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Ethylene-responsive miRNAs in roots of *Medicago truncatula* identified by high-throughput sequencing at whole genome level

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ABSTRACT

Ethylene is one of the classical plant hormones with a diverse function in plant growth and development. Root elongation is sensitive to ethylene such that treatments with ethylene and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) inhibit root growth. MicroRNA as one type of endogenous, non-coding small RNAs, plays an important role in regulation of plant growth, development and hormonal signaling by affecting expression of target genes. However, there has been no detailed study to evaluate the role of microRNAs in mediation of ethylene-dependent physiological processes in plants. *Medicago truncatula* is a model plant widely used for investigation of molecular biology in legume species. In this study, we constructed two small RNA libraries from roots of *M. truncatula* treated with and without ACC. High-throughput sequencing was employed to sequence the small RNA libraries, and more than 30 M raw reads were obtained. We annotated 301 known miRNAs and identified 3 new miRNAs in the two libraries. Treatment of *M. truncatula* with 10 μM ACC led to changes in expression of 8 miRNAs. The targets of the ethylene-responsive miRNAs were predicted by bioinformatic approach. The potential role of the ethylene-responsive miRNAs in the ethylene-induced inhibition of root elongation is discussed. These results are useful for functional characterization of miRNAs in mediation of ethylene-dependent physiological processes in general and root elongation in particular.

1. Introduction

MicroRNAs (miRNAs) are one type of endogenous non-coding small RNAs with the approximate length of 21 nt. miRNAs were initially discovered in *Caenorhabditis elegans* as developmental timing regulators [1]. The existence of miRNAs in organisms such as plants and animals has been widely reported [2,3]. And microRNA-like RNAs have been reported to exist in fungi [4]. There is increasing evidence demonstrating that miRNAs are involved in the regulation of numerous physiological processes in plants by repressing expression of their target genes, ranging from seed germination to plant growth and development such as root formation, legume–rhizobial symbiosis, control of flowering time, and leaf development [5–9]. The involvement of miRNAs in responses of plants to various biotic and abiotic stresses has also been reported [10–14]. In addition, numerous studies revealed that miRNAs are closely associated with the transduction of hormonal signaling in plants, including auxin [15], abscisic acid [7] and gibberellins [16]. Ethylene, a gaseous plant hormone, regulates diverse physiological processes in plants ranging from seed germination to fruit ripening, and nodulation [17]. Ethylene also plays an important role in modulation of root growth [18]. In general, root growth is suppressed by ethylene, and the ethylene-induced inhibition of root elongation may involve disruption of auxin biogenesis and distribution in roots [18,19]. Given the important role played by miRNAs in the regulation of multiple physiological processes in plants [20], the involvement of miRNAs in ethylene signaling cascades is expected. However, there have been no detailed studies to investigate the role of miRNA in ethylene signal pathway.

Medicago truncatula has been used as a model plant to study functional genomics of legume plants due to its small diploid genome and relatively easy transformation [21]. Discovery of new miRNAs in plants on a genome-wide scale is essential to functionally characterize miRNAs. Numerous miRNAs have been identified in different plant species and the regulatory roles of miRNAs are being deciphered in recent years [20,22]. The traditional sequencing of relatively small-size cDNA libraries of small RNAs from the plant species such as Arabidopsis, rice and poplar with Sanger method has revealed that plant miRNAs are highly conserved [23,24]. However, several miRNAs cannot be detected in the genomes of some plant species, implying that miRNAs may

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; miRNA, microRNAs; pri-miRNA, microRNA primary transcript; pre-miRNA, microRNA precursor; qRT-PCR, quantitative real-time polymerase chain reaction.

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have evolved recently [24]. Moreover, the non-conserved miRNAs may often be expressed at a relative low level compared to the conserved miRNAs. Therefore, many non-conserved miRNAs would not be detected in those studies using small-scale sequencing. To overcome this problem, high-throughput sequencing has successfully been used to identify the non-conserved miRNAs in a number of plant species [25,26], including *M. truncatula* [10,14,27]. To evaluate the role of miRNA played in mediation of root elongation by ethylene, miRNAs that are responsive to ethylene were identified by high-throughput sequencing in this study, and their potential function in mediation of ethylene-dependent root development was discussed.

2. Materials and methods

2.1. Plant materials

Seeds of *M. truncatula* (cv Jemalong A17) were soaked in concentrated, anhydrous sulfuric acid for about 5 min to scarify seed coat, and then washed thoroughly with water. The seeds were germinated at 25 °C in dark for 2 days, and thereafter they were grown hydroponically in aerated nutrient solution. The nutrient solution contained 1 mM NH_4NO_3, 2.5 mM KNO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 0.25 mM K_2SO_4, 0.25 mM $CaCl_2$, 100 μM FeNaEDTA, 30 μM H_3PO_3, 5 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$, 0.7 μM Na_2MoO_4, 1 mM NH_4NO_3, 2.5 mM KNO_3 and 50 μM KCl at pH 5.8. Seedlings were grown in a growth chamber under conditions of a 16/8 h light/dark cycle at 25 °C for one week. During seedling growth, the nutrient solution was changed every 2 days. Seedlings were exposed to solution containing 10 μM ACC for 24 h, while the same age seedlings exposed to the identical solution without ACC were used as controls. Roots of approx. 1.5 cm in length with root tips were collected for the construction of small RNA library.

