Virology 523 (2018) 64–73

Contents lists available at ScienceDirect

Virology

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

Small RNA-based interactions between rice and the viruses which cause the tungro disease

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ARTICLE INFO

Keywords:
Small RNA
miRNA
Deep Sequencing
RNA silencing
Gene regulation

ABSTRACT

Rice tungro disease is caused by a complex of two viruses, *Rice tungro bacilliform virus* (RTBV) and *Rice tungro spherical virus* (RTSV). To examine the RNAi-based defence response in rice during tungro disease, we characterized the virus-derived small RNAs and miRNAs by Deep Sequencing. We found that, while 21 nt/22 nt (nucleotide) siRNAs are predominantly produced in a continuous, overlapping and asymmetrical manner from RTBV, siRNA accumulation from RTSV were negligible. Additionally, 54 previously known miRNAs from rice, predicted to be regulating genes involved in plant defence, hormone signaling and developmental pathways were differentially expressed in the infected samples, compared to the healthy ones. This is the first study of sRNA profile of tungro virus complex from infected rice plants. The biased response of the host antiviral machinery against the two viruses and the differentially-expressed miRNAs are novel observations, which entail further studies.

1. Introduction

Rice Tungro Disease is caused by a complex of two viruses; *Rice tungro bacilliform virus* (RTBV, a pararetrovirus, Genus: *Tungrovirus,* Family: *Caulimoviridae*), having a double-stranded DNA genome and *Rice tungro spherical virus* (RTSV, a plant picornavirus, Genus: *Waikavirus,* Family: *Secoviridae*) with a single-stranded (+) RNA genome (Hibino et al., 1978; Jones et al., 1991; Hay et al., 1991; Shen et al., 1993). The vector Green Leaf Hopper (GLH), *Nephotettix virescens*, transmits tungro viruses in a semi-persistent manner. RTBV alone causes severe disease symptoms in the host but is incapable of independent transmission through GLH. RTSV, on the other hand, can be transmitted independently by GLH, but causes only mild stunting symptoms. GLH can efficiently transmit the viral complex (Hibino et al., 1978, 1979; Cabauatan and Hibino, 1985). Cloned RTBV DNA can also be transmitted to rice plants using a method based on Agrobacterium-mediated inoculation, known as agroinoculation (Dasgupta et al., 1991).

RNA silencing is a sequence-specific down-regulation of gene expression, largely conserved in varied eukaryotic organisms and mediated by 21–25 nt small interfering RNA (siRNA) and 21 nt micro-RNAs (miRNAs; Molnar et al., 2005; Qi and Hannon, 2005; Pelisson et al., 2007). RNA silencing pathway in plants involves either the suppression of transcription of the gene (Transcriptional gene silencing, TGS) or the degradation of RNA species (Post-transcriptional gene silencing, PTGS; Vaucheret et al., 2001; Eamens et al., 2008). RNA silencing, in a very distinctive role, acts as a highly potent defence response against foreign genomic material, such as viruses (Wang and Metzlaff, 2005; Eamens et al., 2008).

The production of siRNA involves the processing of double-stranded RNA into primary siRNA by Dicer-Like (DCL) proteins (Bernstein et al., 2001). The RNA induced silencing complex (RISC) then unwinds the siRNA duplex and one of the strands, the guide strand binds to the Argonaute (AGO) protein (Song et al., 2004), subsequently leading to the cleavage of the target RNA transcript (Dunoyer and Voinnet, 2005; Brodersen and Voinnet, 2006). Amplification and maintenance of RNAi signal i.e. production of secondary siRNA is carried out by the activity of host RNA-dependent RNA polymerase (RDR, Dalmay et al., 2000; Mourrain et al., 2000; Garcia-Perez et al., 2004; Himber et al., 2003). Virus infection also causes changes in the expression profile of the endogenous 21 nt miRNAs (Du et al., 2011; Chen et al., 2012). Genes encoding miRNAs are transcribed by plant RNA Pol II to produce primary transcripts (pri-miRNA), which are capped and polyadenylated. The pri-miRNA transcripts form hairpin-like structures which are processed by DCL1 and methylated by HEN1 to generate mature miRNA duplexes for loading into the AGO1 containing RISC, subsequently leading to endonucleolytic cleavage of the complementary transcripts (Voinnet, 2009; Vaucheret, 2008).

