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Research article

Identification of miRNAs and their targets in maize in response to *Sugarcane mosaic virus* infection

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ABSTRACT

MicroRNAs (miRNAs) are endogenous non-coding small RNAs that play essential regulatory roles in plant development and environmental stress responses. Maize (*Zea mays* L.) is a global economically important food and forage crop. To date, a number of maize miRNAs have been identified as being involved in plant development and stress responses. However, the miRNA-mediated gene regulatory networks responsive to virus infections in maize remain largely unknown. In this study, the profiles of small RNAs in buffer- and *Sugarcane mosaic virus* (SCMV)-inoculated maize plants were obtained by high-throughput sequencing, respectively. A total of 154 known miRNAs and 213 novel miRNAs were profiled and most of the miRNAs identified were differentially expressed after SCMV infection. In addition, 70 targets of 13 known miRNAs and 3 targets of a novel miRNA were identified by degradome analysis. The results of Northern blotting and quantitative real-time PCR showed that the expression levels of the selected miRNAs and their targets were mostly influenced by SCMV infection at 12 days post inoculation, including up-regulation of miR168 and miR528, and down-regulation of miR159, miR397 and miR827. These results provide new insights into the regulatory networks of miRNAs and their targets in maize plants responsive to SCMV infection.

1. Introduction

RNA silencing, a widespread mechanism, which functions in part to combat virus infections, is triggered by two major groups of small RNAs: microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Bartel, 2004; Baulcombe, 2004). The miRNAs are a class of endogenous non-coding small RNAs encoded by *MIR* genes (Bartel, 2004). In plants, the primary miRNA (pri-miRNA) is transcribed by RNA polymerase II (Pol II) and cleaved by Dicer-like1 (DCL1), a member of the RNase-III class of enzymes, to generate miRNA precursor (pre-miRNA), which contains miRNA/miRNA* sequences with a stem-loop structure (Bartel, 2004; Kurihara and Watanabe, 2004). The miRNA/miRNA* duplex is diced out from the precursor by DCL1 and transported from nucleus into cytoplasm (Park et al., 2005), where the miRNA guide strand is selectively loaded into an ARGONAUTE1 (AGO1)-containing RNA induced silencing complex (RISC) and the miRNA* strand is usually degraded (Baumberger and Baulcombe, 2005). miRNAs regulate gene expression in a sequence-specific manner at the post-transcriptional level by guiding cleavage or translational inhibition of target mRNAs (Jones-Rhoades et al., 2006).

There is increasing evidence that miRNAs play regulatory roles in plant development and environmental stress responses (Jones-Rhoades et al., 2006; Khraiwesh et al., 2012). A number of miRNAs have been demonstrated to function in biotic and abiotic stress responses in plants and the involvement of miRNAs in plant-pathogen interactions have been widely reported (Khraiwesh et al., 2012; Huang et al., 2016). Virus infections can trigger host RNA silencing as well as interfere with host miRNA pathways through the action of viral suppressors of RNA silencing (VSRs), which contribute to the viral symptom development (Huang et al., 2016; Kasschau et al., 2003; Mallory et al., 2002). *Tobacco mosaic virus* (TMV) infection significantly induced the expression of miR156, 164, 165 and 171, of which the levels were correlated with the severity of symptoms in *Nicotiana tabacum* (Bazzini et al., 2007). miR168 has been shown to be up-regulated by the infection of several plant viruses, which in turn affects the defense response of host by targeting the mRNAs of *AGO1*, an important component of RNA

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silencing (Du et al., 2011; Várallyay et al., 2010; Xia et al., 2016). In rice, miR444, a monocot-specific miRNA, has been reported to regulate resistance against *Rice stripe virus* (RSV) infection by up-regulation of the *OsRDR1* expression (Wang et al., 2016). Recently, it was reported that the induction of miR319 by *Rice ragged stunt virus* (RRSV) infection suppressed jasmonic acid (JA)-mediated defense to facilitate virus infection (Zhang et al., 2016). Moreover, it has been reported that miRNAs in maize and rice were differentially regulated by the infection with *Rice black streaked dwarf virus* (RBSDV) (Sun et al., 2015; Zhou et al., 2016).

In plants, target validation for miRNAs is essential to understand their potential regulatory and biological functions. In the past, 5′-rapid amplification of cDNA ends (RACE) has been employed for cleavage site mapping as target validation after computational prediction (Jones-Rhoades et al., 2006). However, this method is laborious, time-consuming and of low efficiency, making it suitable only for target confirmation on a small scale (Xu et al., 2013). Recently, degradome sequencing combining deep sequencing and bioinformatics has been established and successfully used to identify miRNA targets in a variety of plant species (Addo-Quaye et al., 2008; Sun et al., 2015; Wu et al., 2016; Xu et al., 2013). This approach provides a new and efficient strategy to validate miRNA targets on a large scale in plants.

