Analysis of small RNAs derived from Chinese wheat mosaic virus

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Received: 23 February 2014/Accepted: 19 June 2014/Published online: 6 July 2014
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Abstract The virus-derived small interfering RNAs (vsiRNAs) of Chinese wheat mosaic virus (CWMV), a member of the genus Furovirus, were characterised from wheat plants by deep sequencing. CWMV vsiRNAs of 21–22 nt in length predominated, suggesting that there might be a conserved mechanism of DCL2 and DCL4 involvement in the biogenesis of vsiRNAs, as well as a common RNA silencing pathway in CWMV-infected wheat plants. The 5′-terminal base of vsiRNAs was biased towards A/U, suggesting that CWMV vsiRNAs might be loaded into diverse AGO-containing RISCs to disturb the gene expression of host plants. Possible targets for some of the vsiRNAs were predicted.

RNA silencing is an ancient and conserved mechanism to protect eukaryotic genomes against aberrant endogenous or exogenous RNA molecules [1–3]. Defence mechanisms can be induced by double-stranded RNAs (dsRNA) or single-stranded RNAs (ssRNA) with foldback structures that are processed into small interfering RNAs (siRNAs) of specific size by Dicer-like (DCL) proteins [4, 5]. The siRNAs are integrated into the RNA-induced silencing complex (RISC) and regulate the expression of target genes through base-pairing mechanisms [6]. Virus-derived small interfering RNAs (vsiRNAs) are key elements in plant antiviral strategies [7].

There are at least four DCL (DCL1–4) proteins with distinct functions in plants [8]. DCL2 and DCL4 are the most important plant DICERs involved in virus-induced RNA silencing; their products are 21- and 22-nt vsiRNA, respectively [9–11]. DCL3 is thought to be responsible for 24-nt vsiRNA in the absence of DCL2 and DCL4 in Arabidopsis thaliana [9, 12, 13]. VsiRNAs of some plant viruses and viroids have been characterised by cloning and sequencing since the first discovery of vsiRNA in plants [14–16]. These vsiRNAs share features with host siRNAs and can mediate RNA silencing, resulting in specific antiviral immunity [7]. Interestingly, a few recent reports have shown that some of these vsiRNAs can guide the degradation of the homologous cellular transcripts using base-pairing mechanisms to create cellular conditions suitable for viral proliferation or to induce disease symptoms [17].

Much less is known about the RNA silencing pathways in wheat, but the biochemical framework for small RNA
production and RNA silencing is conserved in plants [18]. Although many different viruses are known to infect wheat, there have been no reports of the origin and function of vsiRNAs in this important plant. Chinese wheat mosaic virus (CWMV) (genus Furovirus, family Virgaviridae) naturally infects wheat [19], typically causing light chlorotic streaking on the young leaves and bright yellow chlorotic streaking or even purple chlorotic stripes on old leaves. Severely infected plants become stunted, wilt, and later die [20, 21]. This typically leads to grain yield losses of 10–30% and, in severe cases, up to 70% [21, 22].

CWMV has a bipartite single-stranded positive-sense RNA genome [19, 20, 23]. RNA1 (7,147 nt) encodes three proteins required for viral replication and movement, while RNA2 (3,564 nt) encodes a 19-kDa major coat protein (CP), a minor CP of 84 kDa produced by the occasional read-through of the UGA termination codon, and a 19-kDa RNA silencing suppressor [19, 23]. To investigate the relationship between CWMV and its host wheat plant, we have characterised the vsiRNAs of CWMV from infected wheat plants using deep sequencing.

