Gene 533 (2014) 332–345

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Identification of 30 MYB transcription factor genes and analysis of their expression during abiotic stress in peanut (*Arachis hypogaea* L.)

Na Chen [a], Qingli Yang [a], Lijuan Pan [a], Xiaoyuan Chi [a], Mingna Chen [a], Dongqing Hu [b], Zhen Yang [a], Tong Wang [a], Mian Wang [a], Shanlin Yu [a,*]

[a] Shandong Peanut Research Institute, Qingdao 266100, China
[b] Qingdao Entry-Exit Inspection and Quarantine Bureau, Qingdao 266001, China

ARTICLE INFO

Article history:
Accepted 28 August 2013
Available online 5 September 2013

Keywords:
MYB
Phylogenetic analysis
Abiotic stress
ABA

ABSTRACT

The MYB superfamily constitutes one of the most abundant groups of transcription factors and plays central roles in developmental processes and defense responses in plants. In the work described in this article, 30 unique peanut MYB genes that contained full-length cDNA sequences were isolated. The 30 genes were grouped into three categories: one R1R2R3-MYB, nine R2R3-MYBs and 20 MYB-related members. The sequence composition of the R2 and R3 repeats was conserved among the nine peanut R2R3-MYB proteins. Phylogenetic comparison of the members of this superfamily between peanut and *Arabidopsis* revealed that the putative functions of some peanut MYB proteins were clustered into the *Arabidopsis* functional groups. Expression analysis during abiotic stress identified a group of MYB genes that responded to at least one stress treatment. This is the first comprehensive study of the MYB gene family in peanut.

1. Introduction

Plant growth and yield are strongly influenced by abiotic stresses such as drought, salt and cold. Plants respond and adapt to these conditions with an array of biochemical and physiological changes (Hsieh et al., 2004; Zhu et al., 2007). Many adaptation processes are regulated by stress-responsive gene expression. Transcription factors (TFs) regulate gene expression, which provides plants with a complicated control mechanism for responding to abiotic and biotic stresses and modulating developmental processes (Mitsuda and Ohme-Takagi, 2009). A previous study has uncovered a group of TF genes – such as DREB, MYB and bZIP – which play important roles in plant molecular stress regulation (Ahuja et al., 2010).

MYB TFs are widely distributed in eukaryotic organisms, and constitute one of the largest TF families in the plant kingdom (Du et al., 2012). The distinguishing property of MYB proteins is a highly conserved MYB domain consisting of 1–4 imperfect tandem repeats (MYB repeat) at the N-terminus. The MYB repeat is 50–53 amino acids in length and contains three regularly distributed tryptophan (or phenylalanine) residues. Each MYB repeat encodes three α-helices, with the second and third helices forming a helix-turn-helix structure, which recognizes and binds to the DNA major groove at the specific recognition site C/TAACG/TG (Lipsick, 1996; Stracke et al., 2001). The MYB family is divided into different types according to the number of MYB repeat(s): 4R-MYB has four repeats, 3R-MYB (R1R2R3-MYB) has three consecutive repeats, R2R3-MYB has two repeats and the MYB-related type usually, but not always, has a single MYB repeat (Dubos et al., 2010; Jin and Martin, 1999; Rosinski and Atchley, 1998).

The first plant MYB gene *C1* was isolated in maize (Paz-Ares et al., 1987). Since then different aspects of the MYB gene family, including gene number, sequence characterization, evolution and functions, have been widely studied in plants (Chen et al., 2006; Du et al., 2012, 2013; Dubos et al., 2010; Matus et al., 2008; Wilkins et al., 2009; Zhang et al., 2011). So far, the MYB proteins have been shown to be involved in many significant physiological and biochemical processes, including regulation of primary and secondary metabolism, control of cell development and the cell cycle, participation in defense and response to various biotic and abiotic stresses, flavonoid biosynthesis, hormone synthesis and signal transduction (Czemmel et al., 2012; Du et al., 2009; Dubos et al., 2010; Feller et al., 2011; Ma et al., 2009; Stracke et al., 2001).