2.2. Construction and sequencing of sRNA libraries

Total RNAs were extracted from the *M. truncatula* roots treated with and without ACC for 24 h using RNAiso Plus (TaKaRa), respectively. Small RNA libraries were constructed by the methods described previously [14]. Briefly, sRNA with length of 18–30 nt was separated and purified on a 15% TBE-urea denaturing PAGE gel. The 5′- and 3′-RNA adapters were ligated to sRNA with T4 RNA ligase (TaKaRa). Thereafter the adapter-ligated sRNAs were transcribed to single-stranded cDNA with superscript II reverse transcriptase (Invitrogen). PCR was conducted using the primer designed according to the adapter sequence to amplify single-stranded cDNA template to double-stranded cDNA. PCR products were sequenced on a Solexa sequencer (Illumina) at the Beijing Genomics Institute (BGI), Shenzhen, China.

2.3. Bioinformatic analysis

The raw reads obtained from the Solexa sequencer were cleaned by removing contaminant reads. The contaminant reads included those reads with 5′-primer contaminants, reads without 3′-primer, reads with poly A, and reads with length less than 18 nt. The clean reads were then used to analyze length distribution, and mapped to *M. truncatula* genome Mt 3.5.1 (http://www.medicagohapmaporg/downloads_genome/Mt3.5/Mt3.5.1_pseudomolecules.tar.gz) using SOAP [28,28].

The clean reads were annotated using several databases such that the known miRNAs were annotated by comparing to miRBase 16 (ftp://mirbase.org/pub/mirbase/CURRENT/miRNA.dat.gz). The annotation of rRNAs, scRNAs, snoRNAs, snRNAs and tRNAs was carried out by BLASTn to NCBI Genbank database and Rfam database ($e = 0.01$). Small interfering RNA (siRNA) is a double-strand RNA with 22–24 nt in length, and each strand is 2 nt longer than the other on the 3′ end. Based on this feature, we aligned tags from the clean reads to each other to identify those sRNAs that meet this criterion. These tags were removed from new miRNA analysis as they may be potential siRNA candidates. The reads that were not annotated were used to predict new miRNA.

Pre-miRNA candidates were predicted by MIREAP (http://sourceforge.net/projects/mireap/). The following parameters were used: minimal miRNA sequence length of 18 nt; maximal and minimal miRNA sequence length of 25 and 20 nt; maximal miRNA reference sequence length of 23 nt. Maximal copy number of miRNAs on reference was 20 nt, and maximal free energy allowed for a miRNA precursor was – 18 kcal/mol. Maximal space between miRNA and miRNA* was 300 nt. Minimal base pairs of miRNA and miRNA* were set as 16; maximal bulge of miRNA and miRNA* was 4. Maximal asymmetry of miRNA/miRNA* duplex was 4 nt. Flank sequence length of miRNA precursors was 20 nt. New miRNAs' pre-miRNA stem-loop structure was constructed by mfold [29]. Predication of target genes was carried out following the protocols proposed by Allen et al. [30]. Pipeline psRNATarget [31] and latest *M. trunacatula* genomic database Mt 3.5.1 was used to search for the targets of miRNAs.

In the analysis of miRNA expression, p value was calculated by chi-square test.

Normalized read count was calculated as:

$$\text{Normalized read count} = \left(\frac{\text{actual miRNA count}}{\text{total count of clean reads}}\right) \times 1,000,000$$

2.4. Quantitative real-time PCR

Total RNAs which were used for construction of small RNA libraries were also used for qRT-PCR. RNAs were reverse-transcribed using the One Step PrimerScript® miRNA cDNA Synthsis Kit (TaKaRa). This led to an addition of a ploy(A) tail to the 3′-end of miRNA, and transcribed by a known oligo-dT ligate. The cDNA products were used for RT-qPCR using SYBR Premix Ex Tag II (TaKaRa). RT-qPCR was performed using Mx3000P™ PCR system (Agilent-Stratagene, USA). The PCR program was set as 30 s at 95 °C followed by 40 cycles of 5 s at 95 °C, 30 s at 60 °C. Small nuclear RNA U6 was used as an internal reference. All primers used in this work were listed in Table S5.

3. Results

3.1. Analysis of small RNA libraries

To identify miRNAs that were responsive to ACC, two sRNA libraries were constructed from the *M. truncatula* roots exposed to solution without ACC (Control) and solution containing 10 μM ACC for 24 h (ACC). Sequenced by Illumina, 17.1 M and 16.6 M raw reads were obtained from the libraries of Control and ACC, respectively. After removal of the contaminant reads like adapter, poly A and those with length less than 18 nt, reads ranging from 18 to 30 nt were obtained, which were designated as 'clean reads' (Table 1). As shown in Fig. 1, reads with length of 24 nt were the most abundant, followed by reads with length of 21 nt. About 11.8 M clean reads from Control (2,869,846 unique reads) and 11.4 M clean reads from ACC (3,197,722 unique reads) were mapped to *M. truncatula* genome sequence (MT3.5.1, released in November 2010) with SOAP [28]. miRNA, tRNA, siRNA, snRNA, snoRNA, rRNA, repeat regions, exon and intron RNA reads were annotated, respectively. Reads which have not annotated by former step that were used for prediction of new miRNAs for Control and ACC were 6,179,361 and