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Research paper

Genome-wide identification, classification and analysis of HD-ZIP gene family in citrus, and its potential roles in somatic embryogenesis regulation

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ABSTRACT

The homeodomain–leucine zipper (HD-Zip) transcription factors, which belong to a class of Homeobox proteins, has been reported to be involved in different biological processes of plants, including growth and development, photomorphogenesis, flowering, fruit ripening and adaptation responses to environmental stresses. In this study, 27 HD-Zip genes (*CsHBs*) were identified in *Citrus*. Based on the phylogenetic analysis and characteristics of individual gene or protein, the HD-Zip gene family in *Citrus* can be classified into 4 subfamilies, i.e. HD-Zip I, HD-Zip II, HD-Zip III, and HD-Zip IV containing 16, 2, 4, and 5 members respectively. The digital expression patterns of 27 HD-Zip genes were analyzed in the callus, flower, leaf and fruit of *Citrus sinensis*. The qRT-PCR and RT-PCR analyses of six selected HD-Zip genes were performed in six citrus cultivars with different embryogenic competence and in the embryo induction stages, which revealed that these genes were differentially expressed and might be involved in citrus somatic embryogenesis (SE). The results exhibited that the expression of *CsHB1* was up-regulated in somatic embryo induction process, and its expression was higher in citrus cultivars with high embryogenic capacity than in cultivars recalcitrant to form somatic embryos. Moreover, a microsatellite site of three nucleotide repeats was found in *CsHB1* gene among eighteen citrus genotypes, indicating the possible association of *CsHB1* gene to the capacity of callus induction.

1. Introduction

Transcription factors, as regulators helping to control response networks, are important for regulating developmental events in higher plants. Homeodomain–leucine zipper (HD-Zip) transcription factors have been reported to play an important role in regulating plant growth and development (Ariel et al., 2007). The HD-Zip proteins containing homeodomain (HD) and leucine zipper (LZ) domains are unique to plants and can be divided into four subfamilies (HD-Zip I, HD-Zip II, HD-Zip III and HD-Zip IV) based on sequence conservation, structural features and functions (Ariel et al., 2007; Elhiti and Stasolla, 2009; Harris et al., 2011). HD-Zip I proteins are generally involved in responses related to abiotic stresses such as water and light stress (Wang et al., 2003), abscisic acid (Olsson et al., 2004; Lechner et al., 2011; Valdés et al., 2012), and de-etiolation (Henriksson et al., 2005). HD-Zip II proteins participate in auxin signaling (Sorin et al., 2009), light response (Ruberti et al., 2012) and leaf polarity (Bou-Torrent et al., 2012). Members of the third group (HD-Zip III) act as master regulators of apical formation during embryo development (Prigge et al., 2005; Izhaki and Bowman, 2007; Smith and Long, 2010). HD-Zip IV proteins play significant roles in differentiation of epidermal cells (Nakamura et al., 2006), trichome formation (Vernoud et al., 2009) and anthocyanin accumulation (Kubo and Hayashi, 2011).

Somatic embryogenesis (SE) is believed to share the similar molecular mechanism with the zygotic embryo development, and is regarded as a useful system to study the molecular and biochemical events during the early stage of embryogenesis in higher plants (Yang and Zhang, 2010). Large numbers of genes involved in this process have been identified and their association with embryogenesis competence was discussed (Braybrook and Harada, 2008; Elhiti et al., 2013; Fujimura, 2014; Smertenko and Bozhkov, 2014). Recently, HD-Zip proteins have been characterized in different plants and found to be important for modifying zygotic embryo development. For example, it was reported that the activity of members of the HD-Zip III protein family was required for proper patterning of the apical region of the globular embryo (Emery et al., 2003; Prigge et al., 2005). In a recent study, Turchi et al. (2013) confirmed the regulatory roles of HD-Zip II protein family in embryonic apical patterning, and found that shoot apical meristem

Abbreviations: HD-Zip, homeodomain–leucine zipper; HD, homeodomain; LZ, leucine zipper; SE, somatic embryogenesis; NEC, non-embryogenic callus; IEC2, induced-calluses for 2 weeks; IEC4, induced-calluses for 4 weeks; EC, embryogenic calluses.

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(SAM) function at least in part was controlled through interaction with HD-Zip III proteins.

The HD-Zip gene family have been identified and characterized in *Arabidopsis* (Ariel et al., 2007), rice (Agalou et al., 2008), poplar (Hu et al., 2012), soybean (Chen et al., 2014), peach (Zhang et al., 2014a, 2014b), and partially in *Cucumis sativus* (Fu et al., 2013; Liu et al., 2013) and tomato (Zhang et al., 2014a, 2014b). However, there is little information on the identification and functional characterization of HD-Zip proteins in *Citrus*, the most important evergreen perennial fruit tree. The objectives of this study were (i) to perform an annotation of the HD-Zip gene family in *Citrus*, (ii) to predict the number of HD-Zip genes associated with the somatic embryogenesis process in citrus, and (iii) to select the best candidate genes for further functional analyses.

In this study, we identified 27 HD-Zip genes (*CsHBs*) based on a genome-wide analysis of the *Citrus sinensis* genome annotation database. Sixteen members of subfamily HD-Zip I, two members of subfamily HD-Zip II, four members of subfamily HD-Zip III and five members of subfamily HD-Zip IV were grouped according to gene structures and additional common motifs. Our results also indicated that six of the *CsHB*s might be involved in somatic embryogenesis, including two of HD-Zip II subclass (*CsHB1* and *CsHB20*) and four of HD-Zip IV subclass (*CsHB7*, *CsHB11*, *CsHB13* and *CsHB27*), exhibited different but relevant expression patterns in *Citrus* somatic embryogenesis. These results provide a basis for further characterization of the physiological functions of *CsHB*s, especially in somatic embryogenesis.