Sugarcane mosaic virus (SCMV), belonging to the genus *Potyvirus* in the family *Potyviridae*, can induce severe disease in maize (*Zea mays* L.), sugarcane (*Saccharum sinensis*) and sorghum (*Sorghum vulgare*), which causes considerable losses in different field crops in the world (Fuchs and Grüntzig, 1995; Shukla et al., 1989). In China, SCMV is the major causal agent of maize dwarf mosaic disease, and the Beijing isolate (SCMV-BJ) belongs to a prevalent strain (Fan et al., 2003). In recent years, research on the interaction between SCMV and maize has mainly focused on protein-protein interactions and the genetic mapping of possible resistance genes (Cao et al., 2012; Tao et al., 2013; Zhu et al., 2014; Liu et al., 2017). Our previous study preliminarily characterized the siRNAs derived from SCMV in infected maize (Xia et al., 2014), while the relationship between SCMV and host endogenous miRNA pathways has not been investigated. In this study, we used small RNA and degradome sequencing to investigate the miRNAs and their targets in maize responsive to SCMV infection at 8 days post-inoculation (dpi). Moreover, we performed Northern blotting and quantitative reverse-transcription PCR (qRT-PCR) to determine the accumulation of several miRNAs and their targets in buffer- and SCMV-inoculated maize plants at 4, 8 and 12 dpi, respectively. Our results contribute to understand the possible roles of miRNAs and their targets in maize plants responsive to SCMV infection.

2. Materials and methods

2.1. Plant growth and SCMV inoculation

SCMV Beijing isolate (GenBank accession number AY042184) was maintained from diseased maize in the northern suburbs of Beijing (Fan et al., 2003). Maize (*Zea mays* L.) inbred line Zong31 was planted in a growth chamber (28 °C day and 22 °C night, 16 h light and 8 h dark cycles) and seedlings were inoculated with 0.01 M phosphate buffer (pH 7.0) or SCMV at the beginning of the 3-leaf stage. Systemic leaves were harvested at approximately 8 dpi for small RNA sequencing, when the newly developed leaves showed viral symptoms. Three replicate experiments were performed to detect the expression of miRNAs and their targets, and the samples were harvested at 4, 8 and 12 dpi, respectively. The collected samples were immediately frozen in liquid nitrogen and stored at -80 °C.

2.2. RNA extraction, small RNA sequencing and bioinformatics analysis

The systemic leaves of at least 15 maize seedlings inoculated with phosphate buffer or SCMV at 8 dpi were pooled for RNA extraction. Total RNA for small RNA sequencing, Northern blotting and qRT-PCR was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) and the concentration was examined with a Nanodrop ND-2000 spectrophotometer (ThermoFisher Scientific, Wilmington, USA).

For high-throughput sequencing, RNA sample integrity was verified by a Bio-Analyzer 2100 (Agilent Technologies, Waldbronn, Germany). Total RNA was separated through 17% denaturing polyacrylamide gels and small RNAs of between 10- and 60-nt were collected. Then, 5′ and 3′ RNA adaptors were ligated to small RNAs and followed by reverse transcription to produce cDNAs. These cDNAs were subsequently amplified by PCR and subjected to Solexa/Illumina sequencing by SBC (Shanghai Biotechnology Corporation, Shanghai, China) as previously described (Xia et al., 2014).

Adapter sequences and low-quality reads were trimmed from raw reads. The clean reads were then used to query miRBase Database (version 19.0, http://www.mirbase.org), of which the reads mapped to miRNA precursors by BLAST were identified as known miRNAs, and Rfam Database (version 10, http://rfam.janelia.org). Novel miRNAs were predicted using miRCat of the UEA small RNA workbench (Stocks et al., 2012).

2.3. Degradome sequencing and analysis

Isolation of mRNA from systemic leaves of SCMV-inoculated maize plants for degradome sequencing was performed as previously described (Addo-Quaye et al., 2008). Degradome cDNA libraries were sequenced on an Illumina Hiseq 2000/2500 (LC Sciences), and categories of sliced miRNA targets were identified and classified using the CleaveLand 3.0 pipeline (Addo-Quaye et al., 2009) and the ACGT301-DGEv1.0 pro-gram (LC Sciences). These sliced target transcripts were grouped into three categories according to the relative abundance of the tags at the target sites (Addo-Quaye et al., 2008). The criteria of different categories were described as previously reported (Sun et al., 2015).

2.4. Northern blotting analysis

Approximately 40 μg total RNA (prepared as described above) was used for the detection of known and novel miRNAs by Northern blotting. Total RNA separated in a 15% urea polyacrylamide gel was transferred to Hybond-NX membrane (GE Healthcare, Buckinghamshire, UK) using a semi-dry transfer apparatus (Amersham Biosciences, Piscataway, USA), which was then chemically cross-linked using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Pall and Hamilton, 2008). Probes were labelled and blots hybridized as previously reported (Xia et al., 2014). Probe sequences used for Northern blotting are shown in Supplementary Table S6.

2.5. Quantitative reverse-transcription PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) and treated with DNase I (TaKaRa Bio Inc., Dalian, China). Subsequently, approximately 2 μg total RNA was reverse transcribed into first-strand cDNA and qRT-PCR was performed as previously reported (Cao et al., 2012). The sequences of the primers used are listed in Supplementary Table S7.

3. Results

3.1. High-throughput sequencing of small RNAs

Virus infections often interfere with endogenous plant miRNA pathways, resulting in changes of accumulation of miRNAs that modulate cellular functions. To investigate the expression of maize miRNAs in response to SCMV infection, two small RNA profiles were obtained from the systemic leaves of phosphate buffer- (Mock-) and SCMV-