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Research article

Identification of miRNA from *Bouteloua gracilis*, a drought tolerant grass, by deep sequencing and their in silico analysis

Perla Lucía Ordóñez-Baquera[a], Everardo González-Rodríguez[a,*], Gerardo Armando Aguado-Santacruz[b,c], Quintín Rascón-Cruz[d], Ana Conesa[e,f], Verónica Moreno-Brito[g], Raquel Echavarria[h], Joel Dominguez-Viveros[a]

[a] Facultad de Zootecnia y Ecología, Universidad Autónoma de Chihuahua, Periférico R. Almada Km. 1C.P. 31453, Chihuahua, México
[b] Programa de Posgrado, Instituto Tecnológico de Roque, Km. 8 Carretera Celaya-Juventino Rosas C.P. 38110, Celaya, Guanajuato, México
[c] BIOqualitum S.A. de C.V., Oriente 7 # 158, Ciudad Industrial Sur, Celaya, Gto. CP 38010, México
[d] Facultad de Ciencias Químicas, Universidad Autónoma de Chihuahua, Campus Universitario No. 2 Circuito Universitario, C.P. 31125, Chihuahua, México
[e] Genomics of Gene Expression Laboratory, Prince Felipe Research Center, Av. Eduardo Primo Yúfera 3, 46012 Valencia, Spain
[f] Microbiology and Cell Science Department, Institute for Food and Agricultural Sciences, University of Florida, Gainesville, FL, United States
[g] Facultad de Medicina y Ciencias Biomédicas, Universidad Autónoma de Chihuahua, Campus Universitario No. 2 Circuito Universitario, C.P. 31125 Chihuahua, México
[h] Conacyt-Centro de Investigaciones Biomédicas de Occidente, Guadalajara, México

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ABSTRACT

Background: MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate signal transduction, development, metabolism, and stress responses in plants through post-transcriptional degradation and/or translational repression of target mRNAs. Several studies have addressed the role of miRNAs in model plant species, but miRNA expression and function in economically important forage crops, such as *Bouteloua gracilis* (*Poaceae*), a high-quality and drought-resistant grass distributed in semiarid regions of the United States and northern Mexico remain unknown.
Results: We applied high-throughput sequencing technology and bioinformatics analysis and identified 31 conserved miRNA families and 53 novel putative miRNAs with different abundance of reads in chlorophyllic cell cultures derived from *B. gracilis*. Some conserved miRNA families were highly abundant and possessed predicted targets involved in metabolism, plant growth and development, and stress responses. We also predicted additional identified novel miRNAs with specific targets, including *B. gracilis* ESTs, which were detected under drought stress conditions.
Conclusions: Here we report 31 conserved miRNA families and 53 putative novel miRNAs in *B. gracilis*. Our results suggested the presence of regulatory miRNAs involved in modulating physiological and stress responses in this grass species.

1. Introduction

MicroRNAs (miRNAs) are small, non-coding RNA molecules, which regulate gene expression via post-transcriptional degradation or translational repression of their target mRNAs Plant miRNAs are between 18 and 25 nucleotides (nt) in length and exhibit a high degree of complementarity with their mRNA targets, which often leads to the target degradation (Alptekin and Budak, 2016; Budak and Kantar, 2015; Esmaeili et al., 2016; Guzman et al., 2012). miRNAs are transcribed in the nucleus by RNA polymerase II as long primary miRNA transcripts (pri-miRNAs), which are subsequently processed into stem-loop secondary structures (pre-miRNA) by endonuclease Dicer-Like 1 (DCL1) (Budak et al., 2015). The generation of the miRNA/miRNA* duplex occurs inside the nucleus within Dicing-bodies in plants. The miRNA/miRNA* duplex is then methylated by the 3 '-methyltransferase Hua enhancer 1 (HEN1) and transported to the cytoplasm possibly through Hasty (HST). Once in the cytoplasm, one miRNA strand of the duplex, usually the 5p arm (miRNA) is

Abbreviations: EST, Expressed Sequence Tag; SOAP, Short Oligonucleotide Alignment Program; MFE, Minimum Folding Energies; UPE, unpaired energy; Osa, *Oryza sativa*; Zma, *Zea mays*; Sbi, *Sorghum bicolor*; Ath, *Arabidopsis thaliana*.