2. Materials and methods

2.1. Plant materials and DNA extraction

The non-embryogenic callus line (NEC) was induced as described by Deng (1987). Calluses at different developmental stages (induced-calluses for 2 weeks, IEC2; induced-calluses for 4 weeks, IEC4) were induced by culturing embryogenic calluses (EC) (*C. sinensis* cv. 'Valencia') on modified Murashige and Tucker (MT) basal medium containing 2% (v/v) glycerol (Liu and Deng, 2002). In addition, calluses of six citrus cultivars with different embryogenic capacity were cultured on MT basal medium. These cultivars were: *C. sinensis* cv. Valencia (V), *C. sinensis* cv. Anliucheng (ALC), *C. sinensis* cv. Newhall (NHO), *Citrus paradisi* cv. Red Marsh (RM), *Citrus reticulata* cv. Changsha (CSC) and *Citrus unshiu* cv. Guoqing No.1 (G1).

Eighteen citrus genotypes were used for the SSR polymorphism analysis (Table S1). These genotypes represent the major groups of citrus which have different callus induction capacities. Total genomic DNA was isolated from young leaves following the procedure as described by Cheng et al. (2003). For the SSR analysis, PCR amplification was performed as described by Chai et al. (2013).

2.2. Identification and physical locations of HD-Zip genes in Citrus

One sequence obtained from the SSH library (Ge et al., 2012) was used to search the Pfam (Protein family) database (http://pfam.sanger.ac.uk/). BioEdit software was used to build local databases from the citrus complete genome nucleotide sequences and protein sequences (Xu et al., 2013). The HD-Zip domain obtained from the Pfam database was used as a standard sequence to isolate all possible homologs. Due to the variation in HD-Zip sequences, we further explored the sweet orange (*C. sinensis*) genome database (http://citrus.hzau.edu.cn/orange/index.php) (Xu et al., 2013) to collect more complete information of putative HD-Zip genes in citrus using the keywords "HD-Zip" and "HB". The predicted HD-Zip genes were confirmed by SMART (http://smart.embl-heidelberg.de/) and Pfam database (http://pfam.sanger.ac.uk/).

A distinctive name was given to each of HD-Zip genes identified in citrus according to its relative location on citrus chromosomes. The chromosome location image of HD-Zip genes was generated by MapInspect software (http://www.plantbreeding.wur.nl/UK/software_mapinspect.html). The exon/intron structures were investigated using the National Center of Plant Gene Research database (NCPGR, http://gbrowse.ncpgr.cn/cgi-bin/gbrowse/japonica/).

2.3. Analysis of phylogenetic relationship and digital expression of HD-Zip proteins in Citrus

Amino acid sequences of HD-Zip proteins were aligned using Clustal W program. Sequences of *Arabidopsis thaliana* HD-Zip proteins were retrieved from TAIR database (http://www.arabidopsis.org/), and a phylogenetic tree was constructed applying the neighbor-joining (NJ) method in MEGA (version 4.0). The analysis of HD-Zip expression profiles was accomplished by searching the *C. sinensis* genome database (http://citrus.hzau.edu.cn/orange/index.php).

2.4. Scanning electron microscope (SEM) analysis

NEC, EC, IEC2, IEC4 of *C. sinensis* cv. 'Valencia' were fixed overnight in 2.5% (v/v) glutaraldehyde in a 0.1 M phosphate buffer (pH 7.2), rinsed three times in the same buffer, and fixed in 1.0% (w/v) OsO_4 in phosphate buffer for 2 h. Tissue samples were dehydrated in a series of ethanol gradient solutions. For scanning electron microscopy (SEM), the critical- point-dried tissues were mounted on steel plates and coated with gold palladium. Samples were observed using a NTC-JSM-6390 Scanning Electron Microscope (Japan).

2.5. Quantitative real-time PCR and reverse transcription PCR

Total RNA was extracted from NEC, EC and different tissues (root, stem, leaf, ovary, stamen and stigma) according to Liu et al. (2006). First strand cDNA was synthesized using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA). Reverse transcription was performed as described previously (Ge et al., 2012). Gene-specific primers used for quantitative real-time PCR were designed based on the gene sequences using an online system (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The target gene primers (Tables S2, S3) and *ACTIN* gene primer (Liu et al., 2009) used in quantitative real-time PCR were diluted in the SYBER GREEN PCR Master Mix according to the protocol of the manufacturer (PE Applied Biosystems, Foster City, CA, USA), and performed in four technical replicates for each sample.

3. Results

3.1. Identification and physical locations of Citrus HD-Zip proteins

The amino acid sequence of HB-type domain was adopted as a query in BLASTP searches for possible homologs encoded in citrus genome (http://citrus.hzau.edu.cn/orange/index.php) (Xu et al., 2013). As a result, 36 candidate HD-Zip protein sequences were identified in citrus. Using keyword search, 27 non-redundant HD-Zip genes were consequently identified and described in citrus genome (Table 1). The number of HD-Zip genes in citrus is smaller than that identified in other plant species, including *Arabidopsis* (47) (Ariel et al., 2007), populus (63) (Hu et al., 2012), soybean (88) (Chen et al., 2014), peach (33) (Zhang et al., 2014a, 2014b) and grape (31) (Jiang et al., 2014).

HD-Zip genes were distributed on every chromosome of the citrus genome (Fig. 1). However, the number of HD-Zip genes on each chromosome varied widely. Up to six HD-Zip genes were detected on Chromosome 1, likewise on Chromosome 2, whereas only one was located on Chromosomes 8 and 9, respectively. The 27 HD-Zip genes identified in our study were designated *CsHB1* to *CsHB27* according to their localization on all nine chromosomes.