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A plant RNA virus suppresses RNA silencing through viral RNA replication

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RNA interference (RNAi) is a post-transcriptional gene-regulatory mechanism that operates in many eukaryotes. RNAi is induced by double-stranded RNA (dsRNA) and is mainly involved in defence against transposons and viruses. To counteract RNAi, viruses have RNAi suppressors. Here we show a novel mechanism of RNAi suppression by a plant virus *Red clover necrotic mosaic virus* (RCNMV). To suppress RNAi, RCNMV needs multiple viral components, which include viral RNAs and putative RNA replicase proteins. A close relationship between the RNA elements required for negative-strand RNA synthesis and RNAi suppression suggests a strong link between the viral RNA replication machinery and the RNAi machinery. In a transient assay, RCNMV interferes with the accumulation of small-interfering RNA (siRNAs) in RNAi induced by a hairpin dsRNA and it also interferes with microRNA (miRNA) biogenesis. An *Arabidopsis dcl1* mutant showed reduced susceptibility to RCNMV infection. Based on these results, we propose a model in which, to replicate, RCNMV deprives the RNAi machinery of Dicer-like enzymes that are involved in both siRNA and miRNA biogenesis.

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Introduction

RNA silencing is an RNA-guided, post-transcriptional gene-regulatory mechanism that operates in eukaryotes from fungi to animals. The mechanism controls processes including development, the maintenance of genome stability and defence against molecular parasites, in which small RNAs (21–26 nucleotides (nt)) play crucial roles. Among these small RNAs, two functionally different RNAs, microRNA (miRNA) and small-interfering RNA (siRNA), have been characterized. miRNAs generated from endogenous hairpin RNA precursors are involved in the regulation of development (Bartel, 2004). On the other hand, siRNA generated from long double-stranded RNA (dsRNA) is mainly involved in defence through RNA interference (RNAi) against transposons and viruses (Lecellier and Voinnet, 2004; Vastenhouw and Plasterk, 2004). Both miRNA and siRNA are processed from their precursors by the RNase III family endonuclease, Dicer. These small RNAs are then incorporated into a multicomponent effector complex known as an RNA-induced silencing complex (RISC) to inactivate homologous mRNA through RNA cleavage or translational repression.

In plants, RNAi is an immune system directed against viruses (Baulcombe, 2004). During RNA virus infection, long dsRNAs derived from the replication intermediates of viral RNAs trigger RNAi. The long dsRNA is converted into functionally different short (21–22 nt) and long (24–26 nt) siRNA duplexes (Hamilton *et al*, 2002) by different Dicer-like enzymes (DCLs) (Tang *et al*, 2003). In *Arabidopsis thaliana*, four DCLs are known. Of these, DCL1 is required for miRNA biogenesis, DCL2 for viral siRNA biogenesis and DCL3 for long siRNA biogenesis (Kurihara and Watanabe, 2004; Xie *et al*, 2004). The short and long siRNAs are amplified through the production of long dsRNA by plant RNA-dependent RNA polymerases (RdRPs; Dalmay *et al*, 2000; Tang *et al*, 2003) and then conversion of the long dsRNAs into siRNAs by DCLs. A strand of the short siRNA duplexes is incorporated into the RISC, and then the siRNA-programmed RISC degrades viral RNA. When RNAi is induced at one site, silencing signals spread from cell to cell and systemically, and subsequently trigger RNAi in the distant tissues of plants. It has been proposed that short siRNA plays a role in the cell-to-cell movement of the silencing signals (Himber *et al*, 2003) and long siRNA in their systemic movement (Hamilton *et al*, 2002). If these signals spread and the silencing condition is established ahead of a viral infection, viral RNAs are degraded before viral replication at the viral infection front (Voinnet *et al*, 2000).

To escape these RNAi-mediated defences, viruses have a counter-defence strategy against RNAi. Over 20 RNAi suppressors have been identified in plant viruses (Moissiard and Voinnet, 2004; Silhavy and Burgyán, 2004) and several suppressors have been identified in animal and insect viruses including *Influenza virus* and *Flock house virus* (Li *et al*, 2002, 2004; Lu and Cullen, 2004). These suppressors are commonly involved in the enhancement of viral pathogenicity and the accumulation of viruses. However, these suppressors do not have obvious sequence similarity and are phenotypically distinct in some assays, suggesting that RNAi suppressors act at different steps in the RNAi pathway. The targets of RNAi suppressors have been unlabelled. For example, tombusviral p19 specifically binds the short siRNA duplexes to interfere with the incorporation of siRNA into the RISC (Vargason *et al*, 2003; Lakatos *et al*, 2004). Similarly, closterovirial p21 binds siRNA duplexes (Chapman *et al*, 2004). Influenza viral NS1 binds to long dsRNA and short siRNA duplexes (Li *et al*, 2004). Adenoviral VA1 RNA binds Dicer

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(Lu and Cullen, 2004). Potyviral HC-Pro interferes with the DCL-mediated cleavage of long dsRNA (Dunoyer *et al*, 2004) and with RISC-mediated mRNA cleavage by inhibiting the unwinding of miRNA and siRNA duplexes (Chapman *et al*, 2004). Potexviral p25 and cucumoviral 2b inhibit the systemic movement of silencing signals (Voinnet *et al*, 2000; Guo and Ding, 2002). Moreover, some viral suppressors, including potyviral HC-Pro, tombusviral p19 and furoviral p15, affect an miRNA pathway that shares common components with the RNAi pathway and induce developmental abnormalities in *A. thaliana* (Chapman *et al*, 2004; Dunoyer *et al*, 2004). Thus, the identification and analysis of viral RNAi suppressors are important not only to elucidate the mechanisms of virus infection but also to dissect RNA silencing pathways.

Red clover necrotic mosaic virus (RCNMV) is a positive-sense single-stranded RNA plant virus and a member of the genus *Dianthovirus* in the family *Tombusviridae*. The genome of RCNMV is divided into two RNA components. RNA1 encodes three proteins, p27, p88 and coat protein (CP), and RNA2 encodes the movement protein (MP) required for virus movement (Lommel, 1999). p27 and p88 are putative replicase components; both p27 and p88 exist in an RdRP fraction purified from RCNMV-infected plants (Bates *et al*, 1995) and localize to the endoplasmic reticulum (ER; Turner *et al*, 2004). p88 is generated via a ribosomal frameshifting from p27 (Kim and Lommel, 1994) and has an RdRP motif (Koonin and Dolja, 1993). RCNMV does not encode proteins containing the helicase motif conserved in eukaryotes (Koonin and Dolja, 1993). CP is translated from a subgenomic RNA transcribed from RNA1 through RNA2-mediated *trans*-activation (Sit *et al*, 1998; Tatsuta *et al*, 2005), whereas RNA2 is not required for the replication of RNA1 in protoplasts (Osman and Buck, 1987). It is not known whether RCNMV has an RNAi suppressor.

Here we show that RCNMV suppresses RNAi by using multiple viral components required for viral RNA replication. We also show that RCNMV interferes with the accumulation of siRNAs and miRNA biogenesis in *Nicotiana benthamiana*, and fails to infect *A. thaliana dcl1-9* mutant. We propose a model that RCNMV deprives the RNAi machinery of a DCL(s) for viral RNA replication.