An isolate of CWMV was collected from Rongcheng County, Shandong Province, China, and maintained on wheat cultivar Yannong 22 in a disease nursery at the Zhejiang Academy of Agricultural Sciences (ZAAS). Leaves with typical mosaic symptoms were collected, and systemic infections were confirmed by ELISA and RT-PCR specific for CWMV [20]. Total RNAs were extracted from CWMV-infected plants using TRIzol Reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA concentrations were estimated using a spectrophotometer (Nanodrop ND-2000, Thermo Fisher Scientific, Wilmington, DE, USA), and the RNA sample integrity was verified using a Bio-Analyser 2100 (Agilent Technologies, Waldbronn, Germany). Small RNA libraries were prepared using the standard Solexa protocol. Briefly, the total RNA was size-fractionated by 15% polyacrylamide gel electrophoresis (PAGE), and small RNAs of 18–30 nt were isolated. Then, the purified small RNAs were ligated with 5’- and 3’-adaptors at both ends. The ligated small RNAs were further purified and reverse transcribed into cDNAs. These cDNAs were finally amplified using the following PCR program: 98 °C for 30 s, followed by 15 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 15 s and, finally, at 72 °C for 10 min. The PCR products were ethanol precipitated and purified using Spin-X filter columns. Finally, the small RNA libraries were sequenced. The sRNA sequences were subjected to a BLAST search against the complete genomic sequence of the CWMV Rongcheng isolate (accession numbers: RNA1, AJ271838; RNA2, AJ271839) [23], and small RNA sequences that did not produce gaps in the alignment and were 18–30 nt in size were selected as candidate vsiRNAs. The output files were searched and summarised using customised scripts in GAWK to identify those with an exact match for CWMV over their entire length. This process allowed us to identify all of the sequences that exactly matched CWMV in the range of 18–30 nt. Further statistical analyses and summaries were performed using Microsoft Excel 2013. Putative targets of the vsiRNAs were identified by psRNA Target (http://plantgrn.noble.org/psRNATarget/) using the following parameters: maximum expectation, 3; length for complementarity scoring, 20; allowed maximum energy to unpair the target site (UPE), 25; flanking length around target site for target accessibility analysis, 17 bp; range of central mismatch leading to translational inhibition: 9–11 nt. The unigene DFCI Gene Index of Triticum aestivum (TAGI) (version 12) was used as the pool for putative target prediction.

A total of 9,751,971 (3,124,607 unique) sRNAs were sequenced from the systemic leaves of infected plants. After the sequences with incomplete matching to the 5’ or 3’ adapter and those resulting from adapter self-ligation were removed, there were 1,899,821 (76,179 unique) sequences from 18 to 26 nt in length. The 21-nt class was the most dominant with 791,121 (18,452 unique) sequences, accounting for 26.75% (42.9% unique) of the total sequences (Fig. 1A). A BLAST search against the CWMV genome yielded a total of 62,292 sequences (19,091 unique) of vsiRNAs, covering almost the entire genome (Table S1). Of these, 39,814 (12,325 unique) sequences mapped to the sense strand of the genome, with 17,723 (6,228 unique) and 22,091 (6,096 unique) sequences corresponding to RNA1 and RNA2 respectively. The other 22,478 (6,766 unique) sequences mapped to the antisense strand of the genome, with 9,616 (3,506 unique) and 12,862 (3,260 unique) sequences mapping to RNA1 and RNA2, respectively. Of the total vsiRNA population, 97.0% (60,413) of the molecules were in the size range of 19 to 24 nt, with 21-nt vsiRNAs being the most abundant, followed by 22 nt and 20 nt (Fig. 1B).