Extensive studies in various plant species have provided a better understanding of the MYB gene family; however, little is known about this family in peanut (*Arachis hypogaea* L.). Until now, only one MYB family gene, whose expression was induced by cold stress, has been reported in peanut (Tang et al., 2011). The cultivated peanut is an important oil crop and its production is severely affected by adverse environmental stresses. Unfortunately, little is known about the network of gene expression regulation related to abiotic stress in peanut, except for several genes shown to be stress regulated (Chen et al., 2012; Dave and Mitra, 1998, 2000; Jain et al., 2006; Rudrabhatla and Rajasekharan, 2002).

Abbreviations: TFs, transcription factors; ABA, abscisic acid; ESTs, Expressed Sequence Tags; ORFs, open reading frames; NJ tree, Neighbor-joining tree; AP2, APETELA2; LCL1, LHY/CCA1-like 1.

* Corresponding author. Tel.: +86 532 87626830.
E-mail addresses: shanlinyu2012@163.com, yshanlin1956@163.com (S. Yu).

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http://dx.doi.org/10.1016/j.gene.2013.08.092

Identifying stress-responsive genes will elucidate the molecular mechanisms of peanut stress response and tolerance, and offer a number of candidate genes as potential markers of tolerance to environmental stresses.

Considering the multiple functions of MYB family proteins, especially their important roles in response to abiotic stresses in plants, research was conducted concerning the evolution and expression properties of the MYB gene family in peanut. In this study, 30 full-length cDNA sequences encoding peanut MYB proteins were isolated. A phylogenetic tree combining peanut and *Arabidopsis* MYB proteins was constructed to examine their evolutionary relationships and the putative functions of peanut MYB proteins based on *Arabidopsis* MYB proteins with known functions. The expression of the MYB family genes' response to abiotic stress and abscisic acid (ABA) was analyzed to identify potential genes that participated in the stress signal transduction pathway in peanut. This is the first comprehensive study of the MYB gene family in peanut and provides valuable information for further exploration of the functions of this significant gene family in this plant.

2. Materials and methods

2.1. Plant materials

Peanut seeds (*Arachis hypogaea* L. cultivar Huayu19) were germinated in a mixture of nutrimental soil and vermiculite (2:1) and grown under conditions of 16 h light/8 h dark (28 °C/22 °C). Seedlings at the trefoil leaf stage were used in subsequent experiments.

The stress treatment processes were performed according to the study reported by Chi et al. (2012). For the cold treatment, seedlings in soil were maintained at 4 °C in a light incubator. For the NaCl, PEG6000 and ABA treatments, the roots of seedlings grown in soil were pulled out carefully to avoid injury, flushed carefully to remove all soil and then dipped into 200 mM NaCl, 20% PEG6000 or 100 μM ABA solution. The leaves and roots for all kinds of treatments were collected at 0 h, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h. All the samples were immediately frozen in liquid nitrogen and then stored at −80 °C until required.

2.2. Identification of peanut MYB genes in the peanut cDNA library using BioEdit software

The cDNA sequences used in this study came from three cDNA libraries from three laboratories (data not shown). All ESTs (Expressed Sequence Tags) of the 36,741 cDNA sequences were saved in FASTA format. The amino acid sequences of the MYB domain of *Arabidopsis* were used to search for homologous genes from the peanut cDNA library. Before searching for the MYB family genes, a local nucleotide database file was created using BioEdit software. A local BLAST procedure was then run to find the homologous genes of the MYB family. We found 74 ESTs that may encode MYB proteins via this method. To remove redundancy, the sequences were assembled using the 'Alignment' function of the DNAman software and adjusted manually. Sequences that shared >95% matches were considered redundant. The ORFs (open reading frames) and putative amino acids of all nonredundant sequences were analyzed using DNAman software and 30 members were shown to contain a complete ORF. Finally, to confirm that the obtained sequences were MYB members, all of the amino acid sequences of the primary that identified 30 MYB members were submitted to the website http://pfam.sanger.ac.uk to predict the MYB domains. Only sequences that contained the conserved MYB domain were confirmed to be MYB members (Coggill et al., 2008).

