Virus Recovery Is Induced in *Brome mosaic virus* p2 Transgenic Plants Showing Synchronous Complementation and RNA-2-Specific Silencing

Lakshminarayan M. Iyer and Timothy C. Hall

Institute of Developmental and Molecular Biology, Texas A&M University, College Station 77843-3155, U.S.A.

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Nicotiana benthamiana plants expressing *Brome mosaic virus* (BMV) p2 protein complemented replication of RNAs1+3 but, surprisingly, supported little or no replication of RNA-2. Despite this, the p2 transgenic plants were able to support systemic migration of RNAs-1 and -3. Kinetic analyses showed identical degradation rates for RNAs-2 and -3, greatly detracting from the concept of an induction of an RNA-2-specific degradation system. Deletion analysis identified a 200-nucleotide sequence that may contribute to silencing in a context-specific manner. When R₁ progeny of a severely silencing p2 transgenic line were tested for virus resistance, three different classes of reactions were observed. In class 1 and class 3 plants, the virus moved systemically and showed various extents of RNA-2 silencing. However, in class 2 plants, there was a stochastic onset of post-transcriptional silencing in the systemic leaves that was reminiscent of virus recovery. Plants showing recovery tended to have a greater number of transgene loci than did those exhibiting component-specific silencing. The induction of silencing did not appear to be dependent solely on the combined steady state levels of the transgene and viral RNA. Some plants transformed with a p2 frameshift construct showed a complete silencing phenotype, but none showed RNA-2-specific silencing. While the relationship between the two types of silencing remains unclear, we speculate that our observations reflect early events in the induction of virus recovery.

Additional keywords: gene silencing, PTGS, RNA degradation, virus-induced gene silencing, virus replication.

Brome mosaic virus (BMV) is a tripartite, positive-strand RNA virus belonging to the alphavirus superfamily (Koonin and Dolja 1993). Although BMV normally infects monocotyledonous plants of the family Gramineae, it is also known to systemically infect the solanaceous plant *Nicotiana benthamiana* (Mise et al. 1993), which has the experimental advantage of being readily transformed. The p1 and p2 proteins, respectively translated from RNA-1 and RNA-2, are vital compo-

nents of the viral replication complex (Kiberstis et al. 1981; French et al. 1986; Pogue et al. 1994). The p2 protein has a central, highly conserved, RNA polymerase domain and the interaction of its N terminus with the C terminus of p1 has been shown to be essential for virus replication (Kao and Ahlquist 1992; Kao et al. 1992). The original focus of our work was to produce transgenic plants expressing p1 and p2 proteins for viral replication studies. However, as will be described, although all lines of transgenic plants expressing the p2 protein were capable of replicating RNAs-1 and -3 *in trans*, they showed severe or partial silencing of RNA-2 replication. This surprising observation stimulated us to investigate the nature of this silencing, leading to observations that some R₁ progeny from a severely silencing line exhibited complete recovery and virus-induced, post-transcriptional gene silencing (PTGS) similar to that seen for *Tobacco etch virus* (TEV) (Lindbo et al. 1993), the tobamovirus *Pepper mild mottle virus* (Tenllado et al. 1995), and green fluorescent protein (GFP) silencing by a chimeric *Potato virus X*-GFP (PVX-GFP) construct (Ruiz et al. 1998b).

Many examples of plant resistance to viruses and recovery from virus infections have been shown to result from PTGS, based on sequence homology to the transgene RNA (de Haan et al. 1992; Lindbo and Dougherty 1992; Mueller et al. 1995; English et al. 1996; Sijen et al. 1996). PTGS is also thought to be responsible for cosuppression of several eukaryotic genes (Romano and Macino 1992; de Carvalho et al. 1992; van Blokland et al. 1994; Kennerdell and Carthew 1998; Fire et al. 1998; Ruiz et al. 1998a; Tabara et al. 1998). Common observations in these systems include the presence of a low steady state level of the transgene RNA, nonfunctional levels of the transgene protein, and the silencing of resident genes, transgenes, or RNA having significant sequence homology. These findings have led to proposals that transgene-specific post-transcriptional degradation systems are activated (Lindbo et al. 1993; English et al. 1996; Waterhouse et al. 1998) that recognize and degrade RNAs that have a high degree of homology to the silenced transgene. While indirect evidence for such degradation is strong (Goodwin et al. 1996; van Eldik et al. 1998), there has been only one report of transcript instability in silencing plants (Holtorf et al. 1999).

The present paper describes investigations into the mechanisms and possible relationship of specific silencing of RNA-2 and the complete silencing of the BMV RNA genome.