Results

RCNMV infection suppresses S-RNAi

We first determined whether RCNMV infection suppresses sense-transgene-mediated RNAi (S-RNAi) using an *Agrobacterium*-mediated transient assay (Takeda *et al*, 2002) in a green fluorescent protein (GFP)-expressing transgenic *N. benthamiana* line 16c. Full-length cDNAs of RCNMV genomic RNAs 1 and 2 were cloned into a binary vector, pBICP35. The resulting plasmids, pBICRC1 and pBICRC2 (Figure 1), were introduced into *Agrobacterium*. For simplicity, we will refer below to *Agrobacterium* strains containing a binary vector plasmid by the name of the plasmid carried. Before S-RNAi-suppression assay, a mixture of pBICRC1 and pBICRC2 (1:1 ratio) was infiltrated into wild-type *N. benthamiana* to test their infectivity. At 14 days post-infiltration (dpi), RCNMV infection was confirmed by the accumulation of RCNMV RNA1 in upper uninfiltrated leaves. The accumulation was similar to that by inoculation with *in vitro* transcripts of RNA1 and RNA2 (Figure 2A). Then, pBICRC1 and pBICRC2 were tested for their ability to suppress S-RNAi by coinfiltration with pBICGFP, which is an S-RNAi inducer (Figure 1). In this assay, if RCNMV infection blocks the onset of RNAi, GFP fluorescence is easily detected under ultraviolet (UV) light in infiltrated patches. Patches receiving a mixture of pBICGFP + pBICRC1 + pBICRC2 (2:1:1 ratio) showed bright green fluorescence at 4 dpi (Figure 2B). Similar bright green fluorescence was observed in patches receiving pBICGFP + pBICNSs (Figure 2B). pBICNSs encodes *Tomato spotted wilt virus* (TSWV) NSs, which is an S-RNAi suppressor (Takeda *et al*, 2002). In contrast, control patches receiving pBICGFP + pBICP35 showed decreased green fluorescence (Figure 2B). These results indicate that RCNMV infection suppresses S-RNAi.

RNA1 is essential for suppressing S-RNAi

To determine the viral components involved in S-RNAi suppression, we investigated which genomic RNA is essential for S-RNAi suppression. Patches receiving pBICGFP + pBICRC1 showed bright green fluorescence at 4 dpi (Figure 2B) with an intensity weaker than that in pBICGFP + pBICRC1 + pBICRC2-infiltrated leaves, whereas patches receiving pBICGFP + pBICRC2 showed decreased green fluorescence similar to that of the control patches infiltrated with pBICGFP + pBICP35 (empty vector, Mori *et al*, 1993) (Figure 2B). To confirm these visual observations, we analysed the accumulation of GFP mRNA and GFP-specific siRNAs in the infiltrated patches at 4 dpi. GFP mRNA levels were higher in patches receiving pBICGFP + pBICRC1 than in those receiving pBICGFP plus either pBICRC2 or pBICP35, and were lower than in those receiving pBICGFP plus either pBICRC1 + pBICRC2 or pBICNSs (Figure 2C). The accumulation of GFP-specific siRNAs was inversely correlated with the accumulation of GFP mRNA: low in patches receiving pBICGFP plus either pBICRC1 + pBICRC2 or pBICNSs, intermediate in those receiving pBICRC1 and high in those receiving either pBICRC2 or pBICP35 (Figure 2C). These results indicate that RCNMV RNA1 but not RNA2 is essential for S-RNAi suppression.

Viral proteins encoded by RNA1 are not sufficient to suppress S-RNAi

RCNMV RNA1 encodes three proteins: p27, p88 and CP (Lommel, 1999). To determine which protein is involved in S-RNAi suppression, pBICp27, pBICp88 and pBICRCCP, which express the mRNAs for p27, p88 and CP, respectively (Figure 1), were tested. Patches receiving pBICGFP plus either pBICp27, pBICp88, pBICRCCP or a mixture of pBICp27 and pBICp88 showed decreased green fluorescence at 4 dpi (data not shown), and the accumulation of GFP mRNA in these patches was lower than that in the patches receiving pBICGFP + pBICRC1 (Figure 3A). A large amount of GFP-specific siRNAs accumulated in patches receiving pBICGFP plus either pBICp27, pBICp88, pBICRCCP (1:1 ratio) or a mixture of pBICp27 and pBICp88 (2:1:1 ratio), as with those receiving pBICGFP + pBICP35 (Figure 3A). Immunoblot analyses using the anti-p27 antibody showed that in patches receiving pBICGFP + pBICp27, the accumulation of p27 was even higher than that in the patches receiving pBICGFP + pBICRC1 at 2 and 4 dpi (Figure 3B, and data not shown). Unfortunately, p88 was not detected in any patches analysed at 2 and 4 dpi (data not shown). p88 may be

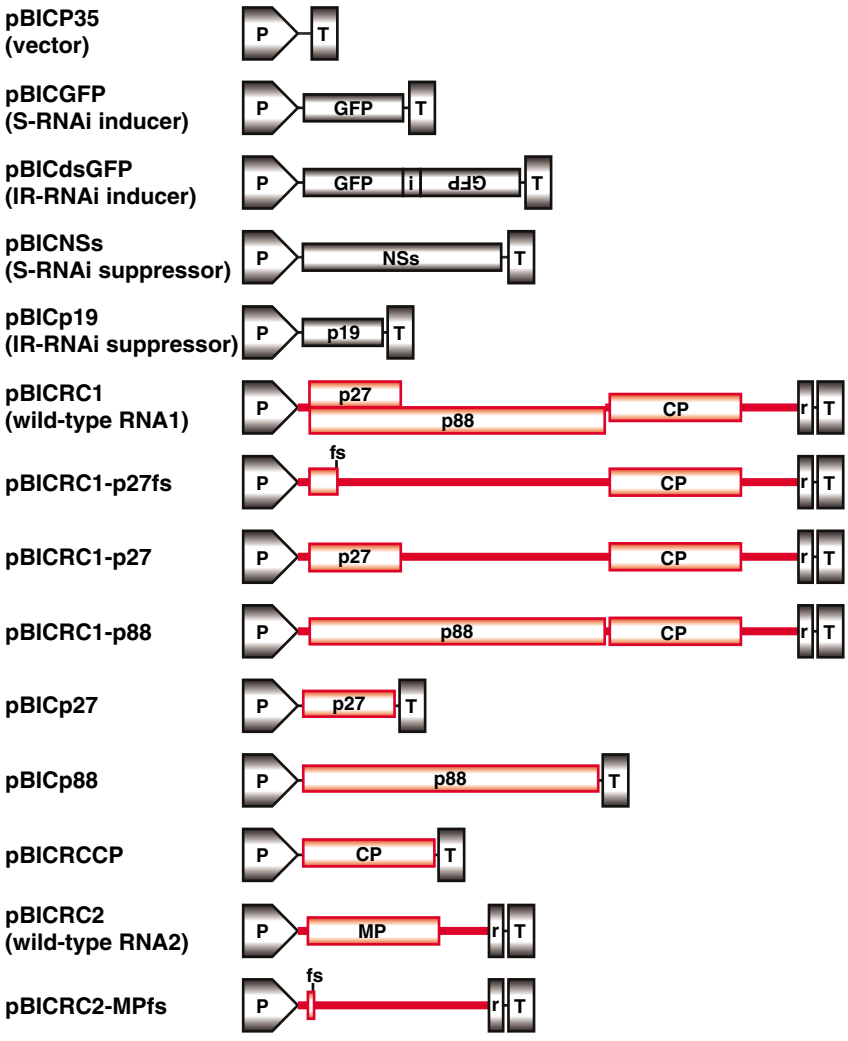

Figure 1 Schematic representation of the transcription cassettes for agroinfiltration. The pentagon labelled P represents the 35S promoter of *Cauliflower mosaic virus* (CaMV). The boxes labelled T, i and r represent the terminator of CaMV, an intron of the *A. thaliana TOM1* gene and the ribozyme of a satellite RNA of *Tobacco ringspot virus*, respectively. The lines and boxes coloured red represent the RCNMV-derived regions, and those coloured black represent non-RCNMV sequences.

unstable *in vivo*. These results suggest that neither p27, p88, or CP, nor a mixture of p27 and p88, alone is sufficient to suppress S-RNAi.

In addition, using a similar assay described above, we tested whether p57 is involved in S-RNAi suppression, because p57, which is the C-terminal part of p88, has been detected in an *in vitro* translation assay (Kim and Lommel, 1994). The results showed that p57 did not suppress S-RNAi (Figure 3A). Collectively, these data suggest that previously reported proteins encoded by RNA1 are not sufficient to suppress S-RNAi.

RNA1-mediated S-RNAi suppression is linked to the replication of RNA1

To delimit the factors required for RNA1-mediated S-RNAi suppression, we used several RNA1 mutants. pBICRC1-ΔCP, in which the CP gene was precisely deleted from pBICRC1, was still capable of suppressing S-RNAi, indicating that the CP-coding region is not essential for RNA1-mediated S-RNAi suppression (see Supplementary data).

