Specific Enrichment of miRNAs in *Arabidopsis thaliana* Infected with *Tobacco mosaic virus*

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Abstract

RNA silencing is a broadly conserved machinery and is involved in many biological events. Small RNAs are key molecules in RNA silencing pathway that guide sequence-specific gene regulations and chromatin modifications. The silencing machinery works as an anti-viral defense in virus-infected plants. It is generally accepted that virus-specific small interfering (si) RNAs bind to the viral genome and trigger its cleavage. Previously, we have cloned and obtained sequences of small RNAs from *Arabidopsis thaliana* infected or uninfected with crucifer *Tobacco mosaic virus*. MicroRNAs (miRNAs) accumulated to a higher percentage of total small RNAs in the virus-infected plants. This was partly because the viral replication protein binds to the miRNA/miRNA* duplexes. In the present study, we mapped the sequences of small RNAs other than virus-derived siRNAs to the *Arabidopsis* genome and assigned each small RNA. It was demonstrated that only miRNAs increased as a result of viral infection. Furthermore, some newly identified miRNAs and miRNA candidates were found from the virus-infected plants despite a limited number of examined sequences. We propose that it is advantageous to use virus-infected plants as a source for cloning and identifying new miRNAs.

Key words: *Arabidopsis thaliana*; *Tobacco mosaic virus*; microRNA; RNA silencing

1. Introduction

Small RNAs play important roles in RNA silencing mechanisms which are involved in many biological processes in eukaryotes. These small RNAs guide post-transcriptional gene silencing by inhibiting translation or degrading the target mRNAs, and guide transcriptional gene silencing by modifying chromatin.[1–3]

In *Arabidopsis thaliana*, functional small RNAs are mostly 21–24 nt long. The best-studied endogenous small RNAs are microRNAs (miRNAs). In *Arabidopsis*, miRNAs are excised from primary miRNA transcripts forming stem-loop structures by the RNase III enzyme DICER-LIKE 1 (DCL1). This enzyme works coordinately with HYL1 and SERRATE to produce miRNA/miRNA* duplexes.[4–9] miRNAs are loaded into the RNA-induced silencing complex (RISC) where the complementary mRNAs are cleaved by ARGONAUTE 1 (AGO1), which has Slicer activity.[10,11] Plants have some unique small RNAs in addition to miRNA. Trans-acting siRNA (tasiRNA) is produced from long double-stranded RNA (dsRNA) by DCL4 with its binding partner, DRB4.[12–14] miRNA and tasiRNA are mainly involved in plant development, response to environmental stresses and so on through regulating gene expression.[1–3] siRNAs derived from natural-antisense transcripts (nat-siRNAs) exist in *Arabidopsis* and function in the stress response and bacterial disease resistance.[15,16] However, the numbers and functions of nat-siRNAs are not fully understood.

The RNA silencing pathway also serves as an anti-viral defense in plants. Viral genome-derived siRNAs are detected in plants infected with RNA viruses[17,18] and DNA viruses.[19,20] The lengths of these siRNAs differ with the type of viruses,[18,21] suggesting the involvement of different DCLs in producing the respective viral siRNAs. It is conceived that the resulting siRNAs are loaded into the RISC as well as endogenous small RNAs, bind the viral genome through complementary sequences and direct the degradation of the viral genome. To counteract the silencing machinery of plants, many viruses have evolved genes which encode silencing suppressor proteins with distinct properties.[22,23] Many suppressors have dsRNA binding activity so that virus siRNAs are trapped, resulting in inhibition of the silencing machinery.[24,25] In contrast to the dsRNA binding strategy, the *Cucumber mosaic virus*-encoded 2b protein directly interacts with AGO1 and inhibits its activity[26] and *Turnip crinkle virus*-encoded P38 protein suppresses DCL4 activity.[18]

