Gene 575 (2016) 498–505

Contents lists available at ScienceDirect

Gene

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

Research paper

Determination of the precise sequences of computationally predicted miRNAs in *Citrus reticulata* by miR-RACE and characterization of the related target genes using RLM-RACE

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ARTICLE INFO

Article history:
Received 29 June 2014
Received in revised form 5 September 2015
Accepted 11 September 2015
Available online 16 September 2015

Keywords:
Citrus reticulata
MicroRNAs
MiR-RACE
Expression
PPM-RACE
RLM-RACE

ABSTRACT

MicroRNAs play vital roles in various biological and metabolic processes by regulating the expression of their target genes in model plants. Since there are limited reports on miRNAs in *Citrus reticulata* (Crt-miRNAs), the determination of precise sequences of miRNAs is essential to further analyze the functions of miRNAs in *Citrus reticulata*. Here, miR-RACE, a recently developed technique for determination of the potential miRNAs computationally, was employed to identify the precise sequences of Crt-miRNAs. Tissue- and development-specific expression of nine miRNAs were identified by quantitative RT-PCR in the leaves, stems, flowers and fruits Subsequently, 10 potential target genes were predicated for the eight Crt-miRNAs, most of which were transcription factors and disease resistance proteins. Four target genes were experimentally validated by Poly (A) polymerase-mediated 3′ rapid amplification of cDNA ends and RNA ligase-mediated 5′ rapid amplification of cDNA ends (PPM-RACE and RLM-RACE). Our findings showed that regulatory miRNAs in C. reticulata may play a key role in regulating growth, development, and response to disease. Future work is required to study the functions of miRNAs and their targets of *C. reticulata*.

1. Introduction

MicroRNAs (miRNAs) are endogenous gene regulators distributed widely in plant genomes which play important roles in plant growth, development, signal transduction and environmental stimuli responses (Bartel, 2004; Chen, 2005; Jones-Rhoades et al., 2006). Recently, it has been demonstrated that a large number of known miRNAs in the plant kingdom are evolutionarily conserved from mosses and ferns to higher flowering plants. The high evolutionary conservation of miRNAs provide powerful evidence for supporting prediction and validation of conserved miRNAs and their target genes in all plant species by bioinformatics and experimental methods (Fahlgren et al., 2007; Song et al., 2009; Wang et al., 2011).

With the advance of bioinformatics, computational methods have been employed for the prediction and identification of novel miRNAs (Unver and Budak, 2009). However, due to miRNAs evolution and the non-availability of miRNA end characteristics that exactly define the start and stop codes of mature miRNAs (Song et al., 2010a; Wang et al., 2011), computational methods make some false predications. This is also the main disadvantage of computational prediction compared to direct cloning of miRNAs. Therefore, determination of precise sequences of predicted miRNAs is essential to lay a solid foundation for some downstream research applications, such as accurate prediction of miRNA targets, miRNA's function on gene expression, miRNA evolution, and the mechanism of miRNA biogenesis. Notably, there are few studies on comprehensive determination of precise sequences of computationally predicted miRNAs. Previous studies on miRNAs were mainly focused on determining the expression using Northern blotting and/or RT-PCR techniques which can only confirm the existence and size, but not the full precise sequence of miRNAs targeted for identification. In contrast, the newly reported miR-RACE can overcome the shortcomings of Northern blotting and qRT-PCR techniques as it is preferentially suitable in the validation of precise sequences, especially both ends, of computationally predicted miRNAs in an organism, which is exemplified by successful utilization in earlier studies on identification of citrus, grapevine, apple and peach miRNA precise sequences (Song et al., 2010a; Wang et al., 2011; Yu et al., 2011; Zhang et al., 2013). Therefore, here we employed miR-RACE to determine the precise sequences of predicted miRNAs in *Citrus reticulata* for further investigating their functions.

Citrus are important nutritional resources for human health and have immense economic values. Production of *Citrus* is among the

Abbreviations: miRNA, microRNA; Crt-miRNAs, *Citrus reticulata* miRNAs; ESTs, expressed sequence tags; RT-PCR, reverse transcription-polymerase chain reaction; qRT-PCR, quantitative RT-PCR; PPM-RACE, poly(A) polymerase-mediated 3′ rapid amplification of cDNA ends; RLM-RACE, RNA ligase-mediated 5′ rapid amplification of cDNA ends.

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http://dx.doi.org/10.1016/j.gene.2015.09.022
0378-1119/

leading fruit crop industries in worldwide. This suggests that research on growth and development of *Citrus* is really important. With the rapid development in plant genomics, the entire genomes of *Citrus* have been completed and released publically, which makes it possible to initiate research on *Citrus* functional genomics, including the prediction and characterization of Crt-miRNAs. Several Crt-miRNAs have been predicted and validated based on the *Citrus* expressed sequence tag (Est) database (Zhang et al., 2005; Sunkar and Jagadeeswaran, 2008; Wu et al., 2011) and high throughput sequencing (Fang et al., 2014; Zhang et al., 2014). Further, some miRNAs in Trifoliate Orange, a citrus related genus, also have been validated by high throughput sequencing (Song et al., 2010c; Zhang et al., 2014), but the number of predicted *C. reticulata* miRNAs still remains low compared with *Arabidopsis* and other plants, and no experimental validation has been carried out, thus calling for identification and characterization of many more Crt-miRNAs. In this study, miR-RACE technology was employed to determine and validate the precise sequences, especially terminal nucleotides of the nine Crt-miRNAs, and their ubiquitous expressions were studied in different tissues of *Citrus* by qRT-PCR. Subsequently, the cleavage site of target mRNAs for Crt-miRNAs and expression patterns of cleaved fragments were mapped by our developed method named PPM-RACE and RLM-RACE (Sun et al., 2012; Wang et al., 2013). Our findings showed that regulatory miRNAs in C. reticulata may play a key role in regulating growth, development, and response to disease. Future work is required to study the functions of miRNAs and their targets of *C. reticulata*.