Corresponding author: Timothy C. Hall; Institute of Developmental and Molecular Biology, Texas A&M University, College Station 77843-3155, U.S.A.; Telephone: 409-845-7728; Fax 409-862-4098; E-mail: tim@idmb.tamu.edu

Toward this, we made extensive use of a protoplast system because it allowed us to observe results from synchronous infection of cells and it permitted a simultaneous investigation and comparison of these silencing processes. Additionally, superinfection of protoplasts from BMV-inoculated plants allowed investigation of both the systemic silencing status of the cell and its ability to support replication of the newly applied inoculum. We also exploited the opportunity provided by the multicomponent BMV system to use individual RNAs as internal controls for comparison of RNA-2 replication and degradation, and have explored the possibility that specific sequences may be involved in the silencing of RNA-2.

RESULTS

Transgenic *N. benthamiana* expressing BMV p2 complement replication of RNAs -1 and -3 while silencing replication of RNA-2.

Transgenic *N. benthamiana* plants expressing the p2 protein ("p2 plants") were made by *Agrobacterium*-mediated transformation of leaf segments (see Materials and Methods; Fig. 1). Of the five primary transformants (plants 2a.2, 2a.3, 2a.4, 2a.5, 2a.6), progeny (R_1) of lines 2a.3 and 2a.4 were extensively analyzed. The presence of a functional p2 protein was directly confirmed by the ability of protoplasts from these

lines to support replication of infectious transcripts of RNAs 1+3 (Dreher et al. 1989) in the absence of RNA-2 (Fig. 2). Since the construct used for transformation (Fig. 1A) does not contain the 5' 98 nucleotides (nt) of RNA-2, the transcript expressed from genomic DNA also lacks these nucleotides. Thus, while it can be translated to give p2, it cannot be replicated, and no progeny RNA was expected. However, the absence of (or reduction in) RNA-2 replication in protoplasts from the p2 plants when virion RNA was supplied as inoculum was unforeseen. Two levels of silencing were discernible: partial silencing (Fig. 2, line 2a.3 and data not shown for lines 2a.5, 2a.6), in which (+) strand RNA-2 levels are lower than those of RNA-1 and severe silencing (Fig. 2, lines 2a.2 and 2a.4), in which RNA-2 (+) strands were not detectable above background or were at trace levels. The levels of BMV RNA (+)-strand accumulation were similar to those seen in barley protoplast experiments, although the ratios for RNA components 1:2:3:4 were closer to 1:3:4:3 than the 1:1:3:3 ratio typi-

Fig. 1. Proportional maps of *Brome mosaic virus* (BMV) constructs showing (A) construct pRAB2a used to express BMV p2 protein in tobacco (see Materials and Methods) and (B) the T-DNA region of *Agrobacterium* vector pGA482 (An 1986), which contains a HindIII site into which pRAB2a was cloned. The 35Sd promoter, together with its enhancer region, was used to drive transcription of an mRNA that includes the BMV RNA-2 sequence from the initiator AUG (nucleotide position 104) to its 3' end (2865). This permits translation of the entire p2 reading frame (104–2569). The TEV leader sequence (Restrepo et al. 1990) was included to enhance translation. Probes used to detect the 35Sd promoter region and a portion of the p2 coding region in genomic DNA blots (Fig. 8) are indicated; sequence elements are denoted in bp. The pRAB2aFS mutant used for transgenic plants expressing a frameshift RNA incapable of making p2 protein contained a frameshift introduced at position 107 in the coding region (position indicated by arrow marked FS).

Fig. 2. Silencing of RNA-2 replication in p2 protoplasts. Protoplasts from wild type (wt) or transgenic *Nicotiana benthamiana* lines 2a.2, 2a.3, and 2a.4 were inoculated with transcripts of RNAs 1+3 or virion RNA (V) and harvested 24 h post inoculation (hpi). As expected, the transgenic lines support replication of RNAs-1 and -3 (and generate subgenomic RNA-4), but wt protoplasts do not. When virion RNA was used as inoculum, replication of both (+) strands (top panels) and (-) strands (bottom panels) of RNA-2 was severely suppressed in protoplasts from lines 2a.2 and 2a.4 and partially suppressed in protoplasts from line 2a.3. Band intensities reflect molar ratios as the 3' probe used is common to each RNA component. From at least three independent experiments, the ratios for accumulation of RNAs-1:2:3:4 in protoplasts were determined to be 1:3:4:3 for wt plants; 1:0.6:4:3 in R_1 progeny of the partially silencing line 2a.3 and 1:0.2:1.5: 1.7 in R_1 progeny of the severely silencing line 2a.4. With respect to wt protoplasts, this corresponds to an RNA-2 decrease in transgenic protoplasts of fivefold in line 2a.3 and 15-fold in line 2a.4.

