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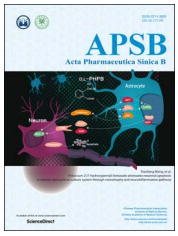

ORIGINAL ARTICLE

Genomic survey of bZIP transcription factor genes related to tanshinone biosynthesis in *Salvia miltiorrhiza*

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Abstract Tanshinones are a class of bioactive components in the traditional Chinese medicine *Salvia miltiorrhiza*, and their biosynthesis and regulation have been widely studied. Current studies show that basic leucine zipper (bZIP) proteins regulate plant secondary metabolism, growth and developmental processes. However, the bZIP transcription factors involved in tanshinone biosynthesis are unknown. Here, we conducted the first genome-wide survey of the bZIP gene family and analyzed the phylogeny, gene structure, additional conserved motifs and alternative splicing events in *S. miltiorrhiza.* A total of 70 SmbZIP transcription factors were identified and categorized into 11 subgroups based on their phylogenetic relationships with those in *Arabidopsis*. Moreover, seventeen *SmbZIP* genes underwent alternative splicing events. According to the transcriptomic data, the *SmbZIP* genes that were highly expressed in the Danshen root and periderm were selected. Based on the prediction of bZIP binding sites in the promoters and the co-expression analysis and co-induction patterns in response to Ag^{+} treatment *via* quantitative real-time polymerase chain reaction (qRT-PCR), we concluded that *SmbZIP7* and *SmbZIP20* potentially participate in the regulation of tanshinone biosynthesis. These results provide a foundation for further functional characterization of the candidate *SmbZIP* genes, which have the potential to increase tanshinone production.

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1. Introduction

Coronary heart disease (CHD) is one of the leading causes of death worldwide, and the number of CHD patients is projected to reach 82 million by 2020[1,2]. *Salvia miltiorrhiza* Bunge (Danshen) is one of the most important traditional Chinese medicines, and it has been widely applied to treat CHD[3]. Tanshinones represent the major bioactive constituents of *S. miltiorrhiza*, and they exhibit strong anti-atherosclerosis, antioxidant, anti-cancer and anti-inflammatory activities[4–7]. More than 40 lipophilic diterpenoids (*e.g.*, tanshinone I, tanshinone IIA, cryptotanshinone and dihydrotanshinone) have been isolated and identified from *S. miltiorrhiza*[8,9]. Tanshinone is synthesized and accumulates in the root periderm of *S. miltiorrhiza*[10]. Many genes encoding key enzymes in the tanshinone biosynthetic pathway have been cloned and analyzed, including *SmGGPPS*, *SmCPS*, *SmKSL*, *SmCYP76AH1*, *CYP76AH3* and *CYP76AK1*[11]. Biotic and abiotic elicitors have been reported to increase the accumulation of tanshinone in *S. miltiorrhiza* hairy roots[12–15]. In addition, a total of 15 *SmSPL*s[16], 110 *SmMYB*s[17], 61 *SmWRKY*s[18], 127 *SmbHLH*s[19], 170 *SmAP2/ERF*s[20] and 25 *SmARF*s[21] from *S. miltiorrhiza* have been identified and characterized. SmMYC2a and SmMYC2b are two SmbHLH transcription factors that have been reported to positively regulate genes in the tanshinone biosynthetic pathway in *S. miltiorrhiza*[22]. Although tanshinone biosynthesis has attracted widespread attention, studies on the basic leucine zipper (bZIP) transcription factors (TFs) involved in the regulation of tanshinone biosynthesis are limited.

The bZIP TF family is one of the largest and most conserved gene families among eukaryotic organisms. The bZIP TFs have a highly conserved bZIP domain composed of a basic region that binds DNA and a leucine zipper region that confers dimerization specificity[23]. The basic region preferentially binds to DNA sequences with an ACGT core, particularly G-box (CACGTG), C-box (GACGTC) and A-box sequences (TACGTA)[24–26]. The TGACG motif is also bound by bZIP TFs[27]. Importantly, bZIP TFs have been demonstrated to participate in the transcriptional regulation of secondary metabolism[28,29]. bZIP TFs have been identified extensively in the plant kingdom based on the availability of whole genome sequences[30–36]. The complete genomic sequence of *S. miltiorrhiza* provides the opportunity to identify the sequences and analyze the features of the bZIP gene family[37].

