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Response of miRNAs and their targets to salt and drought stresses in cotton (*Gossypium hirsutum* L.)

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ABSTRACT

MicroRNAs (miRNAs) are an important gene regulator, controlling almost all biological and metabolic processes, in both plants and animals. In this study, we investigated the effect of drought and salinity stress on the expression of miRNAs and their targets in cotton (*Gossypium hirsutum* L.). Our results show that the expression change of miRNAs and their targets were dose-dependent and tissue-dependent under salinity and drought conditions. The expression of miRNAs in leaf was down-regulated under higher salinity stress while shows variable patterns in other conditions. The highest fold-changes of miRNAs were miR398 in roots with 28.9 fold down-regulation under 0.25% NaCl treatment and miR395 in leaves with 7.6 fold down-regulation under 1% PEG treatment. The highest up-regulation of miRNA targets was AST in roots with 4.7 fold-change under 2.5% PEG and the gene with highest down-regulation was CUC1 in leaves with 25.6 fold-change under 0.25% NaCl treatment. Among seven miRNA-target pairs we studied, five pairs, miR156–SPL2, miR162–DCL1, miR159–TCP3, miR395–APS1 and miR396–GRF1, show significant regulation relationship in roots and leaves under salinity stress concentration.

1. Introduction

Salinity and drought stresses are two of the most severe and wide-range stress factors that affect plant growth and development and therefore lead to significant reduction of agriculture production. Additionally, a great amount of areas cannot be utilized because of high salinity or drought environmental stress to plants. Fortunately, plants have developed a set of strategy to confront such harsh environment and show tolerance in varying degrees. A lot of efforts have been devoted to elucidate the molecular mechanism underlying plant tolerance and it has been recognized that the adaptation of plants to stress environment is achieved partly by regulation of gene expression at the transcriptional and post-transcriptional levels. However, the molecular details about the regulation mechanism of gene expression are still unclear.

MicroRNAs (miRNAs) are a type of small non-coding RNAs with 21–24 nt in length, which play important regulation roles in gene expression at the post-transcription levels by mRNA cleavage or translation repression (Bartel, 2004; Zhang et al., 2006). They have been reported to involve in diverse biological processes in plants by regulating the expression of their targeted protein-coding genes. These biological processes include organ development (Aukerman and Sakai, 2003; Larue et al., 2009), hormone signaling (Mallory et al., 2005), defense against pathogens (Navarro et al., 2006), and response to abiotic stress (Eldem et al., 2013; Lu and Huang, 2008; Sunkar and Zhu, 2004; Sunkar et al., 2007). The abiotic stresses that plants respond to include salinity (Li et al., 2011), drought (Covarrubias and Reyes, 2010; Eldem et al., 2012; Zhao et al., 2007), cold (Zhou et al., 2008), and heavy metals (Huang et al., 2009), and nutrition stresses (Lu and Huang, 2008). Currently, more than 40 miRNA families have been associated with response to abiotic stress in plants (Sunkar, 2010), many of them involve in salt and drought stress response (Covarrubias and Reyes, 2010). Some miRNAs were reported in more than one plant species (Covarrubias and Reyes, 2010; Sunkar, 2010), which indicates their function in response to stress might be conserved among plants.

Upland cotton (*Gossypium hirsutum* L.) is one of widely cultivated economic crops that provide natural textile fiber and edible oil. Studies on the tolerance of cotton to salt and drought environment would benefit the improvement of cotton production and produce great economic value. Both drought and salinity stresses affect cotton growth and development, influence cotton biological and metabolic pathways (Ashraf et al., 2002b), including photosynthesis (Levi et al., 2009a), and adversely affect both yield and quality (Ashraf, 2002; Ashraf et al., 2002a; Basal

Abbreviations: ANOVA, Analysis of variance; AP2, Apetala2-like transcription factor; APS, ATP-sulfurylase; AST, Sulfate transporter; CUC, NAC domain transcription factor; DCL, Dicer-like; GRF, Growth regulating factor; miRNA, microRNA; MS, Murashige and Skoog medium; PEG, Polyethylene glycol; qRT-PCR, quantitative real-time PCR; REV, HD-ZIPIII transcription factor; SPL, Squamosa promoter-binding factor; TCP, TCP transcription factor.

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et al., 2009; Levi et al., 2009b; Onder et al., 2009; Pettigrew, 2004a,b; Vulkan-Levy et al., 1998). Transcriptome and transgenic analysis has shown that drought and salinity stresses also induce differential expression of many protein-coding genes in cotton; some of them have been identified and cloned (Gao et al., 2009; Guo et al., 2009; Kosmas et al., 2006; Li et al., 2009; Lv et al., 2009; Maqbool et al., 2007, 2008; Meng et al., 2009a,b; Nepomuceno et al., 2000; Voloudakis et al., 2002; Xue et al., 2009). Although progress has been made on understanding gene expression during cotton response to abiotic stress, the regulatory mechanisms controlling gene expression remain largely unknown, which limit cotton breeding for improving cotton tolerance to environmental stress. A better understanding of the molecular basis of cotton response to environmental abiotic stress and the regulatory mechanisms controlling gene expression will allow us to design more efficient strategies for using genetic engineering to improve cotton stress tolerance and ultimately improve cotton yield and quality. This will financially benefit cotton farmers, particularly small farmers in developing countries, by decreasing costs and increasing income. Based on previous results from ours and others, we found that miRNAs play an important role in plant response to environmental stress and a miRNA-based biotechnology may be a novel biotechnical strategy for improving crop tolerance to environmental stress. Although miRNAs have been identified in cotton by several technologies, including comparative genomic and deep sequencing (Kwak et al., 2009b; Pang et al., 2009; Zhang et al., 2007), the functions of miRNAs in cotton are still unknown. Quantitative real-time PCR (qRT-PCR) was also employed to analyze the expression of miRNAs in different cotton organs at different development stages; the result shows that miRNAs were differentially expressed in different cotton organs and may regulate the development of cotton and cotton fiber (Zhang and Pan, 2009). While, this study only detected four conserved miRNAs miR156, miR162, miR172, and miR395, and no any data on cotton miRNAs in response to stress conditions has been reported. Based on previous report, many miRNAs were responsive to abiotic stress. In this work, we selected 12 of them to investigate the response of these 12 miRNAs (miR 156, miR 157, miR 159, miR 162, miR 167, miR 169, miR 172, miR 395, miR 396, miR 397, miR 398 and miR 399) and their 10 targets to drought and salinity stresses and found that those miRNAs and their targets responded to salt and drought stresses in a dose- and tissue-dependent manner.