cal for BMV RNAs in barley protoplasts (Duggal et al. 1992). The reason for the substantially different ratios of RNA progeny in tobacco and barley is not known, but they may reflect either an increased requirement for the replicase protein or a decreased requirement for coat or movement protein in the dicot host. For progeny of the partially silencing line 2a.3, the ratio of RNA components was 1:0.6:4:3, and for the severely silencing line 2a.4, the ratio was 1:0.2:1.5:1.7. This corresponds to a fivefold reduction in the partially silencing lines and a greater than 15-fold reduction (i.e., undetectable above background) in the level of RNA-2 (+) strands in the severely silencing line. Moreover, in the severely silencing line, a two-fold decrease, compared with wild type (wt), in the ratios of progeny RNAs 1:3 and 1:4 was observed. In the partially silencing lines, (-)-strand accumulation of RNA-2 was also clearly reduced (Fig. 2).

Time course analysis reveals no preferential degradation of RNA-2.

In protoplast transfection experiments, the synchronous nature of the infection offers the opportunity to follow the degradation of an inoculated RNA transcript over a period of time. Since results from many post-transcriptional silencing studies (English et al. 1996; Goodwin et al. 1996; Metzlaff et al. 1997; Waterhouse et al. 1998) predict the induction of a sequence-specific RNA degradation system, we anticipated that RNA-2 would be preferentially degraded in the p2 plants, especially those that severely silence RNA-2 replication. To examine this possibility, p2 transgenic and wt protoplasts were inoculated with RNAs 2+3 and the level of transcript remaining was monitored over a 24-h period (Fig. 3). The presence of any RNA-specific degradation fragments was evaluated with probes specific for RNA-2 or RNA-3. The RNA-2 probe was 1,125 nt in length, compared with the 200-nt 3' probe used in Figure 2, providing increased detection sensitivity. The degradation kinetics of RNA-2 paralleled those for RNA-3 in both transgenic and wt protoplasts, indicating no increase in the rate of degradation of RNA-2, compared with that of RNA-3. Contrary to the situation in some post-transcriptional silencing systems (Goodwin et al. 1996; Metzlaff et al. 1997; van Eldik et al. 1998), no transgenic, plant-specific RNA fragments were observed.

Time course assays measuring RNA replication in protoplasts inoculated with transcript RNAs 1+2+3 revealed that, in the severely silencing line 2a.4, there was no increase in accumulation of RNA-2 (+) or (-) strands (Fig. 4A). In contrast, the partially silencing line 2a.3 supported an increased accumulation of (+) strands, commencing at about 12 h post inoculation (hpi) (Fig. 4B). This is considerably delayed in comparison with the situation for wt protoplasts, in which accumulation of new RNA-2 (+) strands is evident by 4 hpi in barley (Loesch-Fries and Hall 1980), in wt *N. benthamiana* protoplasts (Fig. 4C), or for RNA-3, which serves as an unsilenced control in the transgenic protoplasts (Fig. 4A and B). Accumulation of RNA-2 (-) strands was at very low levels in the partially silencing line (Fig. 4B).

Evidence that silencing is sequence- and context-specific.

The specificity for silencing of RNA-2 in protoplasts of the p2 plants is likely to result from a phenomenon that is dependent on sequence homology, mediated either by the host or

by the ectopic presence of the p2 protein. We have previously shown that pRNA-2 E/S and pRNA-2 M/S, deletion mutants of RNA-2, can replicate when co-transfected to barley protoplasts with BMV RNAs-1 and -2 (Marsh et al. 1991). When used at high concentration (5 µg), these derivatives reduced the amount of RNA-2 produced and were therefore termed parasitic RNAs (pRNAs). We reasoned that, if a specific sequence in RNA-2 serves as the signal for silencing, then the signal might be absent from such deletion derivatives and they would escape the silencing mechanism. In *N. benthamiana* protoplasts, both Δ1062 and Δ822 (pRNAs-2 E/S and M/S, respectively, in Marsh et al. 1991) replicated in wt protoplasts, and even in p2 protoplasts from the severely silencing 2a.4 line (Fig. 5, note that RNA-2 replication was silenced in the transgenic protoplasts). These results suggested that the sequence recognized by the silencing phenomenon lay between positions 1680 and 2502 on RNA-2. To further define this element, the deleted region between the *EcoRV* and the *StuI* sites was reconstructed in 200-nt increments (see Materials and Methods; Fig. 5) and the derivative constructs were tested

Fig. 3. Comparison of degradation kinetics for RNA-2 and RNA-3 in wild type (wt) and transgenic protoplasts. Protoplasts from (A) wt or (B) transgenic (R_1 progeny of line 2a.4) protoplasts were transfected with RNA-2 and RNA-3 and degradation was followed by RNA blot analysis over a 24-h period with RNA-specific probes. Kinetics of degradation, shown for two independent experiments, were virtually identical for both RNAs, revealing no preferential degradation of RNA-2 in the transgenic protoplasts. Representative RNA blots are shown as inserts.

