The sequence profiles of small interfering RNAs (siRNAs) in Arabidopsis infected with the crucifer tobamovirus tobacco mosaic virus (TMV)-Cg were determined by using a small RNA cloning technique. The majority of TMV-derived siRNAs were 21 nt in length. The size of the most abundant endogenous small RNAs in TMV-infected plants was 21 nt, whilst in mock-inoculated plants, it was 24 nt. Northern blot analysis revealed that some microRNAs (miRNAs) accumulated more in TMV-infected plants than in mock-inoculated plants. The question of whether the TMV-Cg-encoded 126K replication protein, an RNA-silencing suppressor, caused small RNA enrichment was examined. Transient expression of the replication protein did not change the pattern of miRNA processing. However, miRNA, miRNA* (the opposite strand of the miRNA duplex) and hairpin-derived siRNA all co-immunoprecipitated with the replication protein. Gel mobility-shift assays indicated that the replication protein binds small RNA duplexes. These results suggest that the tobamovirus replication protein functions as a silencing suppressor by binding small RNA duplexes, changing the small RNA profile in infected plants.

RNA silencing in plants acts as an immune system, defending against invading nucleic acids such as viruses, transposons and transgenes (Voinnet, 2001). During virus infection, virus-derived small interfering RNAs (siRNAs) are produced (Hamilton & Baulcombe, 1999). These siRNAs are then incorporated into the RNA-induced silencing complex (RISC) and viral RNAs complementary to the siRNA sequences are cleaved. Accordingly, many viruses have developed RNA-silencing suppressors, which act as a counter defence strategy (Voinnet et al., 1999).

Tobacco mosaic virus (TMV), Tomato mosaic virus (ToMV) and crucifer tobacco mosaic virus-Cg (TMV-Cg) belong to the genus Tobamovirus and have positive-sense, single-stranded RNA genomes. Previously, the 126K replication proteins encoded by the TMV and ToMV genomes were shown to act as RNA-silencing suppressors (Kubota et al., 2003; Ding et al., 2004). In the present study, we surveyed the small RNA population in Arabidopsis thaliana infected with the crucifer tobamovirus TMV-Cg.

Three days after inoculation, total RNA was extracted from inoculated leaves by using ISOGEN solution (Nippon Gene). Low-molecular-mass (LMM) RNA was recovered from the total RNA by using anion-exchange chromatography (RNA/DNA Midi kit; Qiagen) according to the manufacturer’s instructions. Small RNAs (21–24 nt) were cloned and sequenced as described previously (Lagos-Quintana et al., 2001; Watanabe et al., 2005).

We determined the sequences of 1700 small RNAs, identifying 210 virus-derived siRNAs (Fig. 1a), 61.9% of which were 21 nt in length. The distribution of the virus-derived siRNA fragments was dispersed over most of the viral genome (Fig. 1b). Moreover, of these 210 virus-derived siRNAs, 140 were derived from the viral positive strand and 70 from the minus strand, which is a viral replication intermediate (Fig. 1b). The fact that positive-strand siRNAs accumulated more than minus-strand siRNAs is consistent with the case for tombusvirus cymbidium ringspot virus, described by Molnar et al. (2005).

We also cloned small RNAs isolated from healthy, mock-inoculated Arabidopsis leaves by using the same procedures. The sequences of 543 cloned small RNAs were determined. The size of the most abundant endogenous small RNAs in mock-inoculated plants was 23 or 24 nt, whilst in virus-infected plants, it was 21 nt (Fig. 1c). MicroRNA (miRNA) is an endogenous small RNA of approximately 21 nt that negatively regulates complementary mRNAs through the function of the RISC (Bartel, 2004). The present results suggest that some miRNAs in
virus-infected plants accumulate to a higher level (25.4% of total small RNAs, excluding TMV-specific siRNAs) than those in mock-inoculated plants (4.6% of total small RNAs sequenced). However, it was difficult to compare accumulation levels precisely, because even if the cloning procedures worked equally well in both cases, the total number of clones analysed was insufficient for definite conclusions. Therefore, we performed Northern blot analysis to compare accumulation of the following miRNAs: miR158, 159, 163, 164, 166, 168 and 172. LMM RNA was extracted from whole plants 10 days after inoculation. The RNAs were resolved on denaturing 15% polyacrylamide gels (7 M urea) in 0.5× TBE buffer and electroblotted onto Hybond-N+ membranes (Amersham Biosciences). Radiolabelled DNA oligonucleotide probes complementary to respective miRNA sequences were constructed by end labelling with [γ-32P]ATP by using T4 polynucleotide kinase (TaKaRa).

