Cloning and Bioinformatics Analysis of SmTIR1 Gene of Salvia miltiorrhiza

Wang Rui 1, Wu Jiwen 1,2, Chang Huixuan 1, Chen Guoliang 1,2, Bai Zhenqing 1,2
1 College of Life Science, Yanan University, Yan'an, 716000, P.R. China
2 Shaanxi Key Laboratory of Chinese Jujube (Yanan University), Yan'an, 71600, P.R. China

Corresponding author email: shanxibzq@163.com
Medicinal Plant Research, 2020, Vol.10, No:01-08 doi: 10.5376/mpr.2020.10.0005
Received: 04 Jul., 2020
Accepted: 19 Jul., 2020
Published: 19 Jul., 2020

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Preferred citation for this article:
Wang R., Wu J.W., Chang H.X., Chen G.L., and Bai Z.Q., 2020, Cloning and Bioinformatics Analysis of SmTIR1 Gene of Salvia miltiorrhiza Medicinal Plant Research, 10(5): 01-08 (doi: 10.5376/mpr.2020.10.0005)

Abstract Auxin receptor plays a pivotal role in plant growth as regulated by auxin in plants. The growth of Salvia miltiorrhiza directly correlates with its quality. Nevertheless, to date gene of auxin receptor in S. miltiorrhiza is still unclear. In the present study, a gene with 1887 bp cDNA sequences was cloned in S. miltiorrhiza, and also performed bioinformatic analysis. This gene deploys the typical structure of auxin receptor TIR1, thus being named SmTIR1 here. By predicating its functions, SmTIR1 is a hydrophilic protein with the typical structure of TIR1. Those results lay a foundation for further study of auxin receptor in S. miltiorrhiza, and provides theories for plant growth and development of S. miltiorrhiza as well.

Keywords Salvia miltiorrhiza, SmTIR1 gene, Cloning, Bioinformatics

Salvia miltiorrhiza is a Chinese herbal medicine of Salvia Linn. Its medical functions involve in promoting blood circulation and removing blood stasis, relieving pain through channels, clearing heart and removing troubles, cooling blood and eliminating carbuncle. It has become one of the most important medicinal plants because of its significant therapeutic effect in modern medicine clinical treatment of coronary heart disease, cardiovascular and cerebrovascular diseases (MA Bingxiang and Dong chongkai, 2014, Chinese pharmacy, 25 (7): 663-665; Wanhxinhuang et al., 2020). lipophilic tanshinone and hydrophilic phenolic acid, which have the pharmacological effects of anti-tumor, prevention and treatment of cardiovascular disease, anti-inflammatory, anti-oxidation, radiosensitization and liver protection, are the main effective components of S. miltiorrhiza, (Li et al., 2009; Dong Fengcai, 2015; Wan Xinhuan, etc., 2020). The accumulation of active components of S. miltiorrhiza is closely related to its growth and development. In the process of plant growth and development, it will be affected by many factors, the external factors are mainly environmental factors, and the internal factors are mainly self-secreted hormones that affect the synthesis and accumulation of metabolites (Li Qian, 2006, Northwest University of agriculture and forestry science and technology, pp.28-39; Bai Fangkun, 2011, Henan Normal University, pp.25-31; Sheng Dongfeng and Chen long, 2013; Liu jingling, 2014, northwest agricultural and Forestry University of science and technology, pp.41-49; Ding et al., 2017; Xing et al., 2018; Zhang et al., 2019).