Fig. 4. RNA-2 replication is delayed in the partially silencing line but completely absent in the severely silencing line. **A**, Protoplasts from R_1 progeny of line 2a.4 were transfected with RNAs-1, -2, and -3 and accumulation of RNAs -2 and -3 was followed over a 24-h period. Although (-) strands of RNA-3 were visible 4 h post inoculation (hpi), (-) strands of RNA-2 were not detected despite the presence of (+) strands. **B**, Protoplasts from R_1 progeny of line 2a.3 were transfected with RNAs-1, -2, and -3 and accumulation of RNA-2 and RNA-3 (+) and (-) strands was followed over a 24-h period. In these lines, an increase in the levels of (+) strands was seen 12 hpi, indicative of a delayed onset of RNA-2 replication. **C**, Protoplasts from wt plants were transfected with RNAs-1, -2, and -3 and accumulation of RNA-2 followed over a 24-h period; in most cases (-) strands were detected 4 hpi.

in wt and transgenic protoplasts. Since RNA-2 and the $\Delta 262$ derivative were silenced, the region recognized by the silencing mechanism appears to lie upstream of $\Delta 262$, i.e., in the 200 nt between positions 2040 and 2240. Since the $\Delta 462$ construct gave only weak expression, nucleotides adjacent to position 2040 may also contribute to the targeting signal.

In order to test if RNA-2-driven silencing can be transferred to a heterologous RNA, four regions of RNA-2 (shaded bars in Figure 5C) were cloned into RNA-3 (see Materials and Methods). Protoplast replication assays revealed no silencing of the chimeric RNA-3 molecules in protoplasts from R_1 progeny of the severely silencing line 2a.4 (Fig. 5D). Additional regions of RNA-2 that overlapped the regions denoted in Figure 5C also failed to be silenced in similar experiments (data not shown). These findings appear to eliminate the possibility that sequence per se is the triggering factor in silencing of RNA-2 replication.

Systemic infection patterns in a silencing line.

As RNA-2 is strongly suppressed in line 2a.4, complete virus resistance or recovery from BMV infection in R_1 progeny of this line was expected. To test this, 14 R_1 progeny of line 2a.4 were infected with BMV and the progression of virus infection and silencing was monitored for up to 30 days. Although it was previously reported that wt BMV systemic infections of *N. benthamiana* were not generally visible (Rao and Grantham 1995), under conditions used in these experiments mild mosaic symptoms were readily discernible on several leaves at 20 to 25 days post inoculation (dpi; Fig. 6A).

An initial step was to establish the silencing status for each plant to be monitored; this was done by preparing protoplasts (from one to two leaves) and inoculating them with virion RNA. As expected, protoplasts from the wt control showed a typical replication pattern for each RNA (Fig. 7A, lanes 1) and RNA-2 replication was silenced in protoplasts from transgenic progeny plants (Fig. 7B–D, lanes 1). However, replication in systemic leaves (and derived protoplasts) of individual R_1 progeny plants revealed three classes of silencing and recovery that could be generalized: class 1, rapid reactivation of RNA-2 replication; class 2, complete silencing of both transgene expression and viral replication such that resistance to all BMV RNAs is generated; and class 3, which includes plants that are intermediate between classes 1 and 2 and display slow reactivation of RNA-2 replication, in which RNA-2 is initially systemically silenced even though the other RNAs are systemically propagated.

In class 1 plants, exemplified by two of the 14 lines, the transgenic plants showed near wt ratios of the BMV RNAs in the inoculated leaf (Fig. 7B, lanes 2), and in protoplasts from systemic leaves (Fig. 7B, lanes 3 and lanes 6), in protoplasts transfected with virion RNAs (Fig. 7B, lanes 4 and lanes 7), and in protoplasts transfected with RNAs 1+3 (Fig. 7B, lanes 5 and lanes 8). Apparently, RNA-2 replication can readily overcome the silencing effect in these plants as normal genomic replication was observed by 10 dpi (Fig. 7B, lanes 3 to 5), masking the fate of exogenously added (superinfected) virion RNA or RNAs 1+3. Mild mosaic symptoms were visible on the systemic leaves 20 dpi (Fig. 6B).