We have examined the crucifer *Tobacco mosaic virus*-Cg (TMV-Cg) infection of *Arabidopsis* as a model system to study plant-*Tobamovirus* interaction.[27,28] TMV-Cg is a positive-sense, single-stranded RNA virus. We have previously cloned and sequenced small RNAs from *Arabidopsis* infected or uninfected with TMV-Cg and demonstrated that miRNAs increased in the virus-infected plants.[28] In TMV and *Tomato mosaic virus* (ToMV), which both belong to the *Tobamovirus* family, 126K replication proteins suppress RNA silencing.[29,30] Thus, it is postulated that the 126K replication protein of TMV-Cg also acts as a silencing suppressor because it binds to small dsRNAs.[28] Owing to this activity, 126K replication protein binds to miRNA/miRNA* duplexes, resulting in the accumulation of miRNAs. In the present study, we examined all small RNA sequences obtained from *Arabidopsis* infected or uninfected with TMV-Cg. We then mapped the region where each small RNA sequence was derived in the *Arabidopsis* genome. Among the small RNAs from virus-infected plants, we identified several newly identified miRNAs and miRNA candidates. Virus-infected plants could be an effective source to find novel miRNAs.

2. Materials and methods

2.1. *Mapping of small RNAs using BLAST searches*

We have analyzed small RNA sequences obtained previously; 1700 and 543 reads from the leaves of *Arabidopsis* infected and uninfected with TMV-Cg at 3 days post-infection (dpi), respectively.[28] Each sequence of non-viral genome origin was mapped to the *Arabidopsis* genome by NCBI BLAST (http://www.ncbi.nlm.nih.gov/ BLAST/) and TAIR BLAST (http://www.arabidopsis. org/Blast/index.jsp) searches. During searches, we allowed one mismatch at its 3′ end, because 3′ ends of

small RNAs in virus-infected plants are possibly unmethylated and unstable.[31] All BLAST searches were performed automatically using BioPython script programs.

2.2. *Other computational analyses*

Secondary structures of miRNA precursors were predicted using the mFOLD program (http://www.bioinfo. rpi.edu/applications/mfold/).[32] Targets of miRNAs were predicted using the miRU: Plant microRNA Potential Target Finder (http://bioinfo3.noble.org/ miRNA/miRU.htm).[33]

To compare the ratio of our sequences to that of other databases, each sequence was searched against the Arabidopsis MPSS Plus Database (http://mpss.udel. edu/at/)[34] and the Arabidopsis Small RNA Project (ASRP) (http://asrp.cgrb.oregonstate.edu/).[35] In Table 2, miRNAs were searched against 4Co10 (Co1-0 wildtype inflorescence tissues, small RNA 454) in MPSS and Co1-0 (Leaf, Inflorescence) in ASRP. In Fig. 2, small RNA sequences were searched against all datasets in both databases.

2.3. *Plant materials and virus inoculation*

The following *Arabidopsis* plants were used; Col-0 (wild type), *dcl1-9* (BC6), *dcl2-2* (SALK_123586), *dcl3-1* (SALK_005512) and *dcl4-1* (BC6). Dr Herve Vaucheret (INRA, France) kindly provided *dcl1-9* (BC6) and *dcl4-1* (BC6), which were backcrossed to Col-0 six times. All plants were grown in a growth chamber at 22°C with photoperiod of 16 h. For the virus inoculation, leaves of 4-week-old plants were dusted with carborundum and gently rubbed with 20 μg/mL of TMV-Cg solution. For mock inoculation, TMV-Cg solution was replaced with 10 mM sodium phosphate buffer.

2.4. *Northern blot analysis*

Total RNA was extracted at 10 dpi from the inoculated leaves, mock-inoculated leaves and inflorescences of *Arabidopsis* using ISOGEN reagent (Nippon gene). Aliquots of 10 μg of total RNA were loaded and resolved on a denaturing 15% polyacrylamide gel (7 M urea). To make the miR847(5′) probe, oligonucleotide (TCGGCTTCCCATTCCTCTTCA) was 5′ end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (TOYOBO). Hybridization was carried out at 40°C using PerfectHyb Plus hybridization buffer (Sigma). The blots were exposed on imaging plates, and signals were visualized using BAS-2500 (Fuji).