Next, to examine whether p27 and/or p88, when expressed from full-length RNA1, are involved in RNA1-mediated S-RNAi suppression, we used pBICRC1-p27, pBICRC1-p88 and pBICRC1-p27fs (Figure 1). pBICRC1-p27 and pBICRC1-p88 are expected to express p27 or p88, respectively (Xiong *et al*, 1993). pBICRC1-p27fs is expected to express no previously known viral proteins. Patches receiving pBICGFP plus either one of these three *Agrobacterium* strains (1:1 ratio) or those receiving pBICGFP + pBICRC1-p27 + pBICRC1-p88 (2:1:1 ratio) showed decreased green fluorescence at 4 dpi (data not shown). With these inoculations, the accumulation of GFP mRNA was similar to that in patches receiving pBICGFP + pBICP35, and GFP-specific siRNAs were easily detected (Figure 3C, and data not shown). These results suggest that an unmodified RNA1, or its replication, but not the individual proteins, is essential for S-RNAi suppression.

The failure of a mixture of pBICRC1-p27 + pBICRC1-p88 to suppress S-RNAi contradicts the S-RNAi suppression activity of RNA1, if RNA1 replicates in the patches receiving the mixture, because pBICRC1-p27 + pBICRC1-p88 contains all the RNA and protein components of RNA1. To investigate RNA1 replication, BY-2 protoplasts were inoculated with *in vitro* transcripts of pUCR1-p27, pUCR1-p88 or both. pUCR1-p27 and pUCR1-p88 have mutations corresponding

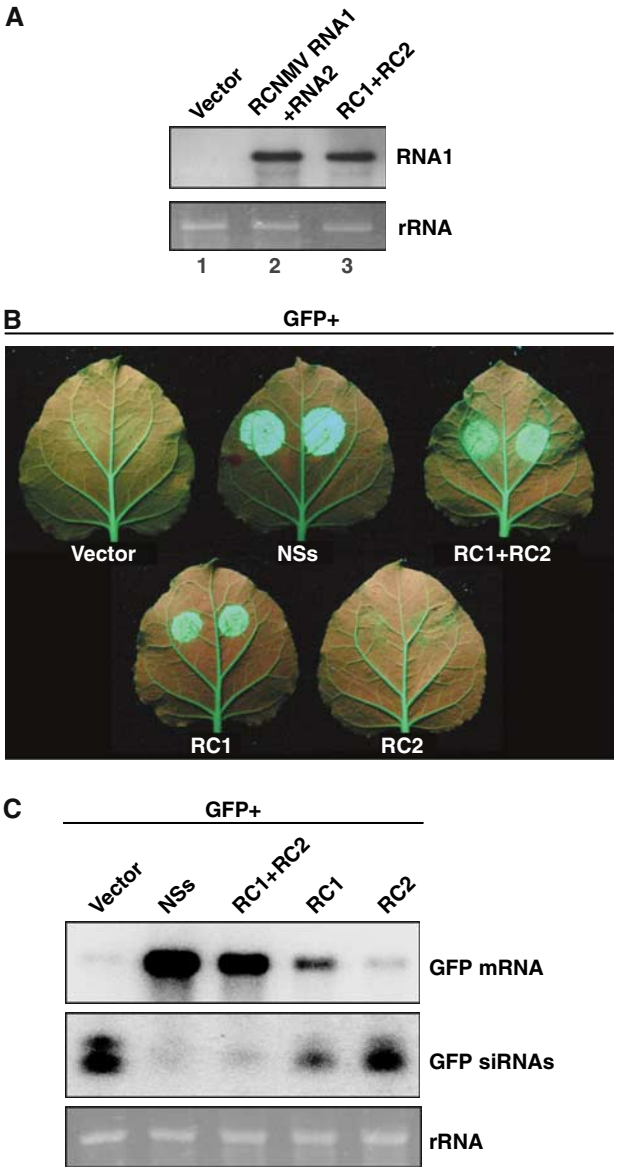

Figure 2 RCNMV RNA1 alone is sufficient to suppress S-RNAi. (**A**) Northern blot analysis of RCNMV RNA1 extracted from the upper uninoculated leaves of wild-type *N. benthamiana* at 14 dpi. Two leaves were inoculated with *Agrobacterium* carrying pBICP35 (lane 1), a mixture of RNA transcripts of wild-type RCNMV (lane 2) or *Agrobacterium* carrying pBICRC1 plus that carrying pBICRC2 (lane 3). (**B**) Leaves of line 16c were viewed under UV light at 4 dpi. (**C**) Northern blot analysis of GFP mRNA (top) and GFP-specific siRNAs (middle) extracted from the agroinfiltrated patches.

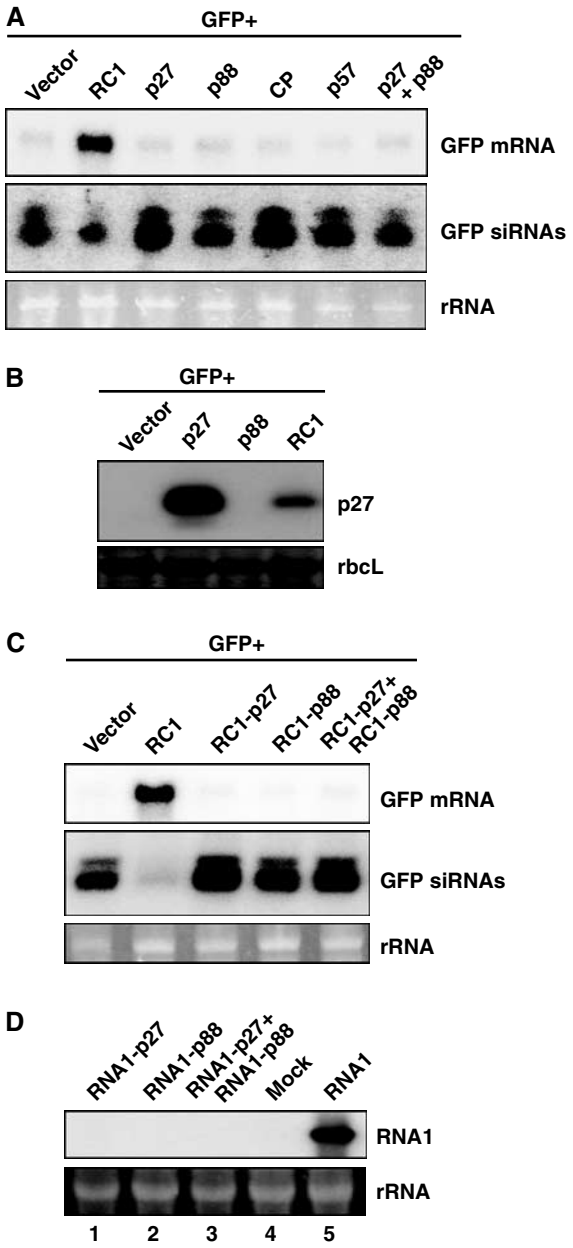

Figure 3 Proteins encoded by RNA1 are not sufficient to suppress S-RNAi. (**A**) Northern blot analysis of GFP mRNA (top) and GFP-specific siRNAs (middle) extracted from the leaves of line 16c infiltrated with *Agrobacterium* carrying plasmids expressing viral proteins and GFP mRNA at 4 dpi. (**B**) Immunoblot analysis of viral proteins extracted from the leaves of line 16c infiltrated with *Agrobacterium* carrying plasmids expressing viral proteins and GFP mRNA using a polyclonal antibody raised against p27 at 2 dpi. (**C**) Northern blot analysis of GFP mRNA (top) and GFP-specific siRNAs (middle) extracted from the leaves of line 16c infiltrated with *Agrobacterium* carrying plasmids containing RNA1 mutants that express p27 or p88 alone at 4 dpi. (**D**) Northern blot analysis of viral RNAs extracted from BY-2 protoplasts inoculated with *in vitro* transcripts of RNA1 mutants that express p27 or p88 alone at 24 hpi.