In this study, we performed the first investigation and analysis of the members of the bZIP TF family. Based on the transcriptome data as well as the results of quantitative real-time polymerase chain reaction (qRT-PCR) analysis and predictions of *cis*-regulatory elements in the promoters, 2 *SmbZIP* genes (*SmbZIP7* and *SmbZIP20*) related to the regulation of tanshinone biosynthesis were selected. Our results are crucial for analyzing the putative functions and regulatory mechanisms of *SmbZIP* genes in *S. miltiorrhiza*.

2. Materials and methods

2.1. Plant materials and Ag^+ treatment

S. miltiorrhiza Bunge (line 99–3) was cultivated in a field nursery at the Institute of Medicinal Plant Development in Beijing. The roots, stems, leaves and flowers were collected in May 2016, and certain root samples were divided into three parts (periderm, phloem and xylem). Three biological replicates of different organs and tissues were frozen for further use.

S. miltiorrhiza hairy roots were cultured in 250 mL beaker flasks with 100 mL 6, 7-V liquid medium on an orbital shaker. Induction was started 18 days after 0.2 g (fresh weight) of the hairy roots was inoculated in each beaker flask. The cultures were treated with Ag^+ at a final concentration of 30 μmol/L and harvested at 0, 0.5, 1.5, 3, 6, 12, and 24 h post-treatment. Each treatment was replicated three times. The methods for Ag^+ preparation were described in a previous study[38]. All samples were stored at –80 °C until further study.

2.2. Identification of SmbZIP genes

A hidden Markov model (HMM: PF00170) of the bZIP domain was used to search all putative *bZIP* genes from the *S. miltiorrhiza* genome database (SRA accession: SRP051524). The presence of a bZIP domain in the selected bZIP proteins was further verified using the online program SMART (http://smart.embl-heidelberg.de/). The sequences of conserved SmbZIP domains were obtained using an NCBI program (http://ncbi.nlm.nih.gov/). In addition, the theoretical isoelectric point (pI) and molecular weight (MW) of the SmbZIP proteins were calculated using the ExPASy program (http://web.expasy.org/).

2.3. Bioinformatic analysis

The AtbZIP protein sequences were downloaded from the Arabidopsis Information Resource (http://Arabidopsis.org/). Multiple alignments of amino acid sequences of the bZIP domain of SmbZIP and AtbZIP proteins were performed by ClustalX 1.83, and an unrooted neighbor-joining tree was generated using MEGA 5.0 with 1000 bootstrap replicates. Conserved motifs outside the bZIP domain were identified by the MEME program (http://meme-suite.org/tools/meme) with the following parameters: any number of repetitions, maximum number of motifs less than 50, an optimum width of 10–200 amino acids, and expected E-values less than 1×10^{-48}. The intron/exon structure of the cDNAs and corresponding genomic sequence of *S. miltiorrhiza* were displayed using the online Gene Structure Display Server analysis tool (http://gsds.cbi.pku.edu.cn/index.php). The alternative splicing isoforms were analyzed using IGV 2.3.34 software (http://www.broadinstitute.org/software/igv/). Additionally, cis-elements for bZIP TFs were searched by analyzing the 1500-bp promoter sequences of 72 genes encoding key enzymes involved in tanshinone biosynthesis using the PLACE database[19] (http://www.dna.affrc.go.jp/PLACE/signalscan.html).

2.4. Analysis of RNA-Seq expression data

A total of 17 sequence libraries were constructed for the expression analyses of the *SmbZIP* genes, including four organs (root, stem, leaf, and flower) and three root tissues (periderm, phloem, and xylem)[10]. RNA-Seq reads were obtained with the Illumina HiSeq. 2000 and 2500 platforms (SRA accessions: SRR1640458, SRP051564, and SRP028388). The RPKM (reads per kilobase per million) values were calculated based on the RNA-Seq reads[10]. For the organ-specific and tissue-specific gene expression profiles of the SmbZIP gene family, heat maps were generated with the R statistical package.

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