Plant hormones can affect the accumulation of plant metabolites. It has been found that abscisic acid, gibberellin, ethylene and other hormones and their interactions can effectively improve the accumulation of phenolic acids in hairy roots of S. miltiorrhiza (Liang et al., 2013). Among many plant hormones, auxin is an indispensable hormone in the process of plant growth, organ development (Ruegger et al., 1998), cell differentiation, etc., and the formation, synthesis, action and signal transduction of auxin have been hot discussed by researchers (Woodward and Bartel, 2005). Auxin mainly interacts with the corresponding receptors, thus mediating the downstream signaling pathway to cause the corresponding growth effect (Kepinski and Leyser, 2004; Winkler et al., 2017). At present, there are three known auxin receptors:ABP, SKP2A and TIR1/AFBs, which are combined with auxin to initiate the degradation of SCFTIR1/AFBs-Aux/IAA ubiquitin proteasome (Salehin et al., 2015).
TIR1 receptor was first found in a Arabidopsis mutant, and its gene was isolated from it. Its product was transport inhibitor protein 1 (TIR1) (Kepinski and Leyser, 2005). TIR1 contains a characteristic domain F-box, which is a part of SCF complex. It can directly bind with Aux/IAA protein and mediate its degradation (Dharmasiri et al., 2005; Shu et al., 2015; Yu et al., 2015; Yamada et al., 2018). The protein encoded by Aux/IAA can pass through auxin response factors, ARFs) specifically bind to form SCF_{TIR1/AFB} Aux/IAA complex, and then the Aux/IAA protein on the complex is ubiquitinated, which mediates the degradation of proteasome, and at the same time activates ARFs to make auxin induced gene expression, leading to auxin response (Gray et al., 2001; Parry et al., 2009; Salehin et al., 2015). As an important component of response to auxin reaction, there is no report about TIR1 gene in S. miltiorrhiza. Therefore, in this study, TIR1 gene of sesame indicum, a relative species of S. miltiorrhiza, was selected to compare with that of S. miltiorrhiza transcriptome measured in our laboratory by using bioedit software. The first ten sequences of similarity scores were screened, and then Seqman software was used for splicing to get a 2,563 bp sequence. Then ORF prediction was carried out, primers were designed for cloning, and finally a 1887 bp sequence was obtained, which was used for later TIR1 of S. miltiorrhiza. The bioinformatics analysis of SmTIR1 (named in this laboratory) will lay a foundation for the research of the related functions of the auxin receptor of S. miltiorrhiza and the breeding of its excellent germplasm resources.

1 Results and Analysis

1.1 Gene cloning and sequence analysis

SmTIR1 has a total length of 1887 bp (Figure 1, Figure 2a), encoding 590 amino acids (Figure 1). The results of blastp showed that the similarity respectively are 92.54% between SmTIR1 and Salvia splendens (StsTIR1, tey30884.1), 87.56% between SmTIR1 and Sesame indicum (SsTIR1, XP 011096196.1), 86.54% between SmTIR1 and Erythrantha gutta (EsTIR1, XP 012849055.1), and 74.07% between SmTIR1 and Daucus Carota subsp. Sativus (DsTIR1, The similarity of XP 017229520.1), 74.79% with Nicotiana tomentosiformis (NsTIR1, XP 00963009.1), 74.03% with Nicotiana sylvestris (NsTIR1, XP 009763253.1), 76.64% with Camellia sinensis (CsTIR1, XP 028113968.1).

Based on the conserved domains analysis of the SmTIR1, the results show that SmTIR1 contain the AMN1 superfamily domain at 135-260 and 316-511 respectively, and it is an antagonist of the MEN pathway (mitotic exit network) (Figure 2C). When the MEN pathway is activated, AMN1 binds with TEM1 (a GTPase) to suppress the MEN pathway and regulate the cell cycle. AMN1 is a leucine rich repeat (LRR) protein. The leucine rich repeat region collects targets by interacting with TIR1, which mainly affects mitosis. In addition, there is an F-box-5 between 26 and 66 bp. It is transp _inhibit domain in 86-131bp. Using softberry to predict the CDs sequence of TIR1 gene, it was found that its CDs region was from 65 bp to 1837 bp (Figure 2B), and its upstream region with 2000bp predicted its upstream regulatory elements by PlantCARE. The results showed

Figure 1 SmTIR1 gene sequence of S. miltiorrhiza
that in addition to the common cis acting elements CAAT-box and core promoter elements in the promoter and enhancer regions in TATA-box, there were also cis-regulatory elements related to meristem expression. In addition, there were gibberellin response elements P-box and GAT-box motif involved in ABSE reaction cis acting elements and some important components (Table 1).