Pre-hybridization and hybridization were performed in 50% (v/v) formamide buffer [10× Denhardt’s solution, 0.5 mg sheared salmon sperm DNA ml−1, 1% (w/v) SDS, 3× SSC and 50 mM phosphate buffer] at 40 °C. The results showed that most miRNAs accumulated to a greater extent in virus-infected plants than in uninfected plants (Fig. 2a). Thus, the general tendency indicated by sequence profiling reflected the actual increase in miRNA accumulation.

To consider the cause of relative enrichment of 21 nt small RNAs in virus-infected plants, we suspected the involvement of the TMV-Cg-encoded replication protein, a putative RNA-silencing suppressor, because previous works indicated that expression of some viral RNA-silencing suppressors leads to high miRNA accumulation (Kasschau et al., 2003; Chapman et al., 2004; Chen et al., 2004; Dunoyer et al., 2004). First, to investigate whether the

![Fig. 1. Characterization of small RNAs in TMV-infected plants. (a) Size distribution of sequenced small RNAs and virus-derived siRNAs in TMV-infected plants. Empty and filled bars indicate numbers of cloned total small RNAs and numbers of virus-derived siRNAs, respectively. (b) Positions corresponding to each siRNA sequence are represented by small dots. Upper and lower dot-plots indicate positive and negative strand-derived siRNAs, respectively, along with a schematic representation of the TMV-Cg genome (6303 nt). Most siRNAs were cloned only once (numbered 1), but some siRNAs were cloned and isolated a few times (numbered 2 or 3) independently. siRNAs cloned a few times are indicated separately. (c) Size distribution of sequenced endogenous small RNAs. Empty and filled bars indicate sequenced endogenous small RNAs from mock-inoculated and TMV-infected plants, respectively. Numbers of virus-derived siRNAs were omitted from the total numbers of small RNAs in the virus-infected plant.]
TMV-Cg replication protein has silencing-suppressor activity (as do the TMV and ToMV replication proteins), the TMV-Cg replication protein and green fluorescent protein (GFP) were co-expressed in *Nicotiana benthamiana* leaves by agroinfiltration (Llave *et al.*, 2000) and total RNA was extracted from the leaves 6 days after infiltration. GFP-specific siRNA levels were lower in the presence of the replication protein and, consequently, GFP mRNAs accumulated at higher levels, consistent with low siRNA levels when they were expressed with the replication protein (data not shown). These results confirmed that the TMV-Cg replication protein suppresses transgene-induced RNA silencing as an RNA-silencing suppressor. Note that all experiments described below were done by agroinfiltration in *N. benthamiana* leaves.

To investigate the effect of expression of the replication protein on miRNA processing and its accumulation, we performed Northern blot analysis to detect miRNA precursors, miRNA and miRNA* (the opposite strand of the miRNA duplex) in the presence and absence of replication protein expression. Replication protein and *Arabidopsis* pri-miR164b were co-expressed in *N. benthamiana* leaves by agroinfiltration using a constitutive promoter. Two days after infiltration, LMM RNA plus, which contains RNAs shorter than 1500 nt, was isolated as described previously (Kurihara & Watanabe, 2004), then resolved on a 7.5 % denaturing gel (8 M urea) for detection of miR164b precursors, or a denaturing 15 % polyacrylamide gel (7 M urea) for miR164 and miR164*, in 0.5 × TBE buffer and electroblotted onto Hybond-N+ membranes. Hybridization and probes were as described previously (Kurihara *et al.*, 2006).