* Corresponding author.
E-mail addresses: plordonez@uach.mx (P.L. Ordóñez-Baquera), evegonzal@uach.mx (E. González-Rodríguez), gaguado@prodigy.net.mx (G.A. Aguado-Santacruz), qrascon@uach.mx (Q. Rascón-Cruz), aconesa@cipf.es, aconesa@ufl.edu (A. Conesa), vmoreno@uach.mx (V. Moreno-Brito), rechavarria.z@conacyt.mx (R. Echavarria), joeldominguez@hotmail.com (J. Dominguez-Viveros).

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selectively incorporated into a RNA-induced silencing complex (RISC) containing proteins from the Argonaute (AGO) family, while the other strand (miRNA-3p) is degraded (Budak and Akpinar, 2015; Kantar et al., 2012). Mallory and Vaucheret, (2006) showed the importance of miRNAs during plant development and function in *Arabidopsis thaliana*, where the absence of DCL1 expression was lethal; however, reduced expression of HYL1, HEN1, HST, or AGO1 resulted in smaller plants compared with their normal counterparts. Furthermore, evidence from functional studies demonstrated that plant miRNAs modulated key cellular processes, such as signal transduction, plant morphogenesis, cell differentiation, metabolism, and responses to various environmental stresses, including temperature, salinity, pathogens, and drought (Akpinar and Budak, 2016; Budak and Akpinar, 2015; Kantar et al., 2011; Lucas and Budak, 2012).

Bouteloua gracilis (*Poaceae*), commonly known as "Blue Grama," is a high-quality forage grass for livestock and other animals that inhabit the semiarid regions of the United States and northern Mexico. The species is valued for its high protein content and digestibility (Morales-Nieto et al., 2009). This warm-season perennial, C_4 photosynthetic grass exhibits a wide geographic distribution and exhibits drought resistance (Ciidir et al., 2010; Plains et al., 2010). The evolution of structural, physiological, and cellular changes together contribute to attributes in *B. gracilis* distinctive from other plants, but the molecular mechanisms underlying these features remain elusive. *B. gracilis* produces highly chlorophyllous cell cultures with in vitro regenerative capacity, which has become an excellent model to study the cellular and molecular mechanisms enabling this species to thrive under drought conditions; and to understand how osmotic stress effects other plant processes, such as metabolism and photosynthesis (Aguado-Santacruz et al., 2002). *B. gracilis* cells were placed under drought stress by the addition of polyethylene glycol and a small number of expressed sequence tags (ESTs) were subsequently isolated and sequenced (GeneBank LIBEST_021123). However, the specific functions of *B. gracilis* ESTs remains unknown and further studies are necessary to understand their role in the species characteristic drought response.

Several miRNAs and their mRNA targets were identified in plants, including some miRNAs induced by drought, including MIR156, MIR159, and MIR162. However to date, miRNA reports for *B. gracilis* in the public miRNA database (miRBase) are not available. Furthermore, the *B. gracilis* genome has not been sequenced and only 256 ESTs have been annotated (GeneBank LIBEST_021123). Therefore, it is difficult to identify conserved *B. gracilis* miRNAs using comparative genomics and/or computational prediction. (Zhao et al., 2010) emphasized the power of deep sequencing as an emerging tool to identify novel small RNA populations, particularly in species where the genome has not been sequenced. In the present study, we employed high-throughput Illumina sequencing technology using a small RNA library constructed from *B. gracilis* chlorophyllous cells to identify conserved and novel miRNAs expressed in this species. Thirty-one conserved miRNA families in *B. gracilis* were detected, including several miRNAs, which have been linked to drought stress responses in other plant species (Akpinar et al., 2015; Kantar et al., 2010; Liu et al., 2008; Lu and Huang, 2008; Zhou et al., 2010). Additionally, 53 potentially novel miRNAs in *B. gracilis* were identified, however additional study remains to elucidate the functions.