1.2 Structure and property analysis of SmTIR1
SmTIR1 has 590 amino acid residues. Its molecular weight and isoelectric point are 66.609 63 kD and 6.08 respectively. The instability coefficient is 45.93, the aliphatic index is 87.73, and the average hydrophilic coefficient (gravy) is -0.172, so SmTIR1 might be hydrophilic, the arginine (Arg) score at 372 is the lowest (-3.200), and the hydrophilicity is the strongest; the glycine (Gly) score at 382 is the highest (1.778), and the

Figure 2 Prediction of CDS region and acquisition of gene SmTIR1
Note: A: Result of amplification; B: Prediction of CDS region of SmTIR1; C: Protein structure domain analysis

Table 1 The prediction of upstream promoter

| Upstream promoter type | Sequence   | Location | Chain (+/-) | Function                                                                 |
|------------------------|------------|----------|-------------|-------------------------------------------------------------------------|
| MBS                    | CAACTG     | 248      | -           | MYB binding site involved in drought-inducibility                       |
| MBS                    | CAACTG     | 1 213    | -           | MYB binding site involved in drought-inducibility                       |
| MRE                    | AACCTAA    | 1 448    | -           | MYB binding site involved in light responsiveness                       |
| GARE-motif             | TCTGTTG    | 80       | +           | Gibberellin-responsive element                                           |
| P-box                  | CCTTTTG    | 228      | -           | Gibberellin-responsive element                                           |
| ABRE                   | CACGTG     | 1 356    | -           | Cis-acting element involved in the abscisic acid responsiveness         |
| ABRE                   | ACGTG      | 1 357    | +           | Cis-acting element involved in the abscisic acid responsiveness         |
| ARE                    | AAACCA     | 1 374    | -           | Cis-acting regulatory element essential for the anaerobic induction     |
| GC-motif               | CCCCCG     | 690      | -           | Enhancer-like element involved in anoxic specific inducibility         |
| CAT-box                | GCCACT     | 979      | -           | Cis-acting regulatory element related to meristem expression           |
| CAT-box                | GCCACT     | 1 054    | +           | Cis-acting regulatory element related to meristem expression           |
| CAT-box                | GCCACT     | 1 801    | +           | Cis-acting regulatory element related to meristem expression           |

Note: Only the main
hydrophobicity is the strongest. Using SOPMA to predict the secondary structure of amino acid sequence of SmTIR1, SmTIR1 contains 47.97% α helix, 35.76% random curl, 12.37% extension chain and 3.90% β corner (Figure 3C). Using NetPhos 3.1 Server to analyze phosphorylation sites, 35 serine sites, 14 threonine sites and 4 tyrosine sites were found (Figure 3B). Using SWISS-MODEL to search and construct the template, we predicted the three-level structure of the amino acid sequence of SmTIR1 (Figure 3A). The three-level structure of SmTIR1 mainly consists of F-box (dark blue area in the middle and lower part of Figure 3A) and LRR, and the similarity with TIR1 model of Arabidopsis (PDB: 3c6o. 1. B) is 54.64%. According to the analysis of SingalP 3.0 Server (Figure 3D) and THHMM 2.0 (Figure 3E), there is no signal peptide in TIR1 sequence, s value (s value of signal peptide region is higher): 0.202; C value (shear site, and one amino acid corresponds to one C value): 0.155; y value (comprehensive analysis of s value and C value to determine the value of shear site): 0.046, which is presumed to be non-secretory protein (D value: 0.027), and there is no transmembrane domain. The subcellular localization results of TargetP 1.1 and Plant-mPLoc (Table 2) show that the gene is predicted to be located in the nucleus.

1.3 Construction of system evolution tree of SmTIR1

Using the NJ method (bootstrap tests value is set to 1000) to predict and analyze the evolutionary relationship of SmTIR1 obtained by MEGA X (Figure 4). The results of the evolutionary tree showed that SmTIR1 is grouped with TIR1 of a string of Salvia splendens Ker-Gawler, Sesamum indicum and Erythranthe guttata and the genetic relationship is the most similar with Salvia splendens Ker-Gawler. It is suggested that SmTIR1 should have similar function with these TIR1 genes. The gene structure determines its functions, so there should be higher similarity in structure.