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https://doi.org/10.1016/j.virol.2018.07.022
Received 22 February 2018; Received in revised form 20 July 2018; Accepted 20 July 2018

Arabidopsis genome encodes four DCLs (DCL1–4, Schauer et al., 2002) whereas the *Oryza sativa* genome encodes five. OsDCL1 and OsDCL3a process hairpin dsRNA structures in the transcript of miRNA genes to produce canonical 21/22 nt miRNAs and non-canonical 24 nt miRNA respectively (Wu et al., 2009, 2010). While 21 nt phased (secondary) siRNAs are generated by OsDCL4 from OsRDR6-dependent dsRNA precursors (Wu et al., 2009; Song et al., 2012), OsDCL3b and OsDCL3a process OsRDR2-dependent dsRNA precursors into 24 nt phased siRNAs and 24 nt repeat associated siRNAs. Further, OsAGO4 protein associates with 24 nt miRNAs and 24 nt siRNAs and directs TGS, while the 21 nt miRNAs associate with OsAGO1 protein which subsequently leads to cleavage of the complementary transcript (Wu et al., 2009; Song et al., 2012; Wei et al., 2014).

RNA silencing pathways play important roles in anti-viral defence, as the viral genome replicates within the host cells. Although this natural defence mechanism has been studied in *Arabidopsis*, the details of the corresponding mechanism in rice are scant. In case of viral infection, dsRNA can be formed during viral replication when both (+) and (-) strand of viral RNA genome are present. This step of replication in plant RNA viruses involves either viral RNA polymerase or the host RNA polymerase II. Alternatively, a stem-loop or hairpin structure in the viral transcript (common with DNA viruses) can trigger the RNA silencing pathway (Ding and Voinnet, 2007). The predominant DCL implicated in antiviral defence in *Arabidopsis* is DCL4, which is evident by the fact that 21 nt siRNA (signature size of DCL4) is the most abundant class detected in virus infected cells. DCL2 plays a surrogate role in this process and produces 22 nt siRNA (Blevins et al., 2006; Bouché et al., 2006; Deleris et al., 2006; Xie et al., 2004). The stem-loop or the hpRNA is acted upon by DCL3, which is shown to play a major role in conferring resistance against DNA viruses and not RNA viruses. Cleavage by DCL3 leads to the production of 24 nt siRNA (Akbergenov et al., 2006; Blevins et al., 2006).

The 21- and 22-nt viral siRNAs (VsRNAs) bind to AGO proteins and direct silencing of viral RNAs. Of the ten Arabidopsis AGOs, AGO1 acts as the leading slicer, however, AGO7 and, more recently, AGO2 have also been found to play a role in anti-viral silencing (Harvey et al., 2011; Jaubert et al., 2011; Qu et al., 2008; Wang et al., 2011). The 24 nt siRNA generated by DCL3 is loaded on AGO4 containing RISC which leads to viral DNA methylation via the RNA-directed DNA methylation pathway (Raja et al., 2008; Zilberman et al., 2003; Xie et al., 2004).

The VsRNA profiles for several viruses of family *Caulimoviridae* have been studied. These include *Cauliflower mosaic virus* (CaMV), *Rubus yellow net virus* (RYNV), *Sweet potato badnavirus* A (SPBV-A), *Sweet potato badnavirus* B (SPBV-B), *Pagoda yellow mosaic associated virus* (PYMAV) and RTBV (Kreuze et al., 2009; Blevins et al., 2011; Kalischuk et al., 2013; Wang et al., 2014; Rajeswaran et al., 2014a). For RTBV, the agroinoculation procedure was used to generate infected rice plants (Rajeswaran et al., 2014a). Similarly, VsRNA profiles have been studied for the six episomal species of *Banana streak virus* (BSV), a badnavirus (Rajeswaran et al., 2014b). The VsRNA for several plant ssRNA viruses including *Citrus tristeza virus* (CTV), *Tobacco rattle virus* (TRV), *Sweet potato chlorotic stunt virus* (SPCSV), *Cotton leafroll dwarf virus* (CLRDV), *Grapevine leafroll associated viruses* (GLRaVs) and *Cucumber mosaic virus* (CMV) have also been studied (Donaire et al., 2009; Kreuze et al., 2009; Ruiz-Ruiz et al., 2011; Silva et al., 2011; Alabi et al., 2012). These viruses show a predominance of 21/22 nt VsRNA and a distinct hot spot at the 3′ terminus of the RNA genome. The 21 nt VsRNA was the most prevalent size class in case of *Rice stripe virus* (RSV), a *Tenuivirus* and showed a significant predisposition towards the genomic (sense) strand of the genomic RNA. VsRNA of all the size classes mapped asymmetrically across the RSV RNA with discernible hotspot at the 5′ terminal of the RNA 3 and RNA 4 of RSV (Yan et al., 2010; Xu et al., 2012).

