Bioinformatics and Expression Analysis of NAC Transcription Factor Genes in *Scutellaria baicalensis*

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**Abstract**

**Background:** NAC, as a unique transcription factor to plants, plays important roles in multiple biological functions, such as regulation of plant growth and development, hormone levels, and responses to various kinds of stresses. However, there is a lack of research of NAC genes in Chinese herbs. **Objective:** The study aimed to evaluate the potential functions of NAC genes in *Scutellaria baicalensis* by bioinformatics and expression analysis, and provide evidence of the molecular regulation mechanism involved in flavonoid biosynthesis in *S. baicalensis*. **Methods:** The genes of NAC transcription factors in *S. baicalensis* were obtained from cDNA library and their functions were explored using bioinformatic methods. The NAC genes were screened from the cDNA library of *S. baicalensis* using BLAST comparison software. Then, the open reading frame (ORF) finder online tool was used to predict the full-length ORFs of NAC genes and their protein characteristics were explored by bioinformatic methods. The expression of NAC genes was then detected by quantitative polymerase chain reaction in different parts of *S. baicalensis* and different leaves treated by gibberellin GA3 treatment. **Results:** Six genes of NAC transcription factors were cloned, two of which had complete ORFs. NAC genes cloned in this study were mainly expressed in the flowers of *S. baicalensis*. The expression levels of NAC2, NAC3, NAC4, NAC5, NAC6 were increased firstly and then decreased gradually after 100 μM GA3 treatment. Meanwhile, some NACs and PAL2 in *S. baicalensis* showed strong correlation. **Conclusion:** This study suggested that NACs cloned in this study were mainly regulated the flavonoid biosynthesis in the flowers of *S. baicalensis*; NAC6 in *S. baicalensis* might be involved in the regulation of PAL2 transcription and affected the accumulation of flavonoids in the root of *S. baicalensis*. Our results provided a basis for further understanding the molecular regulation mechanism of flavonoid biosynthesis in *S. baicalensis*.

**Keywords:** Bioinformatics, expression analysis, NAC transcription factors, *Scutellaria baicalensis*

**Introduction**

*Scutellaria baicalensis* Georgi has been widely used as a clinical medicine in China, the main active components of which are baicalin, baicalein, and other flavonoids. Previous studies showed that high temperature and drought had an important effect on the accumulation of active components in *S. baicalensis*. The content of total flavonoids in the leaves and roots of *S. baicalensis* increased significantly when lacking water. However, the accumulation of baicalin and baicalein was inhibited in *S. baicalensis* suspension cells under the induction of high temperature. Moderate environmental stresses could promote the accumulation of active ingredients of *S. baicalensis* and stimulate the expression of the key genes involved in flavonoid biosynthesis, such as phenylalanine ammonia lyase (*PAL*) and chalcone synthase (*CHS*). Further studies have shown that plant gibberellins (GAs) played an important role during the process in response to...

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environmental stresses. Controlled experiments showed that the GA treatment in vitro could affect the content of active ingredients in S. baicalensis. MYB transcriptional factors were also revealed to activate the expression of flavonoid biosynthesis-related genes.

Transcription factors play an essential role in plant stress resistance and the accumulation of flavonoids. NACs, as unique transcription factors to plants, contain a highly conserved NAC domain in N-terminus of its protein, which were significant in dealing with high temperature, drought, salt, and other abiotic stresses. Meanwhile, NACs could participate in the biosynthesis of GAs and flavonoids. Previous studies showed that overexpression of OsNAC045 could significantly enhance rice drought and salt tolerance, and NAC019 was critical for heat stress resistance in Arabidopsis thaliana. OsNAC2 could affect plant height through mediating the gibberellic acid pathway in rice. Arabidopsis NAC transcription factor JUB1 was able to regulate GA/BR metabolism and signaling through stimulating DELLA domain. In addition, Arabidopsis NAC transcription factor ANAC078 could regulate flavonoid biosynthesis. Thus, it is speculated that NAC is most likely to regulate flavonoid biosynthesis in S. baicalensis.