![Figure 3](http://hortherbpublisher.com/index.php/mpr)

**Figure 3 Structure and properties of SmTIR1 coding protein**

Note: A: 3D-structure of SmTIR1 coding protein; B: Prediction of protein phosphorylation sites; C: Secondary structure of SmTIR1 coding protein; Blue: Alpha helix; Green: Beta turn; Yellow: Random coil; Red: Extended strand; D: The prediction of signal peptide (Because the sequence is too long, the lower amino acid sequence is shown as black); E: The prediction of transmembrane region

**Table 2 The prediction of subcellular localization**

| SmTIR1 | 590 | 0.317 | 0.031 | 0.070 | 0.908 | unknown | 3 | SmTIR1 | Nucleus |
|--------|-----|------|------|------|------|--------|---|--------|--------|
| Name   | Length | cTP  | mTP  | SP   | Other | Location | RC | Query Protein | Predicted location (s) |
|        |       |      |      |      |       |          |    |             |                     |
2 Discussion

The medicinal value and yield of medicinal plants are affected by auxin regulation, auxin regulates the expression of corresponding genes through SCFTIR1/AFBs Aux/IAA-ARF signal pathway, and then regulates various growth processes. Therefore, it is necessary to analyze SmTIR1 to study the growth and development, increase the accumulation of effective components and the yield improvement of S. miltiorrhiza. Previous studies have found that the ORF of MaTIR1 is 1758 bp, encoding 585 amino acids, 65.4387 KD of protein molecular weight, and 6.31 of isoelectric point in Morus alba L. (Tang Zhuang et al., 2014). The ORF of CsTIR1 is 1746 bp, encoding 581 amino acids, 65.18 KD of molecular weight, and 5.64 of theoretical isoelectric point (PI) in Camellia sinensis (L.) O. Ktze. (Cao Hongli et al., 2015). The ORF of DI1TIR1-1 is 1755 bp, encoding 584 amino acids, which are hydrophilic proteins, theoretical isoelectric point is 6.27, the ORF of DI1TIR1-2 is 1926 bp, encoding 641 amino acids, which are hydrophilic proteins, which are hydrophilic proteins with isoelectric point of 5.24 in Dimocarpus longan Lour. (Lai Ruilian et al., 2016, Journal of Tropical Biology, 37 (1): 136-143); the ORF of HcTIR1 is 1761 bp, encoding 586 amino acids in Hibiscus cannabinus (Chen Lihong et al., 2017). The physical and chemical properties of TIR1 in the above species are similar to those of SmTIR1. The properties of these proteins can affect folding, and the formation of protein high-level structure that it is also related to the properties of proteins, which may be closely related to the function and mode of action of TIR1.

Recently, scholars have found that the MaTIR1 may play an important role in the formation of adventitious roots in M. alba L. (Tang Zhuang et al., 2014); the expression of CsTIR1 is closely related to dormancy in C. sinensis (L.) O. Ktze (Cao dividend et al, 2015); DI1TIR1-1 and DI1TIR1-2 may participate in the root differentiation and flowering process of D. longan Lour (Lai Ruilian et al., 2016, Journal of tropical crops, 37 (1): 136-143); SITIR1A affects the fruit setting process, while SITIR1B affects the nutritional growth and fruit formation in Solanum lycopersicum (Lin Dongbo, 2016, Chongqing University), The HcTIR1 gene expression may be related to male sterility in H. cannabinus (Chen Lihong et al., 2017). The SmTIR1 is similar to TIR1 gene in the above species in structure, especially with S. splendens Ker-Gawler, which has a typical highly conserved F-box domain and leucine repeat domain (LRR), and the structure and physical and chemical properties of the protein encoded by TIR1 gene are also similar. Thus, the SmTIR1 might be a potential auxin receptor which can regulate the root development and growth of S. miltiorrhiza.

S. miltiorrhiza is a traditional medicinal medicine for the treatment of cardiovascular and cerebrovascular diseases in modern clinical medicine. Due to the limited planting land area, the limited output is difficult to meet the market demand, and the growth and development process affects the quality of S. miltiorrhiza (Zhou Lili et al., 2012). In this study, SmTIR1, a potential auxin receptor in S. miltiorrhiza, was obtained, and its
structure and function were predicted. The characteristics of *SmTIR1* were preliminarily understood and predicted, which laid a foundation for exploring the function of auxin receptor in the growth and development of *S. miltiorrhiza*, and provided a new idea for the research of excellent germplasm resources selection of *S. miltiorrhiza*. In the future, plant gene editing and other technologies will be used to further confirm the function of *SmTIR1*, so as to lay the foundation for variety improvement of *S. miltiorrhiza* through genetic engineering strategy, and then achieve the goal of improving yield and quality to meet the market demand for *S. miltiorrhiza*.

