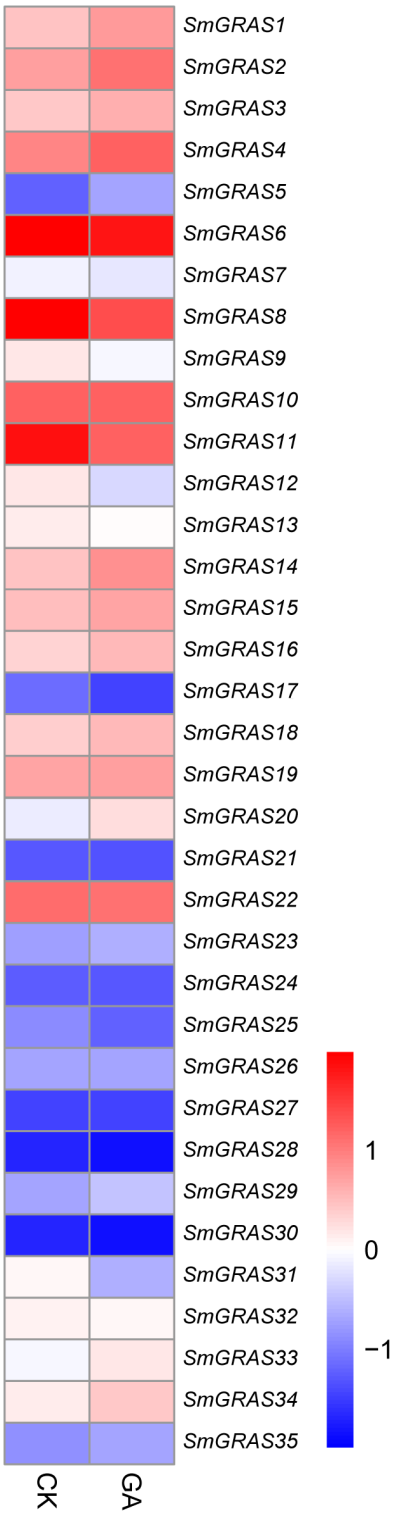

Figure 7

Gene expression profiles of SmGRAS members under control and GA treatment. Genes marked in red indicated they were GA induced, and blue ones indicated they were GA repressed.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS3.xls
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- TableS1.xlsx
- FigureS2.tif
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