2.3. Amino acids conservative analysis of the MYB repeats of R2R3-MYB proteins

To analyze the features of the MYB domain of peanut R2R3-MYB proteins, the sequences of R2 and R3 MYB repeats corresponding to nine R2R3-MYB proteins were aligned respectively with the ClustalW method using BioEdit software, and adjusted manually. The sequence logos for R2 and R3 MYB repeats were obtained by submitting the multiple alignment sequences to the website http://weblogo.berkeley.edu/logo.cgi (Crooks et al., 2004).

2.4. Phylogenetic analysis

The amino acid sequences of *Arabidopsis* (five R1R2R3-MYB, 126 R2R3-MYB and 80 MYB-related members) MYB proteins were downloaded from the Plant TFDB website (http://planttfdb.cbi.edu.cn/) (He et al., 2010). The complete amino acid sequences of MYB proteins were used to construct phylogenetic trees. Sequence alignments were performed with ClustalW using MEGA4.0 software. Neighbor-joining (NJ) trees for 3R-MYB and R2R3-MYB subfamilies and the MYB-related subfamily combining peanut and *Arabidopsis* MYB members were constructed individually using the MEGA4.0 program (Tamura et al., 2007), and internal branch support was estimated with 1000 bootstrap replicates.

2.5. RNA isolation and cDNA synthesis

Total RNA was isolated and purified from samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Before cDNA synthesis, RNA was treated with RQ1 RNase-free DNaseI (Qiagen, Hilden, Germany) to avoid DNA contamination as recommended by the vendor. Only RNA preparations having an A260/A280 ratio of 1.8–2.0 and an A260/A230 ratio >2.0 were used for subsequent analysis. RNA integrity was verified by 2% agarose gel electrophoresis.

The cDNA was synthesized by the use of M-MLV Reverse Transcriptase (Promega, Madison, WI) in a 25-μL reaction system containing 2 μg total RNA. Reverse transcription reactions were carried out at 42 °C for 60 min followed by chilling on ice for 5 min.

2.6. Primer design and quantitative real-time RT-PCR

ACT11 was used as a reference gene to normalize all data (Chi et al., 2012). Primers (Online Resource 1) for *ACT11* and *AhMYB1–30* were designed according to the nucleotide sequences of *ACT11* and *AhMYB1–30* using Beacon Designer V 7.0 (Premier Biosoft International, Palo Alto, CA, USA) with melting temperatures of 58–60 °C, primer lengths of 20–25 bp and amplicon lengths of 60–200 bp.

For real-time PCR, the cDNA samples were diluted to 8 ng μL^{-1}. The qPCR analysis was performed using a LightCycler 2.0 instrument system (Roche, Germany), based on SYBR Premix Ex Taq polymerase (TaKaRa, Toyoto, Japan). Each 20 μL reaction comprised 2 μL cDNA template, 10 μL 2× SYBR Premix and 0.4 μL (200 nM) of each primer. The reactions were subjected to an initial denaturation step of 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 10 s. A melting curve analysis was performed at the end of the PCR run over the range 60–95 °C, increasing the temperature stepwise by 0.5 °C every 10 s. Baseline and quantification cycles (Cq) were automatically determined using the LightCycler Software. Zero template controls were included for each primer pair, and each PCR reaction was carried out in triplicate. The raw Cq values obtained from LightCycler 2.0 were converted into relative quantities via the delta-Cq method.

3. Results

3.1. Isolation of 30 genes encoding MYB family TFs in peanut

74 ESTs that probably encode MYB family proteins were identified from the peanut cDNA library using BioEdit software, of which 44 were removed, as they were either did not contain complete ORFs or