In class 2 plants, exemplified by six of the 14 lines, residual BMV RNA-1 and -3 (no RNA-2) (+) strands but virtually no (-) strands were detected 20 dpi in the inoculated leaves of

most lines (Fig. 7C, lanes 2). Evidence that complete silencing and resistance to BMV infection had been induced was provided by the facts that noninoculated leaves showed no symptoms of infection (Fig. 6C), that BMV RNAs were absent from protoplasts isolated from these leaves (Fig. 7C, lanes 3), and that these protoplasts did not support replication of superinfected virion RNA or RNA 1+3 transcripts (Fig. 7C, lanes 4 and 5).

In class 3 plants, exemplified by six of the 14 lines, although reactivation of RNA-2 replication was seen 20 dpi in the inoculated leaf, in two of these lines RNA-2 was reduced or absent in protoplasts transfected with virion RNA (Fig. 7D, lanes 7) or RNAs 1+3 (Fig. 7D, lanes 8); interestingly, the pattern (reduction or absence of RNA-2) in the superinfected protoplasts maintained that of nontransfected protoplasts: compare lanes 4 and 5 with lanes 3, or lanes 7 and 8 with lanes 6 in Figure 7D). In the other four of the six lines, no virion RNA was detected in the systemic leaves (suggesting that no systemic infection was established), yet protoplasts from these leaves were capable of replicating RNAs -1 and -3 (while silencing RNA-2) when superinfected with virion RNA or RNAs 1+3 (data not shown). Phenotypic symptoms of infection were difficult to detect in the class 3 plants.

Despite the dramatic silencing of RNA-2 in all (except class 1) progeny, in tests of five R₁ progeny of line 2a.4 inoculated with RNAs 1+3, systemic spread of RNAs -1 and -3 was observed (data not shown). Intriguingly, this indicates that the p2 transgene is capable of supporting systemic replication and movement of RNA-1 and RNA-3 in the absence of replicating RNA-2.

DNA blot analysis reveals the stochastic nature of complete BMV genome silencing.

Genomic DNA blot analysis of R₁ progeny of line 2a.4 revealed the presence of two segregating loci (Fig. 8). Since an *Xho*I site is situated between the *Cauliflower mosaic virus* 35S double promoter and the p2 coding sequence (Fig. 1A), probes corresponding to these elements should not hybridize to the same band. Since the 6.9-kb band in Figure 8 is detected by both probes, it appears that locus b is complex and contains a repeat structure. While complete silencing of the BMV genome was observed in the absence of locus a (Fig. 8, line

12) or b (Fig. 8, line 13), its occurrence appeared to be favored by the presence of both loci (Fig. 8, lines 1, 2, 14). However, the presence of both loci did not guarantee complete silencing of the BMV genome (Fig. 8, line 15) and the basis for stochastic onset of silencing in apparently genetically identical plants is perplexing.

Fig. 5. Replication of deletion derivatives of RNA-2 in wild type (wt) and transgenic protoplasts. **A**, Map of RNA-2 showing restriction sites in the p2 coding region, the p2 coding region (shaded bar), and the regions deleted in the pRNA constructs (denoted by the thin line between the hatched regions). The region (1,062 nucleotides [nt]) from the *Eco*RV to the *Stu*I site was reconstructed in 200-nt increments; Δ followed by a number indicates nucleotides deleted from RNA-2 between these sites; number to the left corresponds to lane number in **B** and **C**. **B**, (+) and (-) strand progeny accumulation in wt or transgenic (line 2a.4) protoplasts transfected with RNA-1, RNA-2, and the indicated deletion derivatives (lanes 2-6). Lanes 1 represent protoplasts transfected with RNAs-1+2. Positions of replicated pRNAs (see text) are indicated by filled circles; (*) denotes a degradation product of RNA-1. **C**, Map showing regions of pRAB2a (hatched bars) mobilized into RNA-3 (see Materials and Methods for constructs; restriction enzyme cleavage positions on RNA-2 are indicated). Numbers given beside construct names correspond to lane numbers in **D**. **D**, Accumulation of chimeric RNA-3 (+) and (-) strands in wt and transgenic protoplasts (from R₁ progeny of line 2a.4) inoculated with RNA-1, -2, and chimeric RNA. Lanes 1 show replication of wt RNA-3. RNAs were probed with an RNA-3-specific probe). Protoplasts were harvested and analyzed 24 h post inoculation.

Frameshift plants show complete BMV silencing and no RNA-2-specific silencing.

To test if sequence specific RNA-2 silencing is caused by ectopic expression of the p2 protein, transgenic plants expressing a frameshift p2 RNA were made by *Agrobacterium*-mediated transformation. The frameshift caused a translation termination at codon 7, resulting in the formation of a non-functional polypeptide (see Materials and Methods). Ten putatively independent transformed lines were tested for their ability to either complement replication of RNAs 1+3 (data not shown) or replicate virion RNA by protoplast assays. None of the plants complemented RNAs 1+3 (data not shown), but silencing was seen in three of the 10 plants (exemplified by line 8 in Figure 9). Input (+) strands were barely detected and no (–) strands were seen. None of the plants showed any RNA-2-specific silencing.