3. Results and discussion

3.1. *Increase of miRNAs in Arabidopsis infected with TMV-Cg*

We have previously sequenced small RNAs from the leaves of *Arabidopsis* infected or uninfected with

TMV-Cg at 3 dpi. We obtained 1700 sequences from the virus-infected plants and 543 sequences from mock-infected plants.[28] We reported that total miRNAs were more abundant in virus-infected plants than in mock-infected plants, as shown in the northern blot results of some miRNAs. To further compare the small RNA expression profiles between virus-infected plants and mock-infected plants, we performed extensive BLAST searches to map all small RNA sequences to the *Arabidopsis* genome (Table 1), except for siRNAs derived from the viral genome which were 210 reads (12.4 % of total reads), as previously reported.[28]

The percentage of total miRNAs was 5.5 times higher in TMV-infected plants (25.4%) than that in mock-infected plants (4.6%) (Table 1 and Kurihara et al.[28]). In contrast to the large increase of miRNAs in TMV-Cg-infected plants, the percentage of tasiRNAs was almost unchanged (2.0% in mock-infected plants and 1.6% in virus-infected plants) (Table 1). tasiRNA is another class of small RNAs in plants which is produced by the RNase III enzyme DCL4 protein.[36] This suggests that miRNAs, among other characterized small RNA classes, specifically increased by the TMV infection.

We have recently demonstrated that the increase of miRNAs could be attributed to the activity of the viral-encoded 126K protein, which binds and therefore stabilizes miRNA/miRNA* duplexes.[28] However, we found that there is no big difference between the ratios of miRNA to miRNA* in mock- and TMV-infected plants (6.6 times in mock-infected plants and 10 times in TMV-infected plants). This fact possibly indicates that there is still another mechanism of the miRNA increase or stabilization in the virus-infected plants, in addition to binding activity of 126K protein to miRNA/miRNA* duplexes.

We compared the percentage of each miRNA between mock-infected and virus-infected plants (Fig. 1). The percentages of all miRNAs except for miR160 increased as a result of virus infection. Some miRNAs showed remarkable increases, especially in miR163 (from 1.1 to 9.3%), miR164 (from 0.2 to 5.1%) and miR167 (from 0.4 to 2.7%). Most others increased 2–3 times in virus-infected plants. This result indicates that binding activity of the TMV-Cg replication protein to those miRNA/miRNA* duplexes is specifically high, or that transcription of some miRNAs is also activated.

Inhibition of the miRNA pathway has been reported with many other virus-infected plants and with plants transformed with viral proteins.[26,37–39] Initially we expected that miRNA up-regulation should lead the down-regulation of the target mRNAs and that might work as a defense response to virus infection. However, in the TMV-Cg-infected plants, the target mRNAs of miRNAs were not down-regulated, even though miRNAs increased (Supplementary Fig. S1). The increase of miRNA does not induce target mRNA degradation and the miRNA pathway is completely disrupted in plants infected with TMV-Cg, as is the case during infection with other plant viruses. This supports the notion that the replication protein of TMV-Cg acts as a silencing suppressor.

Table 1. Summary of non-viral small RNA sequences from TMV-infected or uninfected *Arabidopsis*

Class	Mock		TMC-Cg	
miRNA				
Known miRNA	25	(4.6%)	379	(25.4%)
miRNA*	4	(0.7%)	37	(2.5%)
Small RNA drived from miRNA precursor	0	(0%)	5	(0.3%)
tasiRNA	11	(2.0%)	24	(1.6%)
Gene				
Sense	15	(2.8%)	29	(1.9%)
Antisense	3	(0.6%)	15	(1.0%)
Sense and antisense	0	(0%)	2	(0.1%)
Intergenic region	100	(18.4%)	94	(6.3%)
rRNA	164	(30.2%)	486	(32.6%)
tRNA	19	(3.5%)	47	(3.2%)
sRNA	2	(0.4%)	1	(0.1%)
Transposon	11	(2.0%)	9	(0.6%)
Unknown	189	(34.8%)	362	(24.3%)
Total reads	543	(100%)	1490[a]	(100%)