Up to now, NAC transcription factor genes of >129 plant species have been registered in GenBank. Functional studies of NAC transcription factors have been reported mainly in A. thaliana, rice, and other model plants. However, the function and physiochemical properties of NAC transcription factors have not been reported in S. baicalensis. Thus, we analyzed the function of NAC transcription factors by the bioinformatics analysis and gene expression after GA stimulation and in different organs. Our work could lay the foundation to furtherly study the biological function of NAC in S. baicalensis and provided theoretical basis for improvement of the molecular regulation network.

**Materials and Methods**

**Materials**

S. baicalensis seeds preserved in the laboratory of National Resource Center for Chinese Materia Medica, China Academy of Chinese Medical Sciences. After germination of S. baicalensis seeds on wet filter paper, plant seedlings were transferred into flowerpot and followed by incubation at 25°C for 16 h light cultivation. The whole leaves of S. baicalensis were sprayed with 100 μmol/L GA, and then sampled at multiple time points (0 h, 2 h, 1 h, and 3 h). Treated samples were collected and frozen in sealed clear polyethylene plastic bags at −80°C until they were used. Each treatment consisted of three individual plants, and the experiment was repeated in triplicate.

During the flowering period in summer, three S. baicalensis plants were used for collection of tissues. Flowers, roots, stems, and leaves of were sampled for gene expression analysis, and those samples were all cleaned by the distilled water and dried by filter paper. All samples were immediately frozen in liquid nitrogen and stored at −80°C.

**Obtaining of NAC gene sequences in Scutellaria baicalensis**

NAC nucleic acid sequences of A. thaliana were downloaded from the TAIR database and then were designed and used as probes. NAC genes of S. baicalensis were searched by the homologous alignment in S. baicalensis cDNA library. The criterion of identification was as follows: E ≤ 1e−15, score ≥100, and the number of matches: B = 1.

**Bioinformatic analysis of NAC sequences in Scutellaria baicalensis**

The open reading frame (ORF) sequences of NAC gene sequences were predicted by ORF Finder online tool (http://www.ncbi.nlm.nih.gov/projects/gorf/). The physiochemical properties of NAC-encoded proteins in S. baicalensis were predicted by online software Protparam (expasy.ch/tools/protparam.html). Protein domains were analyzed by ExPASy PROSITE (http://www.expasy.ch/prosite) and CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Protein subcellular localization signals were predicted by WOLF PSORT (http://wolfsort.org/). SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP) was used to analyze the protein secretion. Protein secondary structure was analyzed by CFSSP (http://swissmodel.expasy.org/); MEGA 5.0 was used to construct the Neighbor-joining tree (NJ tree) (bootstrap = 1000).

**Total RNA extraction and single-stranded cDNA synthesis**

Total RNA was extracted according to the instructions of Plant Total RNA Kit (TIANGEN), followed by 1% agarose gel electrophoresis to detect RNA integrity. Nuclease protein quantifier (NanoDrop 2000, Thermo) was used to measure RNA concentration, and cDNA was synthesized using the instructions of M-MLV RTase cDNA (TIANGEN) to serve as the template of polymerase chain reaction (PCR) reaction.

**Cloning of NAC genes in Scutellaria baicalensis**

According the NAC gene sequences, the primers of S. baicalensis NACs were designed and synthesized by Sangon Biotech, Shanghai [Table 1]. According the instructions of TransStart® FastPfu DNA Polymerase (TransGen Biotech), PCR reaction system included 5 × TransStart® FastPfu Buffer 10 μL, dNTP 4 μL, 1 μL of 10 μmol/L upstream primer, 1 μL of 10 μmol/L downstream primer, and 1 μL of cDNA template (100 ng/L), and water was added to make a final volume of 50 μL. The reaction procedure was as follows: pre-denaturation for 1 min at 95°C, denaturation for 20 s at 95°C, annealing for 20 s at 56°C, extension for 1 min at 72°C, 40 cycles, and finally, refolding for 5 min at 72°C.