DISCUSSION

PTGS and virus resistance.

A wealth of studies now exists concerning the role of gene silencing during plant virus infection. Our initial finding, that ectopic expression of the BMV p2 polymerase results in the specific silencing of replication of the RNA component from which it derives (Fig. 2), differs from previous studies that describe silencing of the entire viral genome. These differences may reflect the fact that few of the earlier studies used the protoplast techniques that have allowed us to investigate very early stages in silencing events. In contrast, results from our experiments on systemic infection (Fig. 7) have many parallels with findings from other systems. The use of protoplasts appears to provide a unique insight into the processes involved during the development of silencing. For example, they can be used to explore the widely accepted postulate that post-transcriptional silencing yields a low accumulation of viral RNA progeny as a result of an induced degradation process and to consider the alternative speculation that the absence of RNA derives from an active process that specifically inhibits RNA-2 replication. The RNase induction scenario has been postulated in several models of PTGS and, in at least one case, an increased instability of transgene RNA transcripts was observed in time course assays (Holtorf et al. 1999).

However, our studies on the kinetics of RNA-2 silencing (Fig. 3) provide rigorous evidence that there is no increased instability of the RNA-2 transcript in transgenic plants containing a p2 open reading frame as the degradation rates of RNA-2 paralleled those of co-inoculated RNA-3. This greatly detracts from the presence of an “activated” RNase in our lines. An alternative strategy for post-transcriptional silencing of RNA-2 is inhibition of replication and, as discussed below, this can be accomplished in at least three ways.

Inhibition of replication as a silencing strategy.

Many cases of virus resistance appear to involve transgenically expressed virus proteins (Powell Abel et al. 1986; Golemboski et al. 1990; Carr et al. 1992; Beck et al. 1994; Carr et al. 1994; Palukaitis and Zaitlin 1997) that interfere with normal functions of virus replication and movement. Previous experiments have shown that the ratios for accumulation of individual BMV RNA components are strictly maintained, even if one of the RNAs is inoculated at an abnormal level (Rao and Hall 1991). Since the silencing of RNA-2, resulting from ectopic expression of p2 protein, dramatically changes the normal ratio for the accumulation of BMV RNA components, a logical conclusion is that p2 protein controls the level of RNA-2 replication. This appears to be mediated by an inhibition of (–) strand synthesis (Fig. 2). Indeed, in the absence of exogenous p2 protein, 10 transgenic plants containing a frameshift mutation in the transgene RNA failed to show any RNA-2-specific silencing (Fig. 9). Alternatively, control may be mediated solely by aberrant RNA expression levels, resulting in the stimulation of host RNA-dependent RNA-polymerase (RdRp) and the synthesis of RNA molecules complementary to the transgene. The latter concept is supported by its similarity to quelling phenomena in *Neurospora crassa*, where genetic studies have identified the involvement of a host RdRp in PTGS (Cogoni and Macino 1999). A third possibility is that (–) strands are the target of silencing, as proposed in resistance to *Tobacco mosaic virus* by plants expressing the replicase gene (Marano and Baulcombe 1998). However, if (–) strands were the sole target in our system, then p2 transgene expression should be unaffected during systemic silencing, which is not the case (Figs. 2 and 7).

Fig. 6. Mild mosaic infection of *Brome mosaic virus* (BMV) on *Nicotiana benthamiana*. **A**, Infected leaf of a nontransgenic plant (20 days post inoculation [dpi]) inoculated with BMV. **B**, Leaf from a class 1 p2 transgenic plant (20 dpi; see text and Figure 7) infected with BMV. **C**, Leaf from a class 2 p2 transgenic plant (20 dpi) infected with BMV (no systemic infection occurred and the leaf looks identical to a noninfected leaf).

Fig. 7. Virus accumulation and progression of infection in systemically infected *Nicotiana benthamiana*. Accumulation of *Brome mosaic virus* (BMV) RNA (+) and (-) strands is shown for inoculated leaves (I) or protoplasts from (A) wild type (wt) or R₁ progeny of line (B) 2a.4: 2a.4-3 (class 1), (C) 2a.4-1 (class 2), or (D) 2a.4-4 (class 3). Lanes 1 (PI) show accumulation of BMV RNA in protoplast transfection assays prior to inoculation of plants with the virus. Lanes 2 (I) show accumulation of BMV RNA in the inoculated leaf 20 days post inoculation (dpi). Lanes 3 and 6 (S) show accumulation of virus from systemic infection (untransfected protoplasts) at 10 and 20 dpi, respectively. Accumulation of virion RNA and RNAs 1+3 in protoplasts from these 10 dpi systemic leaves superinfected with virion RNA or RNAs 1+3 is shown in lanes 4 (V) and 5 (1+3), respectively, and in protoplasts from 20 dpi systemic leaves superinfected with virion RNA or RNAs 1+3 is shown in lanes 7 (V) and 8 (1+3), respectively. Positions of (-) strand transcripts resulting from use of uncapped RNA-3 transcripts (see Materials and Methods) are shown by arrowheads. Arrows flanking the panels show positions of RNAs-1, -2, -3, and -4.