to those of pBICRC1-p27 and pBICRC1-p88, respectively. In inoculations with transcripts from pUCR1-p27, pUCR1-p88 or both, RNA1s were below detectable levels, whereas RNA1 was easily detected in inoculation with transcripts from pUCR1, which encodes wild-type RNA1 at 24 h post-inoculation (hpi) (Figure 3D). Accumulation levels of viral RNA1 transcripts from pUCR1-p27, pUCR1-p88 and pUCR1 did not differ at 2 hpi, suggesting similar stability of these RNA1s (data not shown). These results suggest that RNA derived from pUCR1-p27 or pUCR1-p88 does not serve as an effective template for replication, implying that replication of RNA1 is essential for S-RNAi suppression. To confirm the activity of p88 expressed without a ribosomal frameshift in RCNMV replication, protoplasts were inoculated with *in vitro* transcripts of RNA2 together with pUBR1-p27 and pUBR1-p88, which express only p27 and p88 from RNA1, respectively. Northern blot analysis showed that RNA2 efficiently accumulated at 24 hpi (data not shown), indicating that p88 expressed without a ribosomal frameshift is competent to

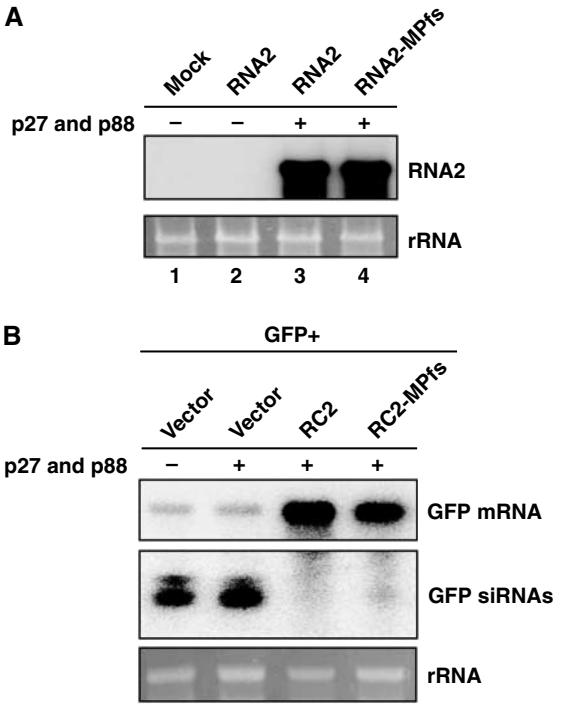

Figure 4 Uncoupled expression of p27 and p88 supports RNA2 replication and suppresses S-RNAi in the presence of RNA2. (**A**) Northern blot analysis of positive-strand RNA2 of RCNMV extracted from cowpea protoplasts inoculated with buffer only (lane 1), *in vitro* transcripts of pRC2|G (lane 2), a mixture of pUBp27, pUBp88 and either *in vitro* transcripts of pRC2|G (lane 3) or pRNA2fsMP (lane 4). (**B**) Northern blot analysis of GFP mRNA (top) and GFP-specific siRNAs (middle) extracted from the leaves of line 16c infiltrated with *Agrobacterium* carrying the plasmids indicated above the panels at 4 dpi.

support RNA2 replication. Similar results were obtained using pUBp27 and pUBp88, which express only p27 and p88, respectively (Figure 4A), confirming the above results. Collectively, these results suggest that RNA1-mediated S-RNAi suppression is strongly linked to RNA1 replication.

The combination of p27, p88 and RNA2 suppresses S-RNAi

To investigate the link between S-RNAi suppression and viral RNA replication, we tested whether a mixture of p27, p88 and RNA2 suppresses S-RNAi. Interestingly, patches receiving a mixture of pBICGFP + pBICp27 + pBICp88 + pBICRC2 (3:1:1:1 ratio) showed bright green fluorescence at 4 dpi (data not shown), and Northern blot analysis for GFP mRNA and GFP-specific siRNAs confirmed S-RNAi suppression (Figure 4B). The suppression of S-RNAi was also observed in patches receiving pBICGFP + pBICRC1-p27 + pBICRC1-p88 + pBICRC2 (3:1:1:1 ratio) (data not shown). Using pBICRC2-MPfs from which MP is not expressed (Tatsuta *et al*, 2005), we confirmed that MP is not required for S-RNAi suppression (Figure 4B). These results indicate that the combination of p27, p88 and RNA2 suppresses S-RNAi. This implies that RCNMV RNA replication is strongly linked to S-RNAi suppression, because p27 plus p88 (Figures 3 and 4B) or RNA2 alone (Figure 2) does not suppress S-RNAi.

Next, we investigated whether the activity of p88 in RNA synthesis is involved in S-RNAi suppression by using pBICp88-GVD. pBICp88-GVD encodes p88 with a mutation in the Gly-Asp-Asp (GDD) motif, which is conserved in RdRP (Koonin and Dolja, 1993) and required for RCNMV RNA synthesis (Bates *et al*, 1995). Patches receiving pBICGFP + pBICp27 + pBICp88-GVD + pBICRC2 (3:1:1:1 ratio) showed decreased green fluorescence at 4 dpi (data not shown), suggesting that p88 competent in RNA synthesis is required for S-RNAi suppression.

The 3′ untranslated region of RCNMV RNA2 is essential for S-RNAi suppression

Replication of positive-strand RNA viruses consists of two main steps: negative-strand RNA synthesis and subsequent positive-strand RNA synthesis (Buck, 1996). To investigate which step in RCNMV replication is required to suppress S-RNAi, we used pBICRC2-5D and pBICRC2-3D (Figure 5A). pBICRC2-5D expresses RNA2 with the 5′ untranslated region (UTR) substituted for a nonviral 5′ leader. The 5′ UTR of RNA2 is required for progeny positive-strand RNA synthesis but not for negative-strand RNA synthesis (Turner and Buck, 1999). pBICRC2-3D expresses RNA2 with a stem–loop structure deleted in the 3′ UTR that is required to produce negative-strand RNA (Turner and Buck, 1999). Interestingly, patches receiving pBICGFP + pBICp27 + pBICp88 + pBICRC2-5D (3:1:1:1 ratio) showed bright green fluorescence, whereas those receiving pBICGFP + pBICp27 + pBICp88 + pBICRC2-3D (3:1:1:1 ratio) showed decreased green fluorescence (data not shown). The accumulation of GFP mRNA and GFP-specific siRNAs in these patches was in accordance with visual observations (Figure 5B). These results indicate that the 3′ UTR but not the 5′ UTR of RNA2 is required for RNA2-mediated S-RNAi suppression.

To further investigate the roles of viral RNA and proteins in RNA2-mediated S-RNAi suppression, we examined accumulations of viral RNAs and viral proteins in agroinfiltrated leaves with pBICGFP + pBICp27 + pBICp88 plus either pBICRC2, pBICRC2-5D or pBICRC2-3D (3:1:1:1 ratio) at 2 dpi. Northern blot analyses showed that RNA2 and a faster migrating RNA were detected in pBICRC2 and pBICRC2-5D inoculations, respectively (Figure 5C). In negative-strand RNA detection, a main RNA band corresponding to positive-strand RNAs in size was detected in pBICRC2 and pBICRC2-5D inoculations (Figure 5C). This indicated that negative-strand synthesis occurred in pBICRC2-5D inoculation. No viral RNAs were detected in pBICRC2-3D inoculation (Figures 5C). Immunoblot analyses showed that accumulation levels of p27 did not vary much between pBICRC2, pBICRC2-5D and pBICRC2-3D inoculations (Figure 5D), confirming that accumulation of p27 alone is not sufficient to suppress S-RNAi. These results suggest that RNA2-mediated S-RNAi suppression correlates with the amount of viral RNAs accumulated or the event of RNA synthesis itself.