The vsiRNAs were mostly clustered in the 5’ half of both the sense and antisense strands of RNA1 but were rather evenly distributed along RNA 2 (Fig. 2B, D). A total of 31 peaks or hotspots (10 on RNA1 and 21 on RNA2) of vsiRNAs with more than 200 reads were identified (Fig. 2B, D). Most of these hotspots occurred within open reading frames (ORFs), notably ORF1 on RNA1, which encodes the viral methyltransferase (MET) and helicase proteins (Hel) (Fig. 2A, B), and in each of the ORFs on RNA2 (Fig. 3C, D), 21-nt vsiRNAs, followed by 22-nt vsiRNAs, predominated within these hotspots (Fig. 2B, D). All vsiRNAs shared a similar bias at their 5’ terminus (Fig. 3A–E), with twice as many A/U as G/C.
The targets of vsiRNAs with more than 50 reads were predicted using the web-based psRNA Target Server (http://plantgrn.noble.org/psRNATarget/). There were 331 target genes that were ultimately predicted (Table S2). Most of the CWMV vsiRNAs were predicted to have one or more targets, but some, such as vsiRNA-4, vsiRNA-5, and vsiRNA-11, had no targets predicted (Table S2), perhaps due to our limited knowledge of the wheat genome. These predicted targets were involved in a broad range of biological processes, such as biological regulation, cellular component organisation, cellular process, localisation, metabolic processes, and responses to stimuli, suggesting that these vsiRNAs might play important roles due to their interactions with their targets during CWMV infection. These findings help to identify wheat genes for further investigation.

Our study is the first report on the characterisation of vsiRNAs produced from a member of the genus Furovirus in infected wheat plants. The majority of vsiRNAs were 19–24 nt long, and 21-nt vsiRNAs were the most dominant. This size distribution pattern is similar to that reported for other plant virus vsiRNA populations [16].
has been reported that DCL4 is the primary sensor of viral dsRNAs and produces 21-nt vsiRNAs as the first antiviral defence in plants of the genus *Arabidopsis* [24]. It has also been shown that DCL2 acts as a DCL4 surrogate to generate 22-nt vsiRNAs but can initiate equally potent RNA-based antiviral immunity independent of DCL4 [12]. The specific vsiRNA composition suggested that there might be a conserved mechanism of DCL2 and DCL4 involvement in the biogenesis of vsiRNAs and a common RNA silencing pathway in CWMV-infected wheat plants. This viewpoint is supported by the findings of both RdRP activity and Dicer activity in wheat plants [12]. By contrast, there were far fewer 23- and 24-nt vsiRNAs, suggesting that DCL3, which is involved in the reception of
the non-cell autonomous silencing signal \[25, 26\], plays only a minor role in the biogenesis of CWMV vsiRNAs in wheat plants.

Most CWMV vsiRNAs (63.9%) were localised on the sense strand of the RNA segments, as found in several other positive single-stranded RNA viruses \[16, 27, 28\]. These results consistently suggest that the highly structured sense RNAs of positive ssRNA viruses are preferentially processed by the DCLs. Most of the 31 hotspots (Fig. 3) along the genome of CWMV were formed by 21- and 22-nt vsiRNAs, suggesting that these hotspots could be preferential cleavage sites for DCLs and that DCL-2/DCL-4 might play a major role in vsiRNA formation from these hotspots. All of the flanking sequences of these hotspots were further predicted to form stem-loop-like secondary structures (see supplementary Figure S1), similar to those of host-plant pre-miRNA. These data support the viewpoint that the pre-miRNA-like structural regions present in viral ssRNAs are the precursors of the vsiRNAs that trigger RNA-based antiviral immunity \[29–31\].

Previous reports have shown that the loading of plant siRNAs into a particular Argonaute (AGO)-containing RNA-induced silencing complex (RISC) is largely preferentially dictated by their 5’-terminal nucleotides \[32–35\]. Here, a strong bias of CWMV vsiRNAs for a 5’ terminus with A and U supports the view that most vsiRNAs may be loaded onto diverse AGO-containing RISCs to disturb the gene expression of host plants.

Acknowledgments This work was funded by the Special Fund for Agro-Scientific Research in the Public Interest of China (201303021), China Agriculture Research System (CARS-3-1) from the Ministry of Agriculture of China, Project of New Varieties of Genetically Modified Wheat of China (2011ZX08002-001), and the Zhejiang Provincial Foundation for Natural Science (Y3090657). We thank Professor M. J. Adams, Rothamsted Research, Harpenden, UK, for help in correcting the English of the manuscript.

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