Why do RNA-2 deletion derivatives escape silencing?

RNA-2 possessing a 200 nt (or greater) deletion upstream of the *StuI* site (Fig. 5) replicated in protoplasts of the severely silencing transgenic line 2a.4. This implicates the 200-nt region as a recognition sequence for silencing. However, chimeric RNA-3 containing a 1,062-nt region from the *EcoRV* to the *StuI* site (which includes this 200-nt region) is not silenced in transgenic plants. These results suggest that, although this region is involved in triggering silencing, its context with respect to the rest of the RNA is probably a vital component in the observed silencing of RNA-2 replication. An alternative explanation is that these deletion derivatives replicate faster due to their reduced size, resulting in higher accumulation and hence their detection in protoplasts. However, in several instances of PTGS and virus resistance, regions as small as 60 nt have been imputed as targets on the silenced transgene, or viral RNA (Hellwald and Palukaitis 1995; English et al. 1996; Sijen et al. 1996; Marano and Baulcombe 1998).

Induction of systemic silencing.

Two patterns of virus resistance in transgenic plants have been attributed to PTGS: complete virus resistance and recovery (Lindbo et al. 1993; Goodwin et al. 1996). While our systemic infection studies showed three different infection patterns in transgenic plants, all the plants had the propensity to silence RNA-2 in protoplast assays prior to virus inoculation (Fig. 7B–D, lanes 1). The ability to recover from RNA-2-specific silencing probably reflects the net accumulation of RNA-2, resulting from the opposing events of replication and inhibition. The RNA-2 replication rate is higher than the inhibition rate for class 1 plants infected with BMV, but lower in class 3 plants, resulting in a delayed accumulation of RNA-2 in class 1 plants and none in class 3 plants. Similarly, in the partially silencing lines (Fig. 2, lines 2a.3), where the replication rate is probably somewhat greater than the inhibition rate, a delayed increase in RNA-2 accumulation was seen in the protoplast assays (Fig. 4B). Interestingly, in class 1 and class 3 plants, protoplasts obtained from systemic leaves 20 dpi showed more severe silencing of RNA-2 than those obtained 10 dpi (Fig. 7B and D, compare lanes 3–5 with lanes 6–8), suggesting that the inhibition rate can be modified during infection. Some of these observations parallel virus resistance studies in *Cucumber mosaic virus* (CMV), where plants expressing CMV RNA-1 showed differential accumulation levels of RNA-1 when infected with CMV, while being able to complement replication of RNA-2 and RNA-3 (Canto and Palukaitis 1998).

The most striking infection pattern was seen in the class 2 R_1 progeny of line 2a.4 (Fig. 7C), where it resulted in a complete cessation of virus replication and p2 transgene expression. This pattern is reminiscent of virus recovery and virus-induced gene silencing observed in several systems (Lindbo et al. 1993; Kumagai et al. 1995; Tenllado et al. 1995; Anandalakshmi et al. 1998; Kjemtrup et al. 1998; Ruiz et al. 1998b; Ratcliff et al. 1999). Interestingly, systemically silenced class 2 plants did not show a relative increase in degradation of RNA-2 in time course assays (data not shown). However, in these plants the severity of silencing was increased many fold. This may reflect the transmission of a silencing signal from the inoculated leaves, as has been proposed by others (Palauqui et al. 1997; Voinnet et al. 1998; Palauqui and Balzergue 1999). In kinetic studies of sys-

Fig. 9. Complete silencing of the *Brome mosaic virus* (BMV) genome in protoplasts from *Nicotiana benthamiana* plants transformed with an RNA-2 frameshift mutant. Lack of replication of viral RNA shown for line 8 is representative of three silencing lines of 10 independent transformants tested; no line was obtained that showed RNA-2-specific silencing. Lines 7 and 11 are representative of seven nonsilencing lines.

Fig. 8. Genomic organization for the (A) 35S promoter and (B) p2 coding region in segregating R_1 progeny of line 2a.4. Arrows denote restriction fragments from two independently segregating loci; progeny were designated as class 1, 2, or 3 according to systemic silencing characteristics (see text).

temic infection, we observed that the spread of systemic silencing typically takes between 4 and 10 dpi (data not shown).