Direct involvement of negative-strand RNA in RNA2-mediated S-RNAi suppression was tested using pBICRC2(−), which expresses full-length negative-strand RNA2. Patches receiving pBICGFP + pBICp27 + pBICp88 + pBICRC2(−) (3:1:1:1 ratio) showed decreased green fluorescence, and the accumulation of GFP mRNA and GFP-specific siRNAs in these patches was in accordance with visual observations (data not shown). This suggests that negative-strand RNA is not directly involved in RNA2-mediated S-RNAi suppression.

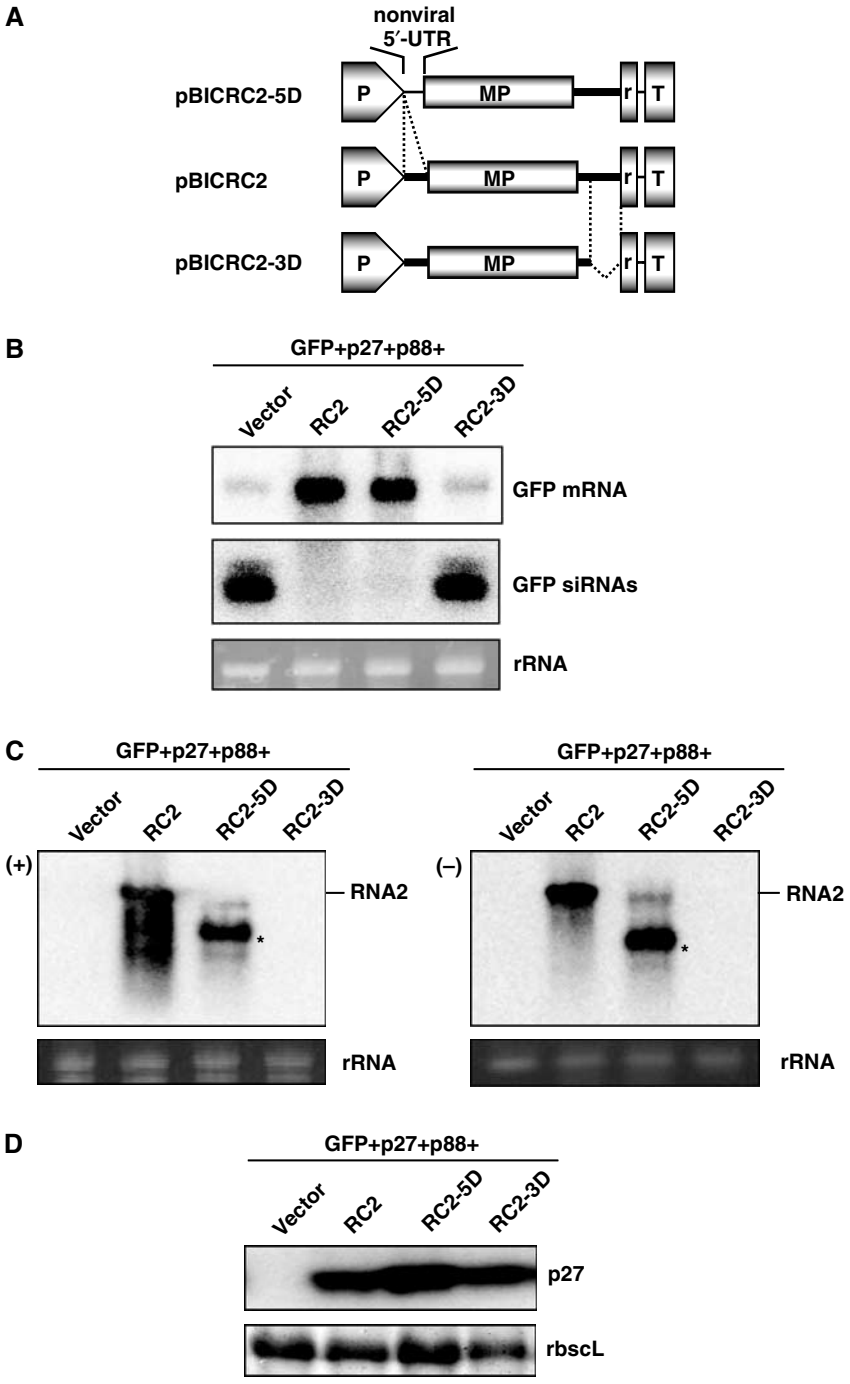

Figure 5 The 3′ UTR but not the 5′ UTR is required for RNA2-mediated S-RNAi suppression. (**A**) Schematic representation of the transcription cassettes for agroinfiltration. In pBICRC2-5D, the 5′ UTR of RNA2 (81 nt) was substituted with a nonviral 5′ leader sequence. In pBICRC2-3D, the 25 nt stem–loop structure required for RNA2 replication at positions +1421 to +1445 was deleted from pBICRC2. Nonviral sequences are shown as a thin line. (**B**) Northern blot analysis of GFP mRNA (top) and GFP-specific siRNAs (middle) extracted from the leaves of line 16c infiltrated with *Agrobacterium* carrying the plasmids indicated above the panels at 4 dpi. (**C**) Northern blot analysis of viral RNAs extracted from the leaves of line 16c infiltrated with *Agrobacterium* carrying plasmids indicated above the panels at 2 dpi. (+): positive-strand RNAs, (−): negative-strand RNA2s. A band with asterisk is an RNA migrating faster than full-length RNA2. We used DIG-labelled full-length RNA2 and RNA complementary to 3′ UTR of RNA2 to detect negative- and positive-strand RNA2s, respectively. (**D**) Immunoblot analysis of viral proteins extracted from the leaves of line 16c infiltrated with *Agrobacterium* indicated above the panels using a polyclonal antibody raised against p27 at 2 dpi.

RCNMV reduces the accumulation of siRNAs in an RNAi induced by a hairpin dsRNA

To investigate which steps in plant RNAi are suppressed by RCNMV, we tested whether RCNMV suppresses the RNAi induced by a hairpin dsRNA expressed from an inverted-repeat (IR) transgene. Here we refer to this type of RNAi as IR-RNAi. IR-RNAi is distinct from S-RNAi because plant RdRP is not required to form dsRNA in IR-RNAi (Beclin *et al*, 2002). At 2 and 4 dpi, the patches of wild-type *N. benthamiana* receiving pBICGFP + pBICdsGFP plus either pBICP35 or pBICRC1 + pBICRC2 did not show GFP fluorescence, whereas those receiving pBICGFP + pBICdsGFP + pBICp19, which suppresses IR-RNAi (Takeda *et al*, 2002) through binding siRNA duplexes (Vargason *et al*, 2003; Lakatos *et al*, 2004),

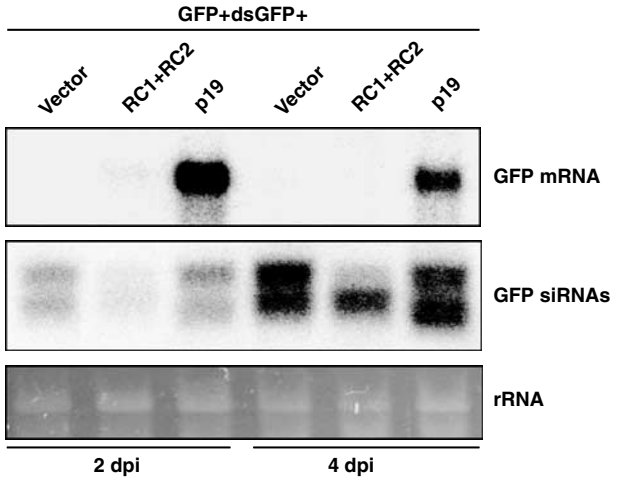

Figure 6 RCNMV inhibits siRNA accumulation in IR-RNAi. Leaves of line 16c were infiltrated with *Agrobacterium* carrying the plasmids indicated above the panels. Northern blot analysis of GFP mRNA (top) and GFP-specific siRNAs (middle) extracted from agroinfiltrated patches.

showed bright green fluorescence (data not shown). Visual observations were confirmed by the undetectable accumulation of GFP mRNA in RCNMV inoculation at 2 and 4 dpi (Figure 6). However, interestingly, at 2 dpi, the accumulation of siRNAs was extremely low in RCNMV inoculation compared with that in other inoculations, suggesting that RCNMV interferes with DCL-mediated dsRNA cleavage like carmoviral CP (Chapman *et al*, 2004). At 4 dpi, the accumulation level of long siRNAs remained very low compared to that of short siRNAs in RCNMV inoculation (Figure 6A). This suggests that RCNMV preferentially inhibits long siRNA biogenesis, although RCNMV does not inhibit GFP mRNA degradation in this IR-RNAi assay.