miRNAs are produced through multi-step processing of their precursors with a Dicer-like enzyme (Kurihara & Watanabe, 2004; Kurihara *et al.*, 2006). The miRNA primary transcript, pri-miRNA, is processed to a second precursor, pre-miRNA, which is then processed to an miRNA–miRNA* duplex and a remnant containing the loop structure. The results showed that the presence of the TMV replicase protein did not affect the accumulation of pri-miR164b, pre-miR164b or the remnant fragment (Fig. 2b, upper panel). However, in sharp contrast, the accumulation of miR164b* was much higher in the presence of the replication protein than in its absence, although the accumulation of miR164b was similar with and without the replicase protein (Fig. 2b, lower panels). A similar result was obtained when *Arabidopsis* pri-miR171a and replication protein were co-expressed (Fig. 2c). Here, the accumulation of miR171a* was also much higher in the presence of the replication protein than in its absence.

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**Fig. 2.** Enhanced miRNA accumulation in TMV-infected plants and effect of the replication protein (Rep.) on accumulation of miRNA and miRNA* in a transient-expression assay. (a) Northern blot analysis of the representative miRNAs in uninoculated and TMV-Cg-infected plants. The same amount of LMM RNAs were loaded in each lane. (b, c) Northern blot analysis of miR164b precursors, miR164 and miR164* (b) and miR171 and miR171* (c), in a transient-expression assay. 5S rRNA and tRNAs were used as loading controls. The TMV-Cg replication protein expressed by the 35S promoter was used.
From the results shown in Fig. 2(b, c), it is suggested that the replication protein or replication complex binds small RNA duplexes. We have examined this possibility by performing the following experiments. Firstly, we performed co-immunoprecipitation experiments of the replication protein to see whether the protein bound miRNA–miRNA* duplexes. Replication protein tagged with the C-terminal haemagglutinin (HA) epitope and pri-mi171a were co-expressed in the leaves of *N. benthamiana* by agroinfiltration. As a positive control, we used a tomato bushy stunt virus (TBSV)-encoded silencing suppressor, p19 protein, tagged with the C-terminal HA epitope, which was shown previously to be able to bind both miRNA and the corresponding miRNA*, possibly as small RNA duplexes (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004). Here, to express the replication protein at a much higher level for immunoprecipitation, we used a dexamethasone (DEX)-inducible promoter (Aoyama & Chua, 1997), whilst other constructs were driven by the constitutive 35S promoter. For induction of replication protein expression, 30 μM DEX (Wako) was sprayed onto the plants twice at 27 and 45 h after agroinfiltration. Forty-eight hours after agroinfiltration, 2 g infiltrated leaves was ground in liquid nitrogen and homogenized in 3 vols extraction buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, 5% glycerol, complete proteinase inhibitor cocktail (Roche)] by using a mortar. Cell debris was pelleted by centrifugation and the supernatant was incubated with 100 μl anti-HA antibody conjugated to agarose beads (Sigma) for 2 h at 4 °C. The immune complexes were collected by centrifugation, washed four times in 1 ml wash buffer [25 mM Tris/HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, complete proteinase inhibitor cocktail] and mixed with 200 μl 1% SDS solution for denaturation. RNA was extracted from the immune complexes by using ISOGEN solution (Nippon Gene) and subjected to Northern blot analysis. It can be seen that miR171 and miR171* co-immunoprecipitated with the replication protein, as with the p19 protein (Fig. 3a).

Next, to examine whether the replication protein bound siRNA, the replication protein was co-expressed with a...
double-stranded RNA (dsRNA) hairpin of GFP sense and antisense sequences, which was used previously to produce siRNA (Takeda et al., 2002). Small RNA in the immune complexes was analysed as described above. Northern blot analysis showed that GFP siRNA of approximately 20–25 nt in length co-immunoprecipitated with the replication protein, as well as with the p19 protein (Fig. 3b). These results indicate that the replication protein or its complex binds miRNA–miRNA* and siRNA duplexes in vivo.