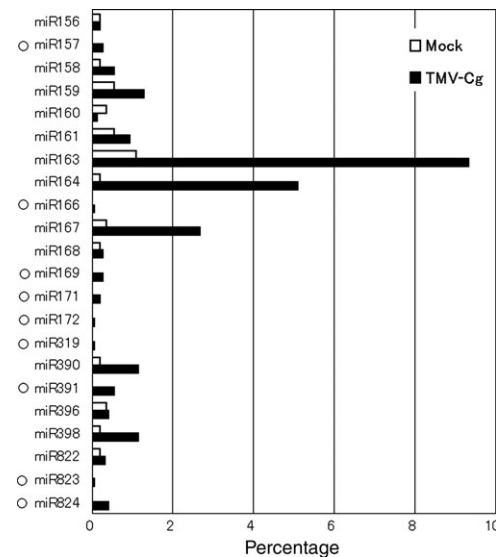[a]Viral genome-derived siRNA are not included.

Figure 1. Most miRNAs increased in TMV-Cg-infected plants. Percentages of each miRNA cloned in non-viral small RNAs are shown. All miRNAs except for miR160 increased in the virus-infected plants. Circle indicates miRNA that was detected only in the small RNA dataset from the virus-infected plants.

3.2. Unique small RNAs in gene and intergenic regions in TMV-infected and mock-infected plants

In addition to the increase of miRNA percentage in plants infected with TMV-Cg, some miRNAs were cloned and sequenced only in the virus-infected plants (Fig. 1; indicated with circles). This led us to speculate that the virus-infected plants could be a good source for finding new miRNAs.

If there are new miRNAs in our small RNA sequence reads, they are likely to be mapped in the gene or intergenic region (Table 1). Therefore, we checked whether there are new sequences in the small RNAs mapped to the gene and intergenic regions compared with other small RNA databases; ASRP[35] and MPSS[34] (Fig. 2). Interestingly, not only in TMV-Cg-infected plants but also in mock-infected plants, most small RNAs were not detected in ASRP and MPSS databases and were unique to our reads, although there are 34 times more sequences in MPSS and 260 times more in ASRP than in our dataset from virus-infected plants. This is possibly due to the differences in RNA sources and cloning methods between ours and other groups' which performed deep sequencing using the 454 sequencing method. This suggests that many small RNAs remain to be discovered. They will be found if the cloning condition is changed even though the total reads are less than those using 454 sequencing. Thus, it is quite possible that there are new functional small RNAs in our resources of virus-infected and mock-infected plants. Especially in the TMV-infected plants in which many kinds of miRNAs were more abundant, we can expect new miRNA reads.

3.3. miRNA candidates in plants infected with TMV-Cg

Three miRNAs (miR822, miR823, and miR824) reported very recently[40] were found in our reads of virus-infected plants and, moreover, their appearances were more frequent in our total small RNAs from virus-infected plants than those in MPSS and ASRP databases
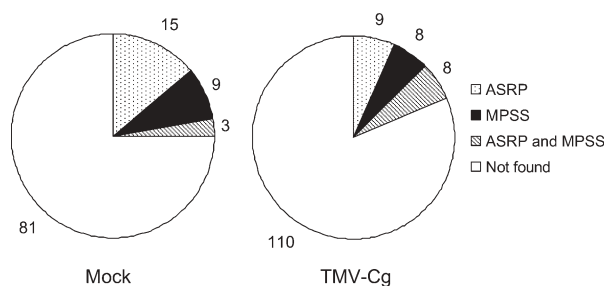
Table 2. Newly identified miRNAs and miRNA candidate are more abundant in TMV-Cg-infected *Arabidopsis* than in other databases

miRNA	Number of read (percentage%)			
	TMV	MPSS	ASRP Leaf	ASRP Inflorescence
miR847 (5′)	2 (0.12%)	0	0	0
miR822	5 (0.29%)	0	0	0
miR823	1 (0.06%)	1 (0.01%)	7 (0.04%)	9 (0.01%)
miR824	6 (0.35%)	15 (0.13%)	15 (0.09%)	42 (0.05%)
Total	1700 (100%)	11 631 (100%)	15 826 (100%)	78 583 (100%)