3 Materials and Methods

3.1 Materials

*S. miltiorrhiza* leaves (seeds purchased from Hebei An guo Pharmaceutical Group Co., Ltd.); Tiangen polysaccharide polyphenol plant RNA Extraction Kit (purchased from Tiangen Biological Reagent Co., Ltd., cat.#DP441, Beijing, China); nanodrop one micro spectrophotometer; Takara reverse transcription Kit (Takara, Code No. RR047A, Beijing, China).

3.2 RNA extraction and cDNA synthesis

Take 100 g of *S. miltiorrhiza* leaves and put them into a mortar, grind them with liquid nitrogen, and then extract the total RNA of *Salvia miltiorrhiza* with RNA Extraction Kit (polysaccharide polyphenol) (Tiangen, cat.# DP441, Beijing, China). The RNA concentration was measured by nanodrop. The RNA was stored in -80 °C refrigerator for downstream cDNA synthesis. The total RNA of *Salvia miltiorrhiza* was reverse transcribed by Takara (Code No. RR047A, Beijing, China), and the cDNA of *Salvia miltiorrhiza* was obtained.

3.3 Cloning of *SmTIR1* gene

According to the obtained *SmTIR1* gene fragment, PCR specific primers were designed and polyacrylamide gel electrophoresis (Figure 2A), *SmTIR1*-F: CTTGGTAGGCCTAAATGAATCCATCC, *SmTIR1*-R: GGGTTTC CCTTCCTGTCAAAGT were used. The amplification conditions were: 95 °C for 5 min, (95 °C for 45 s, 58 °C for 45 s, 72 °C for 2 min), 35 cycles, 72 °C for 10 min, 4 °C.

3.4 *SmTIR1* bioinformatics analysis

Putting the full length sequence of cDNA in ORF finder of NCBI database (http://www.bioinformatics.org/sms2/orf_find.html). The ORF of the sequence is analyzed in the softberry website (http://linux1.softberry.com/berry.phtml) The HMM based gene structure prediction module in NCBI is used to predict the gene structure of the target gene, verify and obtain the corresponding amino acid sequence, and then use blast in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Search for similar proteins, then use ClusterW (https://www.genome.jp/tools-bin/clusterw) The amino acid sequences were compared and the phylogenetic tree was constructed by MEGA X. The software bioedit was used to compare the genome of *S. miltiorrhiza*, search and obtain the gene sequence of 2 000 bp upstream of *SmTIR1*, and then use PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) forecast the upstream control elements. Then, the amino acid sequence encoded by *SmTIR1* was analyzed by CDD online analysis website, and the conserved domain was verified by Pfam and MEME online analysis website (https://web.expasy.org/compute_Pi) was used to predict the physical and chemical properties of protein. Then TMHMM 2.0 and SignalP 3.0 server were used to predict and analyze transmembrane domain and signal peptidie respectively. WOLFPSORT is used for subcellular location prediction analysis (https://wolfsort.hgc.jp/) and TargetP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP), Plant-mPLoc and other online analysis websites. On line prediction and analysis of secondary structure is carried out by SOPMA website,. The disulfide bond and phosphorylation sites were predicted respectively with NetPhos 3.1 Server and Disulfindis (http://disulfind.dsi.unifi.it/). The three-level structure model of the protein was searched and constructed by using the online website Swiss-Model, and the protein properties and hydrophobicity were predicted and analyzed by ProtParam and ProtScalein of the ExPASY website.
Author's contribution

Rui Wang and Huixuan Chang are the experimental designers and executors of this study, who complete the data analysis and the writing of the first draft of the thesis; Jiwen Wu and Guoliang Chen participate in the experimental design and the analysis of the experimental results; Zhenqing Bai is the conceiver and director of the project, who guides the experimental design, data analysis, and the writing and modification of the thesis. All the authors read and agreed to the final text.

Acknowledgements

This research is co-sponsored by the research project of Yan'an University (2003/205040217), the research project of Yan'an University (2003/205110027) and the research project of Yan'an University (YCX201928).

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