2. Materials and methods

2.1. Plant materials

The samples were collected from five-year-old 'Gongben' (Mandarin orange, *Citrus reticulata*) trees that were grown in Suzhou Evergreen Fruit Tree Research Institute, China. Young leaf, mature leaf, old leaf, young stem, mature stem, old stem, flower bud, half and fully flower, as well as fruits at different developmental stages (15, 45, 75, 105 and 145 days after full bloom, DAFB) were collected. After collection, all the samples were immediately frozen in liquid nitrogen and stored at −80 °C until used.

2.2. Low molecular weight RNA extraction and construction of cDNA libraries of small RNAs

Total RNA was isolated from 100 mg of the selected plant tissues using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA). Low and high molecular weight RNA were separated with 4 M LiCl (Adai et al., 2005; Song et al., 2010a). The small RNA fraction was dissolved in 50 μl of RNase-free water. The concentration of the RNA was measured by the UV-1800 spectrophotometer (Shimadzu, Japan) and visually checked in a 2.0% agarose gel. The larger molecular weight RNA samples were used to study the expression patterns of the target genes of Crt-miRNAs.

We generated miRNA-enriched library that has been popularly used to clone miRNAs and to measure the expression of miRNAs via RT-PCR (Lewis et al., 2005), in which 5′- and 3′-end adaptors were linked to the miRNA molecules (Song et al., 2009). Then they were further reverse transcribed using Superscript III reverse transcriptase (Invitrogen) in the presence of random nonamers (Sigma), according to the protocols provided by the manufacturer. After preparation of the miRNA libraries from various *C. reticulata* organs and tissues, we pooled similar quantities of these library samples for further PCR amplification reactions.

2.3. Verification of Crt-miRNAs precise sequence by miR-RACE

The cDNA was amplified with the mirRacer 5′ primer (5′-GGACACTG ACATGGACTGAAGGAGTA-3′) and the mirRacer 3′ primer (5′-ATTCTA GAGGCCGAGGCGGCCGACATG-3′) to generate a pool of non-gene-specific product. 5′ end reactions were performed with the mirRacer 5′ primer and miRNA-gene-specific forward primers (GSP1), and 3′ end reactions were carried out with the mirRacer 3′ primer and miRNA gene specific reverse primers (GSP2). GSP1 and GSP2 were complementary to seventeen nucleotide length sequences of the potential Crt-miRNAs and a part of Poly (T) and 5′ adaptor (Table S1). The 5′ end and 3′ end clones with PCR products about 56 bp and 87 bp in length, were sequenced respectively.

2.4. C. reticulata miRNAs and prediction of potential target mRNAs

Blast searches of mRNA database for plants were used to successfully select potential miRNA targets in mRNA sequences to predict newly identified miRNA targets, while BlastX was conducted for the potential functional analysis of their target genes. The comparative software (BLAST-2.2.14) was downloaded from NCBI GenBank and BLASTX from the web site http://www.ncbi.nlm.nih.gov/BLAST/ used for analysis of potential targets. Putative Crt-miRNAs were first blasted against the *Citrus* unigene database. BLASTn hits with less than four nucleotide mismatches (plus/minus) were chosen as the candidate targets, and were then searched in the genome sequence of clementine mandarin (http://www.phytozome.net/clementine.php) (Song et al., 2010a, 2010b).

2.5. Real-time PCR of C. reticulata miRNAs and their target genes

The template for RT-PCR was the miRNA-enriched library mentioned above. RT-PCR was conducted with the fluorescence quantitative polymerase real-time quantitative PCR (Bio-Rad) and the Rotor-Gene software version 6.1 (Wang et al., 2004). SYBR green reaction mix (SYBRH Green qRT-PCR Master Mix; Toyobo, Osaka, Japan) were used in Real-time PCR reactions, according to the manufacturer's instructions. The comparative quantification procedure was used to determine relative expression levels as previously described (Wilson et al., 2005). The 5.8S rRNA was used as a reference gene in the qPCR detection of miRNAs following the work in Arabidopsis (Shi and Chiang, 2005). Data were analyzed with an R2 above 0.998 using the LinRegPCR program (Ramakers et al., 2003). The primers used are listed in Tables S1 and S2.

2.6. mRNA cleavage sites mapping with PPM-RACE and RLM-RACE

Low molecular weight RNA (LMW RNAs) and high molecular weight RNAs (HMW RNAs) were separated with 4 M LiCl (Adai et al., 2005). For mapping miRNA-mediated cleavage products, the modified method developed in our laboratory with the key steps being nested RLM-RACE and PPM-RACE being employed. After construction of libraries of poly(A)-tailed HMW RNA and adapter-ligated HMW RNA, the productions of reverse transcription of poly(A)-tailed HMW RNA and adapter-ligated HMW RNA were performed with PPM-RACE and RLM-RACE (Wang et al., 2013) using corresponding common primer and specific primers, respectively (Table S2). The amplification products were gel purified, cloned, and sequenced, and at least eight independent clones were sequenced.

3. Results

3.1. Validation of precise sequences of Crt-miRNAs

Validation of the exact sequences of the miRNAs is necessary for further studies on the functions, biogenesis, and sequence evolution of the predicted miRNAs. MiR-RACE has been successfully used to verify the precise sequences of miRNAs in citrus, apple, grape, and peach (Song et al., 2010a; Yu et al., 2011; Wang et al., 2011; Zhang et al., 2013). In this study, 14 Crt-miRNAs (Song et al., 2010b) belonging to