RCNMV does not efficiently accumulate viral RNA in A. thaliana dcl1-9 mutant

The results obtained above tempted us to test whether RCNMV infects *A. thaliana dcl* mutants, because RCNMV replication is linked to RNAi suppression and because it may be linked to DCL-mediated dsRNA cleavage step. Four DCLs are known in *A. thaliana*, and DCL1, DCL2 and DCL3 are involved in miRNA biogenesis, in viral siRNA biogenesis and in long siRNA biogenesis, respectively (Kurihara and Watanabe, 2004; Xie *et al*, 2004). Three *A. thaliana dcl* mutants (*dcl1-9*, *dcl2-2* and *dcl3-1*) were inoculated with RCNMV, and RNA1 accumulation in inoculated leaves was analysed at 4 dpi. RNA1 accumulated in *DCL1-9/DCL1-9*, *dcl2-2*, *dcl3-1*, Col-0 (parental accession of *dcl2-2* and *dcl3-1*), WS and Ler plants (parental accessions of *dcl1-9*), whereas RNA1 accumulation levels in *dcl1-9/dcl1-9* plants were extremely low compared to those in other lines (Figure 7, and data not shown). Accumulation levels of *Tobacco mosaic virus*-Cg (TMV-Cg) RNA did not significantly differ between *DCL1-9/DCL1-9* and *dcl1-9/dcl1-9* plants (Figure 7). These results indicate that RCNMV requires DCL1 to accumulate viral RNA in the inoculated leaves of *A. thaliana*.

RCNMV inhibits miRNA biogenesis

Next, we investigated whether RCNMV inhibits miRNA biogenesis, because DCL1 is required for miRNA biogenesis

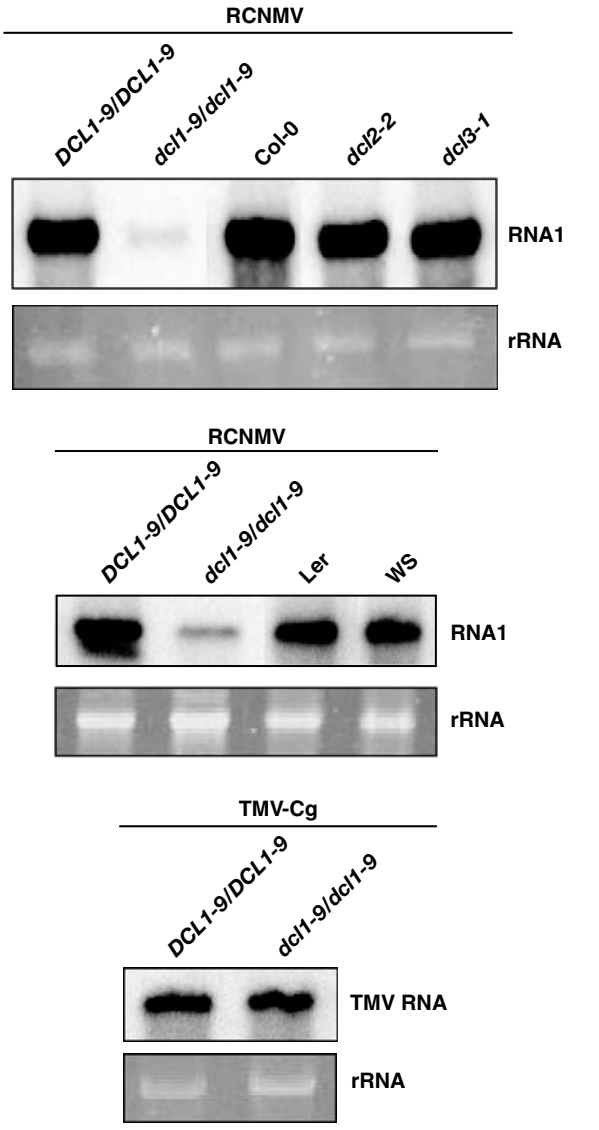

Figure 7 RCNMV does not effectively accumulate viral RNAs in *A. thaliana dcl1-9/dcl1-9* mutant. Three *dcl1-9*, *dcl2-2* and *dcl3-1* mutants and wild-type *A. thaliana* Col, Ler and WS were inoculated with a mixture of *in vitro* transcripts of RCNMV RNA1 and RNA2, and RNA1 accumulation in the inoculated leaves was analysed at 4 dpi. Northern blot analyses for RCNMV RNA accumulation are shown in the top and middle panels. The bottom panel shows TMV-Cg RNA accumulation in *A. thaliana* leaves inoculated with virion RNA of TMV-Cg at 4 dpi.

(Reinhart *et al*, 2002; Kurihara and Watanabe, 2004). We carried out a transient miRNA assay in *N. benthamiana* (Kasschau *et al*, 2003). In patches receiving pBICP35, endogenous *N. benthamiana* miR171 was detected (Figure 8). Accumulation level of miR171 was higher in patches receiving pBICmiR171prec + pBICP35 (17:3 ratio) than that in patches receiving pBICP35 because of the miR171 produced from a synthetic precursor RNA (Parizotto *et al*, 2004), which is transcribed from pBICmiR171prec (Figure 8). However, the accumulation of miR171 in the patches receiving pBICmiR171prec + pBICRC1 + pBICRC2 (34:3:3 ratio) was similar to that in the patches receiving pBICP35, and, moreover, the accumulation level of endogenous miR171 was low in patches receiving pBICP35 + pBICRC1 + pBICRC2 (34:3:3 ratio) compared to patches receiving pBICP35 alone

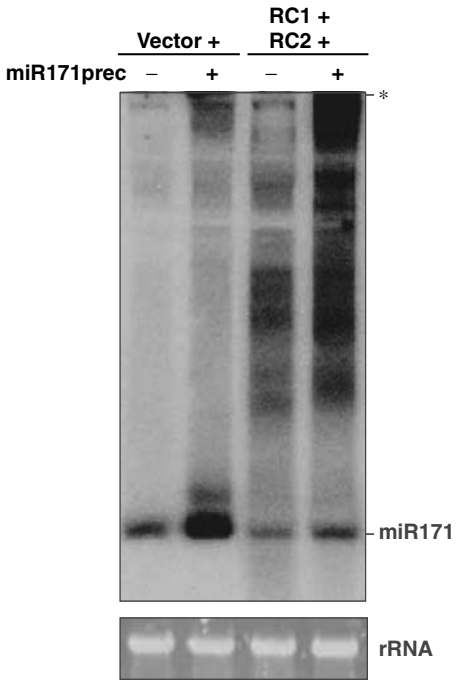

Figure 8 RCNMV suppresses miRNA biogenesis. *N. benthamiana* was infiltrated with *Agrobacterium* carrying plasmids indicated above the panels, and total RNAs extracted form the leaves were analysed at 2 dpi by Northern blot method using a probe that hybridizes miR171. Asterisk shows the bottom of wells, in which total RNAs were loaded.

(Figure 8). These results indicate that RCNMV inhibits the miR171 production either from endogenous miR171 precursor or from transiently expressed miR171 precursor derived from pBICmiR171prec, and suggest that RCNMV inhibits miRNA biogenesis.

Discussion

We have shown here that RCNMV has the ability to suppress RNAi, the suppression of which requires three viral components: two replication proteins (p27 and p88) and viral RNAs with *cis*-acting RNA elements that are strongly linked to negative-strand RNA synthesis (Figure 5; Turner and Buck, 1999). RNAi suppression by RCNMV was consistently correlated with viral RNA accumulation accompanied with RNA replication or RNA synthesis. There are three possible explanations for the mechanism of RNAi suppression by RCNMV: (i) novel RCNMV proteins expressed during viral RNA replication suppress RNAi, (ii) RCNMV RNA itself suppresses RNAi and (iii) both RCNMV RNA and proteins coordinately function and suppress RNAi.