2. Materials and methods

2.1. Collection of cotton samples

Before sterilization, the coat was removed from the seeds of *G. hirsutum* L. cultivar TM-1. Surface sterilization of seeds was performed with 70% (v/v) ethanol for 60 s, 6% (v/v) bleach for 6–8 min, followed by washing at least 4 times using sterilized water. The sterilized seeds were cultivated on 1/2 Murashige and Skoog (MS) medium (pH 5.8) containing 0.8% agar under a 16 h light/8 h dark cycle at 24 °C for 7 d. The media were supplemented with 0.1%, 0.25%, 0.5% NaCl and 1%, 2.5%, and 5% polyethyleneglycol (PEG) 6000 to simulate salinity and drought stresses, respectively. Roots and leaves were separately collected from the 7-days-old seedlings and were immediately frozen in liquid nitrogen.

2.2. RNA isolation and real-time RT-PCR analyses

Total RNA was isolated from the grinded samples using the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA) following the user manual except an additional ultrasonic step for 15–20 s just after lysis of cells. The quantity and quality of total RNAs were assessed using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and gel electrophoresis.

Real-time PCR was used to identify the expression fold change of 12 miRNAs and 10 miRNA targets in leaves and roots under salt and drought stress treatments (for sequences, see Table S1 and S2). The reason for selecting these 12 miRNAs was that these 12 miRNAs are drought/salinity stress responsible miRNAs or related to plant development according to previous reports (Eldem et al., 2013; Lu and Huang, 2008; Sunkar and Zhu, 2004; Sunkar et al., 2007). Briefly, reverse transcription was carried out using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The mixture of 12 miRNA specific primers (Table S1) and a poly-T primer were used as RT primers to obtain the cDNAs of miRNAs and protein-coding genes, respectively.

qRT-PCR was run on an Applied Biosystems 7300 Sequence Detection System (Foster City, CA, USA) according to the manufacturer's protocol using gene specific primers. Briefly, a single-stranded miRNA cDNA sequence was generated from 1 μg of the total RNA collected from each treatment. The cDNA was generated using reverse transcription with the Applied Biosystems TaqMan microRNA Reverse Transcription Kit and the miRNA-specific stem-looped RT primers. Then, qRT-PCR was run in a total of 20 μL PCR reaction, which included 10 μL Syber green PCR mixture, 2 μL RT products, 2 μL primers and 6 μL water. The reaction mixtures were initially heated at 95 °C for 10 min to activate the polymerase, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Three technical replicates were run for each reaction. The gene specific primers of miRNAs were designed and their sequences were shown in Table S1. The gene specific primers for the targets of miRNAs were designed based on the first hit sequences of Blast search using targets of miRNAs in *Arabidopsis* as queries. The detailed information was shown in Table S2.

The expression levels of miRNAs and their targets were normalized using the mean C_T values of ten common house-keeping genes as the reference gene. The 10 housekeeping genes included GheIF5 (eukaryotic translation initiation factor 5), GhHis3 (histone H3), GhACT14 (actin 14), GhUBQ7 (ubiquitin extension protein 7), GhUBI1 (ubiquitin gene family), GhCYP1 (cydophilin 1), GhGAPDH (glyceraldehyde-3-phosphate dehydrogenase C subunit), GhTUA10 (alpha-tubulin 10), GhPP2A (protein phosphatase 2A catalytic subunit), GhEF1A8 (translation elongation factor 1A-8). These 10 genes have been widely used as reference genes in cotton. ΔΔCt method was used to calculate the fold change of gene expression for each miRNA and its target. Analysis of variance (ANOVA) of single factor was used to compare the expression difference of miRNAs under different salt and drought treatments in leaves and roots.

3. Results

3.1. The expression level of miRNAs and miRNA targets in leaves and roots

3.1.1. Comparison of the expression of miRNAs and miRNA targets with that of the reference genes in roots and leaves

The expression level of five miRNAs, miR156, miR157, miR159, miR172 and miR397, was higher than that of the reference genes in both leaves and roots (Fig. 1A). Three miRNAs, miR169, miR395, and miR399, show lower expression level than the reference genes in both leaves and roots. The expression level of miR162, miR167, miR397, and miR398 in roots was lower than that of the reference genes but in leaves was higher than that of the reference genes. Of these miRNAs, miR157 and miR169 show the highest, 82-fold expression level in leaves and the lowest, 0.001-fold expression level in roots than that of the reference genes, respectively.

The expression levels of all 10 detected miRNA targets were far lower than that of the reference genes (Fig. 1B). Of these miRNA targets, REV shows the lowest expression level in both leaves and roots with 0.001-fold and 0.004-fold than that of the reference genes, respectively. ARF shows the highest, with 0.5-fold expression level in roots than that of the reference genes.