**Quantitative real-time polymerase chain reaction**

According to NAC sequences of S. baicalensis, quantitative real-time PCR (qRT-PCR) primers were synthesized by Sangon Biotech (Shanghai) [Table 2]. The lengths of qRT-PCR products were 100–250 bp. According the instructions of SYBR Premix Ex Taq™ Kit (Takara Company), qRT-PCR reaction system included 5 μL
of SYBR Premix Ex Taq enzyme, 0.5 μL of 10 μmol/L upstream and 0.5 μL of 10 μmol/L downstream primer, and 1 μL of 100 mg/L cDNA template, and water was finally added to make the volume to be 10 μL. Each reaction system was repeated for at least three times. The amplification procedure was as follows: predenaturation for 3 min at 95°C, denaturation for 5 s at 95°C, annealing extension for 34 s at 60°C (fluorescence signal was collected after each cycle), 40 cycles, and finally, denaturation for 10 s at 95°C. The melting curve was analyzed at 65°C–95°C. The temperature was increased by 0.5°C per step and stayed for 5 s for each temperature. \( C_t \) of each gene was obtained for calculating relative expression.

**Calculation of gene relative expression levels**

The expression levels of NACs after 100 μmol/L GA \( _3 \) treatment were calculated using sample with 0-h treatment as reference site, and the expression levels in different S. baicalensis tissues were calculated using stems as reference site. Sb18S was used as a reference to determine the relative expression of NAC genes.[19] Data of the qRT-PCR were collected and analyzed to calculate the relative expression levels of each gene. The formula was as follows: 

\[
2^{-\Delta \Delta C_t} = 2^{-([C_t \text{Target gene} - C_t \text{Housekeeping gene}]_{\text{Test group}} - [C_t \text{Target gene} - C_t \text{Housekeeping gene}]_{\text{Control group}})}
\]

**Correlation analysis of gene expression**

Pearson correlation analysis was used to analyze the correlation of the expression of NAC genes and flavonoid biosynthetic genes of S. baicalensis by SPSS 23.0 software. \(|r| > 0.7\) showed that there was a high degree of linear correlation, \(0.7 \geq |r| \geq 0.5\) showed moderate linear correlation, \(0.5 \geq |r| \geq 0.3\) showed low linear correlation, and \(|r| < 0.3\) showed extremely low linear correlation. \(P \leq 0.05\) was the significance level, and \(P \leq 0.01\) indicated an extremely significant difference.[20]

**RESULTS**

**Characterization analysis of NAC transcription factors in Scutellaria baicalensis**

By BLAST alignment and splicing of the obtained gene fragments, six NAC gene sequences were obtained from the cDNA library of S. baicalensis. Then, the obtained genes were cloned and verified by sequencing. The electrophoresis image of NAC gene products is shown in Figure 1. ORF Finder prediction showed that NAC3 and NAC4 were full-length cDNAs, but the rest NACs did not have complete ORF. Physical

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**Table 1: Sequences of primers used in NAC cloning**

| Gene name | Forward primer sequences (5'-3') | Reverse primer sequences (5'-3') | Product length (bp) |
|-----------|----------------------------------|----------------------------------|---------------------|
| NAC1      | GCGGCAGGCAGCTATGGC               | GGATTCACATCTATCACCAGGCA          | 746                 |
| NAC2      | GGGGGGAAGAAAAACTGAAATG           | CATGTTGAGCTTACTAAGCCCCACT        | 828                 |
| NAC3      | TCTTTCAATTTTTCTATTTGCAGTTG      | CGGAGATTACCCCCGGTCG             | 762                 |
| NAC4      | CAAATCTCTTCTTTCTTTTCTATTTTCA    | CCAATGCTTAAGCATCAGGAGGTACCA     | 1242                |
| NAC5      | GGGGAGAGAAAAAGAAGAAAAAT          | TTCATTACATCAACCGGAAACACAG       | 1556                |
| NAC6      | GTGTCACCAAAATTAAGTATGGTAGC      | CGCTCTACCTGTCTTCTCTGGT         | 480                 |