(Table 2). This supports that virus-infected plants are good source for identifying novel miRNAs.

Next, we tried to find out novel miRNAs in the small RNA data of the virus-infected plants. Before confirmation and validation of new miRNAs, we performed secondary structure predictions using the mFOLD program. We used sequences ∼300 nt in length from around the matched stretch where small RNAs mapped to the gene and intergenic regions. Three different sequences formed hairpin structures, which are common structures to miRNA precursors (Fig. 3 and Supplementary Fig. S2), suggesting that these sequences are candidates of miRNAs.

Out of them, we found that one candidate could be mapped to the 5′ arm of miR847 (Fig. 3). Though it may be a variant of miR847*, it is possible that the small RNA is also functional because it has an 8 nt overhang instead of 2 nt, which the DCL product generally has (Fig. 3). Thus, we named it miR847(5′) and analyzed further. In our small RNA data, there was no miR847 sequence while miR847(5′) was read twice. miR847(5′) was not cloned in ASRP and MPSS databases (Table 2). The other two sequences forming hairpin structures were named candidates A and B (Supplementary Fig. S2). Each of them was read once in the small RNA data from virus-infected plants.

We performed northern blot analyses to detect these miRNA candidates and confirm their expression patterns. Out of three miRNA candidates, only miR847(5′) could be detected (Fig. 4). Candidates A and B were not detected.

miR847(5′) was not expressed in flowers but in rosette leaves. It was more abundant in TMV-Cg-infected plants than in mock-infected plants (Fig. 4). This result reflected the tendency observed in the cloning frequency discussed earlier and northern blot results of other miRNAs reported previously.[28] Furthermore, miR847(5′) was expressed at a similar level in mutants except in the *dcl1* mutant. In *Arabidopsis*, most miRNAs are produced by DCL1 in general, whereas, for example, tasiRNAs require both DCL1 and DCL4 for their productions. Therefore, if the expression of a small RNA is reduced only in *dcl1* mutant plants, the small

Figure 2. Most sequences in this study are not found in other databases. Each small RNA sequence was searched against the ASRP and MPSS databases. Numbers of different sequences in gene and intergenic regions are shown, which are also found in ASRP and/or MPSS database, or not found in both. In both mock-infected and TMV-Cg-infected plants, most sequences are unique in this study.
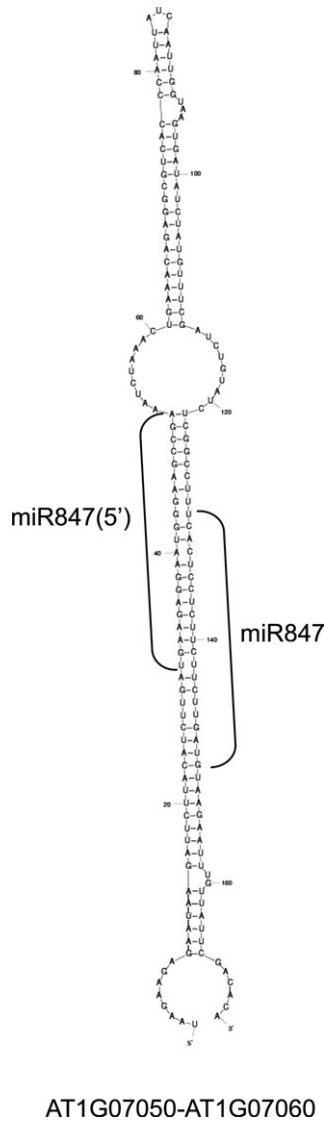
AT1G07050-AT1G07060

Figure 3. Predicted secondary structure of new miRNA candidate's precursor. Secondary structure of new miRNA candidate found in this study was predicted using the mFOLD program. miR847(5′) is derived from the 5′ arm of miR847 but they are not in miRNA/miRNA* relationship.