Lastly, to examine whether the replication protein has small RNA duplex-binding activity in vitro, gel mobility-shift assays were performed as described previously (Silhavy et al., 2002; Mérai et al., 2006). HA-tagged 126 kDa replication protein was expressed constitutively by the 35S promoter. Protein extract was prepared from leaves 2 days after infiltration. Leaf tissue (0.25 g) was ground and homogenized in 4 vols band-shift buffer (83 mM MgCl₂, 66 mM KCl, 100 mM NaCl, 10 mM dithiothreitol), then centrifuged twice (15 000 r.p.m., 15 min, 4 °C), and the supernatant was used for experiments. Two duplexes were tested: a small RNA duplex containing a few bulges, comprising a synthetic miR171 and the complementary miR171* (UGAUUGAGCCGCGCCAAUAUC and UAUU-GGCCUGGUUCACUCAGA), and a small RNA duplex containing no bulges, comprising an siRNA and the complementary siRNA* (the opposite strand of the siRNA duplex; UCGAAGAUUCCCGGUACGUU and CGUACCGGGAUAUUCGUU). Both have 19 nt duplex and 2 nt 3’ overhangs. miR171* and siRNA* were end labelled with [α-³²P]ATP whilst miR171 and siRNA were phosphorylated with unlabelled ATP by using T4 polynucleotide kinase. To generate dsRNA, the same amounts of unlabelled small RNA (miR171 and siRNA) and labelled complementary small RNA (miR171* and siRNA*), respectively, were annealed by incubation at 94 °C for 1 min in annealing buffer [10 mM Tris/HCl (pH 7.5), 20 mM KCl] and cooling down to room temperature. In the binding reaction, 1 pmol labelled small RNA duplex and extracts containing 2 μg protein were incubated in the band-shift buffer for 20 min at room temperature, and then the reaction was stopped by adding dyes. Samples were resolved on a 7.5% native polyacrylamide gel. In supershift assays, 1 μg antibody (anti-HA and anti-FLAG; both from Sigma) was added to the protein extracts and incubated for 30 min before binding with the small RNA duplexes. Consequently, both kinds of small RNA duplex showed mobility shifts by the replication protein (Fig. 3c, lanes 4 and 11), whereas single-stranded small RNA did not (Fig. 3c, lanes 5 and 12). HA antibody, but not FLAG antibody, caused supershift of small RNA duplex binding by HA-tagged replication protein (Fig. 3c, lanes 6 and 13). These data suggest that the replication protein or its complex has an ability to bind small RNA duplexes, regardless of the existence of bulges in vitro.

It was revealed that replication protein or its complex forms a complex with small RNA duplexes, leading to suppression of RNA silencing through inhibition of small RNA function. The binding activity to a 21 nt small RNA duplex was recently identified in extracts from TMV-infected leaves (Mérai et al., 2006). Our results are consistent with those of a previous general model in which several viral suppressors of RNA silencing were shown to bind small RNAs (Lakatos et al., 2006; Mérai et al., 2006).

In virus-infected plants, the replication protein might preferentially capture small RNA duplexes of approximately 21 nt, because the size-distribution peak of endogenous small RNAs was 21 nt, whereas that of mock-inoculated plants was 23 or 24 nt (Fig. 1c). Replication protein co-immunoprecipitated with siRNAs of 24 nt as well as 21 nt in the transient-expression assay (Fig. 3b). Replication proteins are located on cytoplasmic membranes and in the cytoplasm, but not in nuclei (Hagiwara et al., 2003; Nishikiori et al., 2006). It is suggested that the majority of 21 nt endogenous small RNAs such as miRNA are located in the cytoplasm (Park et al., 2005), whilst the majority of 24 nt endogenous small RNAs are found in nuclei, because several of the latter have been shown to function in the chromatin-modifying nuclear siRNA pathway (Li et al., 2006; Pontes et al., 2006). Therefore, these findings together suggest that the replication protein contacts and preferentially recruits cytoplasmic 21 nt small RNA duplexes.

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