**Table 2: Real-time polymerase chain reaction primer sequences**

| Gene name | Forward primer sequences (5'-3') | Reverse primer sequences (5'-3') | Product length (bp) |
|-----------|----------------------------------|----------------------------------|---------------------|
| NAC1      | CAGTTGTCGATGAGTATGGG             | CGCTGTCTGAGTGATGTTAGG           | 262                 |
| NAC2      | TGTTCAGGATGGATGGGG               | GGGTTAGCATGTTGTTGAGTC           | 165                 |
| NAC3      | CAGTTGTAATCAGCCCCCAACT           | TATCCACCACCCAACACCA             | 155                 |
| NAC4      | TAGTTGTTGATGTTGAGGGG             | CTGCAATGATGGAAGGGGTG            | 197                 |
| NAC5      | GCACTTTGTTGCTTTCTTATG           | GAAATGCTAACCCCAAT               | 257                 |
| NAC6      | GAAATGTTGTTGCTTTCTTATG          | GTCGTACCTGGCTTGGGA              | 178                 |
| PAL1      | GCAATAATGGGTGCGCTTG              | GGTTCTGGCAGTTGTG               | 148                 |
| PAL2      | GATTCTCGTCCCGTACCA              | GATGCAATGGCCATTTCCCC            | 182                 |
| PAL3      | GCCCAACAAGATGTCG                | GGTTCTGGCAGTTGTG               | 143                 |
| CHS       | GAGGTTGCTGGGCTTCTTACCCTG        | GATGCAATGGCCATTTCCCC            | 156                 |
| MYB2      | GATGTCAGCTTCAGGAAAACTCACC       | TCTAAAAACTAAACTAAAGC            | 233                 |
| MYB8      | GATGGAATCTCCAAGAGCAACA          | GGGTTACCTGCTTCAGG               | 174                 |
| Sh18S     | GGGTTACCTGCTTCAGG               | GGGTTACCTGCTTCAGG               | 130                 |

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and chemical properties of NAC3 and NAC4 in *S. baicalensis* were analyzed by ProtParam software [Table 3]. Using the CDD database, both NAC3 and NAC4 had NAC family unique NAM domains. WOLF PSORT was used to predict the protein subcellular localization of the above NAC transcription factors containing the full-length cDNA of *S. baicalensis*. Our results showed that both NAC3 and NAC4 were localized to the nucleus. SignalP 4.1 Server software analysis showed that the full-length cDNA of NAC3 and NAC4 was predicted to be nonsecreted proteins in *S. baicalensis*.

**Prediction of the secondary structures and phylogenetic analysis of NAC in Scutellaria baicalensis**

The secondary structure of NAC3 and NAC4 transcriptional factors was predicted by CFSSP software. The properties of their α-helices, β-sheet, β-turn, and random coils are showed in Figure 2. Both NACs in *S. baicalensis* had low properties of β-sheet.

Phylogenetic tree was constructed based on NAC sequences retrieved from *A. thaliana* and *S. baicalensis* using MEGA 5.0 neighbor-joining (NJ) method [Figure 3]. One hundred and five NAC transcriptional factors of *A. thaliana* have been divided into two groups (I and II). The phylogenetic analysis indicated that NAC1 and NAC2 of *S. baicalensis* and AT1G33060.1, AT4G35580.1, AT1G52890.1, and AT3G15500.1 of *A. thaliana* were divided into Group I, which might participate in resistance of abiotic stresses, such as high temperature and drought stress. In addition, NAC3, NAC4, NAC5, and NAC6 in *S. baicalensis* and AT3G56560.1 and AT1G03490.1 in *A. thaliana* belonged to the Group II, which may be transcriptional activators and take part in multiple transcriptional regulations in plants.

**Gene expression analysis**

To characterize the transcription pattern of *S. baicalensis* NAC and genes involved in flavonoid biosynthesis in response to GA₃, the leaves of *S. baicalensis* were treated with 100 μmol/L GA₃ for 0, 1, 2, and 3 h, respectively. NAC2, NAC3, NAC4, NAC5, NAC6, and PAL2 were upregulated after 1 h 100 μmol/L GA₃ treatment [Figure 4]. After simple correlation analysis and partial correlation analysis, NAC2, NAC3, NAC4, NAC5, NAC6, and PAL2 were significantly positively correlated with 100 μmol/L GA₃ treatment, respectively ($r = 0.9566$, $0.7024$, $0.8697$, $0.8196$, $0.7834$).