Are novel viral proteins involved in RNAi suppression?

If novel viral proteins are involved in RNAi suppression, RCNMV must encode at least two novel proteins, each of which is sufficient to suppress RNAi. One of these must be encoded by RNA1 and the other by RNA2, because known RCNMV proteins alone were not sufficient to suppress RNAi (Figures 3 and 4, and data not shown). One of the smallest RNAi suppressors identified is cucumoviral 2bs, which consists of over 80 amino acids (Ding *et al*, 1994). However, there are no open reading frames encoding over 50 amino acids on RCNMV genomic RNAs (data not shown). Therefore, it is unlikely that any unidentified novel RCNMV proteins are involved in RCNMV RNAi suppression.

Are viral RNAs involved in RNAi suppression?

RNAi suppression activity by RCNMV components was consistently correlated with viral RNA accumulation, suggesting central roles of RCNMV RNAs in RNAi suppression. In this case, putative RNA elements must be contained in both RNA1 and RNA2 in either negative- or positive-strand RNAs that exclude the 5′ UTR of RNA2 (Figures 3–5, and data not shown). In addition, putative RNA elements must be present in large amounts through RNA replication or synthesis with p27 and p88, because RCNMV RNAs constitutively supplied from the 35S promoter were not sufficient on their own to suppress RNAi (Figures 3–5). If RCNMV RNAs directly suppress RNAi, the mechanism seems different from that in adenovirus, which uses VA1 noncoding small RNA (Lu and Cullen, 2004). This is because small RNAs similar in size to VA1 were not detected in the RCNMV RNAi suppression assay (Figure 5, and data not shown). Although our experimental results cannot exclude the direct involvement of viral RNAs as a mechanism of RNAi suppression by RCNMV, we believe that the coordinate involvement of RNA and protein factors is more likely to occur in RNAi suppression by RCNMV as discussed below.

A link between RNAi suppression and RCNMV replication

Correlations between RNAi suppression activity by RCNMV and viral RNA accumulation suggest a link between suppression activity and the event of RNA synthesis itself. p27 and p88 are required for RCNMV RNA replication (Figures 3 and 4), and are thought to form RNA replication complexes with RCNMV RNAs (Bates *et al*, 1995; Turner *et al*, 2004).

The replication of positive-stranded RNA viruses can be divided into two main processes: synthesis of a negative-strand RNA using the genomic positive-strand RNA as a template and the synthesis of a progeny positive-strand RNA using the negative-strand RNA as a template (Buck, 1996; Ahlquist *et al*, 2003). The requirement of the 3′ UTR of RNA2, but not that of 5′ UTR, for RNA2-mediated S-RNAi suppression (Figure 5) implies that RNA replication complex formation at least needed to initiate negative-strand RNA synthesis is important for RNAi suppression by RCNMV. The failure of negative-strand RNA2 to suppress S-RNAi with p27 and p88 suggests that the negative-strand RNA2 alone has no RNA elements required to initiate RNA synthesis and to suppress RNAi. In addition, the failure in suppressing S-RNAi of a p88 with a mutation in the GDD motif required for viral RNA synthesis together with p27 and RNA2 suggests that a stable RNA replication complex of RCNMV cannot be formed by the inactive p88. Studies of viral RNA synthesis using *Brome mosaic virus* (BMV) and its RdRP *in vitro* show that negative-strand viral RNA synthesis consists of three steps with distinct stability levels of RdRP–template RNA interactions. A final stable RdRP complex tightly associated with the template RNA occurs upon formation of between 3 and 13 phosphodiester bonds in RNA synthesis (Sun *et al*, 1996; Sun and Kao, 1997). The BMV data indicate that RNA synthesis with active RdRP activity is required for the formation of a stable RdRP complex, and suggest the requirement of a stable RdRP complex for RCNMV RNAi suppression.

We cannot completely rule out the possibility that p88 alone has RNAi suppressor activity because of the failure in detecting p88 using the anti-p27 antibody in all inoculation tests in this study. The requirement of p27 and replication-competent viral RNA with p88 for S-RNAi suppression suggests that at least an interaction of p88 with these factors is required to stabilize p88 or target it properly, when p88 functions in suppressing RNAi. This scenario does not contradict the requirement of stable RdRP complex formation for RCNMV RNAi suppression.

A possible mechanism for RNAi suppression through RNA replication

In the replication of positive-strand RNA viruses, host factors as well as viral proteins play important roles in initiating RNA synthesis, which include the assembly of viral RNA replication complexes (Ahlquist *et al*, 2003; Noueiry and Ahlquist, 2003). It is tempting to assume the involvement of the same host factor in both the RCNMV replication complex and RNAi machinery because the recruitment and sequestration of the host factor into the viral replication complex should interfere with RNAi. In this model, recruitment of the host factor into the replication complex from the RNAi machinery is mediated by RCNMV RNAs together with p27 and p88. This is because neither p27 or p88, nor viral RNAs that do not express viral proteins, alone suppressed RNAi (Figures 3 and 4, and data not shown). Putative host factors involved in both RCNMV replication and RNAi are discussed below.

RNAi processes affected by RCNMV

RCNMV reduced the accumulation of siRNAs in a transient IR-RNAi, although it failed to block the degradation of GFP mRNA in this assay (Figure 6). A possible explanation for the failure of RNAi suppression is that the suppressor activity of RCNMV is incomplete, and the small number of short siRNAs generated is sufficient to clear GFP mRNA through RISC-mediated cleavage. RCNMV also inhibited miRNA biogenesis (Figure 6). These results suggest that RCNMV affects a process in dsRNA cleavage, which is mediated by DCLs.

Roles of DCLs in RCNMV replication and RNAi suppression

RNAi is a defence system against virus infection in plants (Baulcombe, 2004). Therefore, mutant plants defective in RNAi-related genes may show increased susceptibility to virus infection as reported for *Cucumber mosaic virus* infection in *sgs2*, *sgs3* and *sde3* plants (Mourrain *et al*, 2000; Dalmay *et al*, 2001) and for *Turnip crinkle virus* infection in *dcl2* plants (Xie *et al*, 2004). However, surprisingly, infection tests of RCNMV using three *A. thaliana dcl* mutants (*dcl1*, *dcl2* and *dcl3)* showed a reduced susceptibility of *dcl1* plants against RCNMV infection as assessed by virus RNA accumulation in inoculated leaves (Figure 7). This suggests that DCL1 is required for RCNMV infection in either virus replication or virus movement in *A. thaliana*. Among four DCLs in *A. thaliana*, DCL1 is required for miRNA biogenesis (Reinhart *et al*, 2002; Kurihara and Watanabe, 2004), but not for siRNA biogenesis (Finnegan *et al*, 2003). Therefore, RCNMV may suppress RNAi through inhibition of other DCL functions. Alternatively, DCL1 may function in siRNA biogenesis as well as miRNA biogenesis. Further characterization of DCL function in *A. thaliana* contributes to clear the mechanism of how RCNMV suppresses RNAi.

DCL1 contains several domains including a nuclear localization signal, helicase, PAZ, two RNase III and two dsRNA binding (dsRBD) domains (Schauer *et al*, 2002). Although the molecular mechanism of DCL1 in RCNMV infection is unclear, an attractive hypothesis is that RCNMV utilizes a DCL1 as an RNA helicase, because the carmo-like plant viruses including RCNMV lack an RNA helicase domain in their coding proteins (Koonin and Dolja, 1993). To utilize DCL1 or its homologues as a host factor, RCNMV must inhibit the RNase activity of the protein to avoid cleavage of the viral dsRNA of the replication intermediate. Interestingly, the PIWI domain of human Ago2 interacts with the RNase III domain of Dicer to inhibit its RNase activity (Tahbaz *et al*, 2004). Such a protein with a PIWI-like domain may be another candidate involved in the RCNMV RNA replication complex, as well as in RNAi suppression.