RNA is possibly a miRNA. Therefore, the northern blot result supports that miR847(5′) is an miRNA. In contrast, miR847 could not be detected by northern blot analysis neither in leaves nor in inflorescences (data not shown).

The predicted targets of these three miRNA candidates and miR847 are shown in Supplementary Table S1. However, we have not yet detected their possible cleaved fragment by the 5′ RACE method.[38] On the basis of the available data, we could not conclude which of small RNAs is a functional miRNA; miR847 and miR847(5′) at present. Further analyses will be needed to make conclusion.

Recently, many results of comprehensive small RNA sequencing by 454 sequencing have been reported one after another in *Arabidopsis.*[40−43] It has been demonstrated that miRNAs are enriched in loss-of-function mutants of RNA-dependent RNA polymerase 2 (RDR2) and RNA polymerase IV because they are required for the majority of endogenous siRNAs.[41,43] By analyzing these mutant plants with the 454 sequencing method, dozens of miRNAs were discovered. In this study, we showed that plants infected with TMV-Cg are good materials for cloning and finding novel miRNAs frequently because cloning percentages of many miRNAs increase and newly identified miRNAs are found in virus-infected plants. Moreover, considering that the number of small RNA sequences read were tens to hundreds less than those of other groups, TMV-infected plants are very efficient materials to discover new miRNAs. It is anticipated that new miRNAs would be found from small RNAs in the virus-infected leaves if extensive reads were performed.

Most *Arabidopsis* miRNAs, expressed abundantly and conserved, have already been identified. However, there are many kinds of miRNAs which are less-expressed and non-conserved[42] and it is possible that some are yet to be discovered. These miRNAs will be hereafter identified using plants under various conditions or mutant plants, which will leads to the identification of overall miRNAs
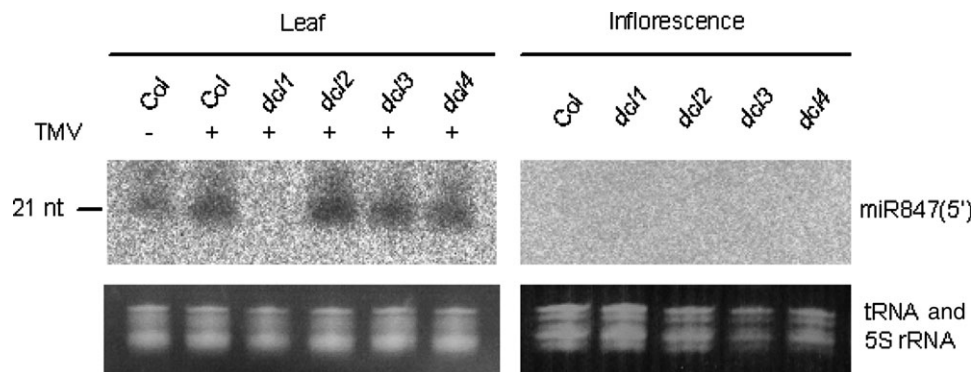

Figure 4. Northern blot analysis of miR847(5′). Total RNA (10 μg) from rosette leaves (mock-infected and infected with TMV-Cg) and inflorescences were used for northern blot analyses. miR847(5′) increased during TMV-Cg infection but was not detected in the *dcl1* mutant, indicating that miR847(5′) is miRNA. miR847(5′) was not detected in inflorescences.

and siRNAs and the understanding of how small RNAs are involved in many biological processes in *Arabidopsis*.

Supplementary Data: Supplementary data are available online at www.dnaresearch.oxfordjournals.org.

Funding

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