To compare gene expression levels of NAC and genes involved in flavonoid biosynthesis in different *S. baicalensis* parts, flowers, stems, leaves, and roots of *S. baicalensis* were collected and sampled [Figure 5]. Most above genes have highest expression levels in flower, higher than those in stems and leaves. After simple correlation analysis and partial correlation analysis, NAC2, NAC3, NAC4, NAC6, and PAL2 were significantly positively correlated in different plant organs, respectively ($r = 0.8084$, $0.9855$, $0.9778$, $0.7095$).

**Table 3: The characteristics of Scutellaria baicalensis NACs**

| Name | Length/bp | Full length of cDNA | Relative molecular mass/kDa | Amino acid number | Isoelectric point |
|------|-----------|----------------------|----------------------------|-----------------|------------------|
| NAC1 | 815       | No                   | -                          | -               | -                |
| NAC2 | 888       | No                   | -                          | -               | -                |
| NAC3 | 786       | Yes                  | 64,567                     | 137             | 5.16             |
| NAC4 | 1378      | Yes                  | 113,518                    | 119             | 5.02             |
| NAC5 | 887       | No                   | -                          | -               | -                |
| NAC6 | 710       | No                   | -                          | -               | -                |
NAC5 had no obvious gene expression characterization in various plant organs, and thus, it was not showed expression correlation with PAL2. In addition, NAC6 might be the key regulator in flavonoid biosynthesis of the root in S. baicalensis, for it mainly expressed in the flowers and roots which were also the main flavonoid accumulation parts of S. baicalensis.

**DISCUSSION**

NAC transcription factors are one of the largest families of transcription factors, which play an important regulatory role in response to plant abiotic stresses. In our previous studies, high temperature, low temperature, drought, and other abiotic stresses had an important impact on the accumulation of active ingredients in S. baicalensis. Based on the obtained cDNA library sequences of S. baicalensis, we studied the relationship between the function of NAC transcriptional factors and the accumulation of flavonoids by the bioinformatics analysis and gene expression analysis.

In this study, a total of six NAC genes of S. baicalensis were obtained, named NAC1-6, respectively. Phylogenetic tree analysis revealed that NAC1 and NAC2 of S. baicalensis and AT1G33060.1, AT4G35580.1, AT1G52890.1, and AT3G15500.1 of A. thaliana were clustered in the Group I, which could regulate the response in high temperature stress, lacking water, and other abiotic stresses. For the drought stress was very crucial for the content of flavonoids, it might be involved in the abiotic stresses, which could affect the accumulation of active components in S. baicalensis. In addition, NAC3, NAC4, NAC5, and NAC6 in S. baicalensis and AT3G56560.1 and AT1G03490.1 in A. thaliana belonged to the Group II, most of which were transcriptional activators and might take part in flavonoid biosynthesis regulation in plants.

Meanwhile, some NACs and PAL2 in S. baicalensis showed strong correlation. PAL was the key enzyme in the baicalin synthesis pathway and catalyzed the first-step reaction of phenylpropanoid metabolism, which also connected the primary metabolism and phenylpropanoid metabolism. Our previous study demonstrated that MYC2 transcription factor could affect the accumulation of flavonoids by regulating the transcription of PAL, and the regulation of MYB2 on flavonoid accumulation was affected by GA signaling. The expression levels of CHS, MYB2, and MYB8 showed the opposite expression trend with the one of NAC and PAL2 after GA treatment, which showed that CHS and MYB8 were negatively regulated by GA. In addition, CHS, PAL, and MYB were highly expressed in the roots and flowers of S. baicalensis, which were the most active flavonoid accumulation parts. This study suggested that NACs cloned in this study were mainly regulated the flavonoid biosynthesis in the flowers of S. baicalensis; NAC6 in S. baicalensis might be involved in the regulation of PAL2 transcription and affected the accumulation of flavonoids in the root of S. baicalensis, and GA should have a significant impact on this process. Our work laid a foundation for further understanding of the molecular mechanism of flavonoid biosynthesis regulated by transcriptional factors, which could provide a basis for improving the molecular regulation network of flavonoid active ingredients in S. baicalensis.

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**Conflicts of interest**

There are no conflicts of interest.

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