2. Methods

2.1. Plant materials

Chlorophyllic cells from *B. gracilis* were cultured on MPC medium containing MS basal medium supplemented with 2,4D (1 mg/l), BAP (2 mg/l), adenine (40 mg/l), and sucrose (3%). The medium pH was adjusted to 5.8 and cells were maintained under light conditions (fluorescent lamps, 77 $mols^{-1}m^{-2}$) at 33 ±1 °C (Aguado-Santacruz et al., 2002). After 5 d, the cells were harvested through filtration and immediately stored in liquid nitrogen at −70 °C until total RNA extraction was performed.

2.2. RNA extraction

Total RNA was extracted using the mirVana kit (Life Technologies); we also employed Sample Grinding (GE Healthcare Life Sciences) and Plant RNA Isolation aid (Life Technologies), according to each manufacturer's instructions. Briefly, 150 mg of *B. gracilis* cells were placed into extraction tubes and macerated for 2 min before adding 600 μl of Lysis/Binding Buffer and 100 μl of Plant RNA Isolation aid. The tubes were subsequently centrifuged for 10 min at 10,000 rpm at room temperature and 800 μl of supernatant was recovered following centrifugation. The supernatant was divided into two 1.5 ml tubes and 40 μl of homogenate additive was combined with the supernatant and incubated for 10 min on ice. Four hundred microliters of acid-phenol:chloroform was added to each tube, vortexed for 1 min, and centrifuged at 10,000 rpm for 5 min; 800 μl of the aqueous phase was recovered and separated into two 1.5 ml tubes. Each tube received 500 μl of ethanol and the mixture was placed into mirVana extraction columns, centrifuged at 10,000 rpm for 15s, and washed three times using wash solutions 1 and 2/3 from the mirVana kit. RNA was eluted from the column using the elution solution pre-heated to 95 °C and centrifuged at 10,000 rpm for 30 s. RNA quality was verified on an agarose gel (1.5%) separated at 90 V by electrophoresis for 45 min. Finally, RNA concentration was determined using NanoDrop 2000TM. Thirty microliters RNA (624.8 ng/μl), 3 μl sodium acetate (3 M, pH 5.2), and 90 μl ethanol were added. The mixture was homogenized and stored at −80 °C.

2.3. High-throughput illumina sequencing

RNA extracted from *B. gracilis* chlorophyllic cells was sent to the LC Sciences Laboratory (Houston, TX, USA), where its quality was verified through a series of methods, including Bioanalyzer, spectrophotometry, and gel electrophoresis. The RNA was subsequently separated in a vertical polyacrylamide gel (PAGE) and the fraction corresponding to RNAs of 14–40 nt in length were recovered. An adaptor sequence (5′-CCTTGGCACCCGAGAATTCCA-3′) was ligated to both ends of the RNA sequences, followed by cDNA synthesis and PCR amplification. The cDNA was then sequenced using a Genome Analyzer Instrument (GAI, Illumina, San Diego, CA, USA).

2.4. B. gracilis *conserved and specific miRNA identification*

Sequencing data quality was determined with the program FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw data was imported to FastQC to evaluate its quality using the algorithm PHRED, as previously reported (Ewing et al., 1998). Raw sequences were processed using CLC Genome Workbench version 5.5.2 (CLC bio, Aarhus, Denmark). The data were analyzed by selecting the option "Transcriptomics analysis," then "Small RNA analysis," and finally "Extract and count". The first step was elimination of the sequence corresponding to the Illumina adaptor (5′-CCTTGGCACCCGAGAATTCCA-3′). Following adaptor sequence removal, all sequences15-55 nucleotides in length and at least one count were considered mappable small RNAs and used in further analyses.

Once we obtained all mappable *B. gracilis* sequences, we proceeded to search for miRNAs homologous to those previously