Apart from siRNA mediated viral defence recent studies have also implicated miRNA in plant defence against pathogens including viruses. miRNAs in plants regulate a number of developmental pathways. Hence, a positive correlation between the development of symptoms and alteration in miRNA levels in case of virus infections cannot be ruled out. Modulation in host-specific miRNA populations in infection has been studied in many viruses, some of which are CTV, CLRDV, GLRaV-3 and RSV (Ruiz-Ruiz et al., 2011; Silva et al., 2011; Alabi et al., 2012; Guo et al., 2012; Romanel et al., 2012).

In this study, we have characterized the VsRNAs and miRNA populations in tungro infected rice plants by Deep Sequencing. Our results reveal for the first time that the VsiRNAs produced from the two viruses differ greatly in abundance. In addition, a number of miRNAs show differential accumulation.

2. Material and methods

2.1. Detection of virus infection

Rice plants (Taichung Native 1; TN1) showing symptoms of tungro disease were obtained from Indian Council of Agricultural Research-Indian Institute of Rice Research, Hyderabad, India. The presence of RTBV (primers MP F-5′TATGGATCCATGAGTCTTAGACCG 3′ and MP R-5′GGAGCTCTTCATCAGAATTTATTTC 3′) and RTSV (primers N7000F-5′GTATACGGCAGAT GATCATTCTTTTGAGCG 3′ and N9000R-5′CTGA CGTGCATTCCAATTATCTTCCTG 3′) was confirmed using PCR. For inoculation of rice plants, non-viruliferous GLH were given an acquisition access period of approximately 18 h from a rice plant infected with both RTBV and RTSV. Twenty day old Pusa Basmati-1 (PB1) rice seedlings were inoculated with three of these viruliferous GLH per plant by giving an inoculation access period of approximately 24 h. The GLH were removed from the plants, following the above inoculation access period and the plants were kept GLH-free till the symptoms of tungro became apparent in the inoculated plants. The inoculated plants which showed symptoms of tungro disease were screened for the presence of both RTBV and RTSV at 25 days post inoculation (dpi) by conventional PCR and RT-PCR, following which, infected leaf tissue was collected. For non-infected (control), uninoculated 45 days old rice seedlings were used.

2.2. sRNA library construction and analysis

The infected rice leaf tissue (screened and confirmed for the presence of viruses) and non-infected (confirmed for the absence of viruses) were used for sRNA library construction. Total RNA was isolated from 50 to 100 mg of four plant samples, using the RaFlex RNA isolation kit (GeNei) as per the manufacturer's instructions. The samples with intact 28S and 18S ribosomal bands on the agarose gel and A $_{260/280}$ ratio of 1.9–2.1 and A $_{260/230}$ ratio of 2.0–2.1 were used for sRNA library preparation. Further, the quality of the total RNA was then analyzed for their RIN (RNA Integrity Number) using Bioanalyzer 2100 (Agilent Technologies) using Pico chip as per manufacturer's instructions. The samples with a RIN 5.4–5.6 were used for library construction (Supplementary File S1). Four sRNA libraries were generated separately from leaves of uninfected (C1 and C2) and infected plants (E1 and E2) with different barcodes. The sRNA cDNA libraries were multiplexed and sequenced on Illumina Hi-Seq. 2000 Genome Analyzer. Then, the amplified libraries were analyzed on Bioanalyzer 2100 (Agilent Technologies) for their size distribution, quantification and quality using High Sensitivity (HS) DNA chip as per manufacturer's instructions (Supplementary File S1). The sequencing of the sRNA libraries and the bioinformatic analysis was outsourced to Xcelris Genomics Ltd., Ahmedabad, India who used CLC workbench flow for adapter trimming and read length filtration. RTBV- and RTSV-derived VsRNA sequences were identified using a local BLAST database of the RTBV complete genome, isolate West Bengal, GenBank: AJ314596.2 (with zero mismatches) and RTSV complete genome, West Bengal isolate, GenBank: AM234049.1 (with zero mismatches). Characterization of the libraries and mapping to the viral genome were performed using locally developed Perl script at Xcelris Genomics Ltd. SearchSmallRNA