How does RCNMV inhibit miRNA biogenesis? In plants, miRNA is processed in the nucleus (Park *et al*, 2005), in which a majority of DCL1 localizes (Papp *et al*, 2003). However, most plant RNA viruses are thought to replicate in the cytoplasm. Interestingly, Turner *et al* (2004) observed that both a GFP-fused form of RCNMV p27 and p88 localized in the ER in close contact or extended through the nucleus in *N. benthamiana* cells. RCNMV may use DCL1 or its homologue for RNA replication in the perinuclear region, which inhibits miRNA biogenesis.

Enhancement of RNA1-mediated RNAi suppression by RNA2

It is worth noting that the RNAi suppression activity of RNA1 is enhanced by the presence of RNA2 (Figure 2). The mechanism whereby RNA2 enhances the suppressor activity of RNA1 is unknown. However, the replication of RNA2 itself is likely to play a role in the enhancement, because replicable RNA2 can serve as the core of the RNA replication complex assembly, which is essentially required for RNAi suppression by RCNMV, as discussed above.

In summary, we present a model of RNAi suppression by RCNMV (Figure 9). In this model, the RCNMV replication complex in the ER suppresses RNAi through the deprivation of DCL1 or its homologue. Novel aspects of our results are that (i) RCNMV requires multiple viral components (p27, p88 and viral RNAs) to suppress RNAi, probably through virus RNA replication, (ii) RCNMV inhibits both miRNA and siRNA biogenesis and (iii) RCNMV requires DCL1 for efficient infection in plants.

Materials and methods

Plasmids

The plasmids containing the prefix 'pBIC' were used for agroinfiltration. The plasmids containing the prefix 'pUCR' or 'pRC' were used as templates for *in vitro* transcription. The plasmids containing the prefix 'pUB' were used as inocula for transient gene expression in protoplasts. See Supplementary data for detailed information on the plasmids used in this study.

S-RNAi and IR-RNAi suppression assays and transient miRNA assay

An S-RNAi and an IR-RNAi suppression assays were performed as described (Takeda *et al*, 2002). A transient miRNA assay was performed as described elsewhere (Kasschau *et al*, 2003). See Supplementary data for detailed information on these assays.

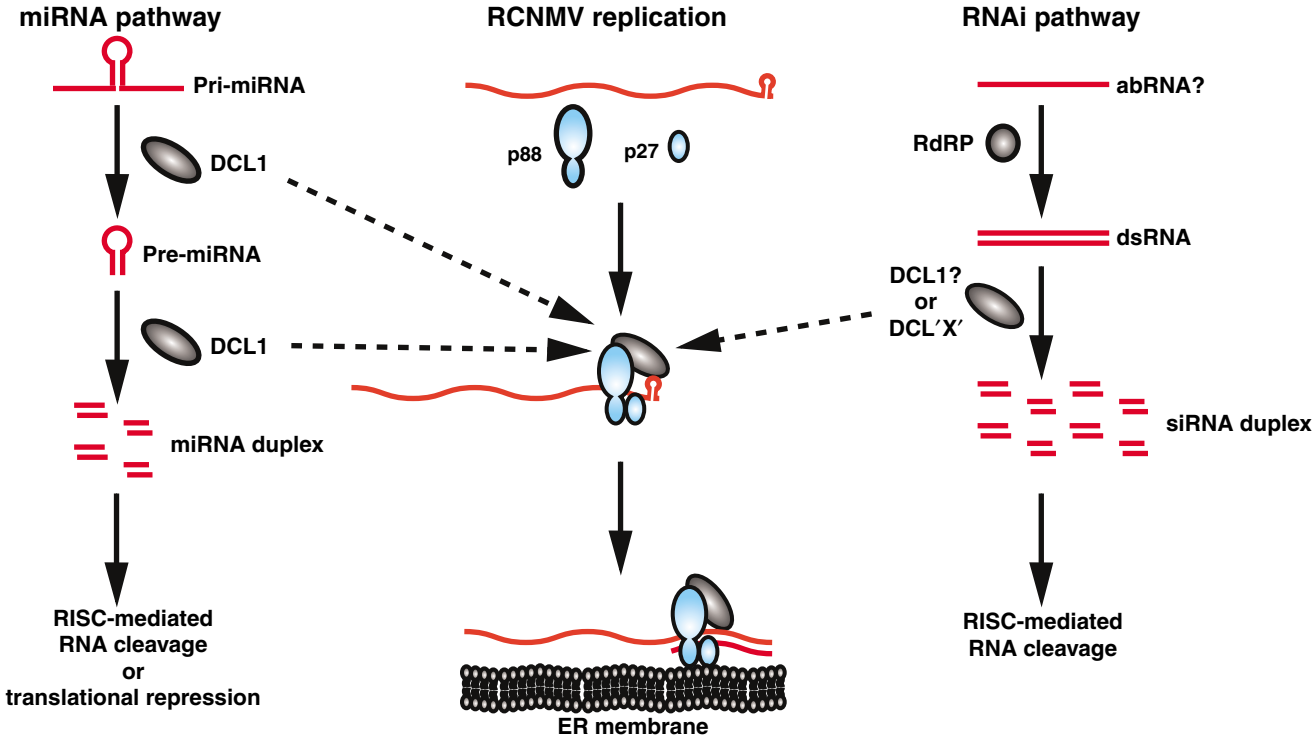

Figure 9 A model for RNAi suppression by RCNMV. p27 and p88 form RNA replication complexes with RCNMV RNA together with a host factor including DCL1 or its homologues on the ER. Recruitment of DCL1 or its homologues, which is involved in siRNA and miRNA generation, for the formation of RNA replication complexes leads to RNAi suppression.

In vitro transcription, inoculation of protoplasts and plants and analysis of viral RNA

pUCR1, pRC2|G and their derivatives were digested with *Sma*I, from which RNA transcripts were synthesized as described (Mizumoto *et al*, 2003). Protoplast experiments using BY-2 suspension-cultured cells were performed as described elsewhere (Watanabe *et al*, 1987) with minor modifications. Cowpea protoplasts were prepared as described (Mizumoto *et al*, 2003) and were inoculated as described elsewhere (Matsuda *et al*, 2004) with minor changes. The inoculated BY-2 and cowpea protoplasts were incubated at 17°C, and total RNA was extracted at 24 hpi as described (Mizumoto *et al*, 2003). Inoculation of the transcripts or the virion RNA of RCNMV onto *N. benthamiana* (Mizumoto *et al*, 2002) and *A. thaliana* (Fujisaki *et al*, 2003) and inoculation of the virion RNA of TMV-Cg (0.1 mg/ml) onto *A. thaliana* (Kurihara and Watanabe, 2003) were performed as described. *DCL1-9/DCL1-9* homozygous, *DCL1-9/dcl1-9* heterozygous and *dcl1-9/dcl1-9* homozygous plants were distinguished by both PCR analysis using appropriate primers as described (Kurihara and Watanabe, 2003) and by plant morphology (Schauer *et al*, 2002). Northern blot analyses for the detection of positive-strand RNA1 and RNA2 of RCNMV (Mizumoto *et al*, 2002) and TMV-Cg RNA (Kurihara and Watanabe, 2003) were performed as described. Negative-strand RNA2 of RCNMV was detected using DIG-labelled RNA probe transcribed by T7 polymerase from *Sma*I-linearized pRC2|G. See Supplementary data for more detailed information on these experiments.

Antibody production and immunoblot analysis

A PCR fragment was amplified from pRCAN1 (Mizumoto *et al*, 2002) using the primers p27-5Sma and p27-3Bam and then digested with *Nde*I and *Bam*HI. The 0.6 kb *Nde*I–*Bam*HI fragment of the DNA fragment was inserted into pET-15b (Novagen) to construct pET15b-550. An N-terminal His-tagged p27 expressed from pET15b-550 in *Escherichia coli* was used to prepare a rabbit anti-p27 antiserum. Immunoblot analysis was performed as described (Mizumoto *et al*, 2003).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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