The trigger for systemic gene silencing remains unclear. It is not the level of RNA-2 alone, as RNA-2 increased substantially in class 1 plants during infection without triggering systemic silencing. Moreover, systemic silencing was also induced by inoculating RNA-2 alone, indicating that replication of RNA-2 is probably not necessary for induction (data not shown). Analysis of genomic DNA blots showed that the presence of multiple loci gave a greater propensity for induction of systemic silencing than did few loci (Fig. 8); this is commonly seen in PTGS, where inverted repeats and multiple loci have a high affinity for silencing (Jorgensen et al. 1996; Sijen et al. 1996; Vaucheret et al. 1998). However, two lines with similar patterns showed different reactions to virus infection (Fig. 8, lines 14 and 15). This stochastic nature of induction is puzzling but has been seen in other systems (Smith et al. 1994; English and Baulcombe 1997), where isogenic lines showed variability in virus resistance, and it may be related to the ploidy of the locus. It is likely that, as found by others (English and Baulcombe 1997; Que and Jorgensen 1998; Elmayan et al. 1998), the expression levels of aberrant transgene RNA play an important role in the onset of systemic silencing of the entire viral genome. As discussed elsewhere, this situation probably differs from that responsible for gene-specific silencing of RNA-2.

Does a relationship exist between RNA-2-specific and systemic silencing?

It is possible that the initial silencing of RNA-2 seen in protoplasts and the systemic silencing induced by virus inoculation are two unrelated silencing events, even though both may work by targeting RNA-2. Whereas the CMV system described by Hellwald and Palukaitis (1995) involved transgenic plants expressing a nonfunctional replicase and our observations for BMV involve plants expressing a functional replicase component, they may be similar in exhibiting two distinct mechanisms that lead to resistance: one that affects replication and another that affects replication and movement. This hypothesis is supported by the findings from frameshift transgenic plants in which multiple plants were obtained in which the BMV genome was completely silenced, but no plants showing RNA-2-specific silencing were encountered (see above and Figure 9), implicating the p2 protein in silencing. However, the lack of RNA-2-specific silencing in these plants is not conclusive proof of the absence of a relationship between the two processes. It is possible that all the p2 plants initiated PTGS prior to virus inoculation (RNA-2 silencing) but only some of them (class 2) triggered a catalytic, systemic, silencing signal that resulted in complete silencing of the p2 transgene and BMV resistance. It would be interesting to see if, in other cases of transgene-mediated virus recovery or virus-induced gene silencing, a basal level of PTGS exists that is insufficient to silence the transgene or to mediate virus resistance and is capable of being amplified several fold following virus or nucleic acid inoculation.

MATERIALS AND METHODS

Plasmid constructs and *Agrobacterium*-mediated transformation.

The region of BMV RNA-2 encoding protein p2 and the complete 3' end was amplified by polymerase chain reaction

(PCR) with a forward primer (5'CTTTCTACTATGGCCAA GATGTCTTCGAAAACC3') containing a site that can be digested with *MscI* to release the underlined sequence, which includes the 5' coding region of p2 (bold), and a reverse primer (5'CAGGATCCCGACATGGTCTCTTTTAGACAT TTACAGTG3') containing a *BamHI* site to release the underlined sequence, which includes the 3' end of RNA-2 (bold). The *MscI*-*BamHI* fragment was cloned into the vector pRTL-2 (Restrepo Hartwig and Carrington 1994; kindly provided by Jim Carrington), which had been digested with *NcoI* and blunt-ended with mungbean nuclease and then digested with *BamHI*. This yielded clone pRTLB2a, which has a 35S double promoter (35Sd), a TEV leader, the p2 coding region with its 3' 200 noncoding sequence, and a 35S terminator (Fig. 1A). Partial digestion of pRTLB2a with *HindIII* yielded a 3,912-bp fragment containing the 35Sd promoter, the p2 coding region, and the 35S terminator. This was transferred to the *HindIII* site of the plant transformation vector pGA482 (An 1986) to give the vector pRAB2a (Fig. 1) and used in *Agrobacterium* (strain LBA4404)-mediated transformation (Horsch et al. 1984) of *N. benthamiana*. For construction of pRAB2aFS, a PCR fragment was amplified (Pfu polymerase; Stratagene, La Jolla, CA) from pRTLB2a with a forward oligonucleotide primer complementary to the 5' portion of the TEV leader that contained a unique *EcoRI* site (underlined) (5'GGAGAGGAC CTCGAGAATTCAAC3') and a reverse oligonucleotide primer (5'TTTTCGAA(GA)CATCTTGGGCTATCGTTC3') that had a unique *BstBI* site (underlined). This resulted in the deletion of nucleotides 107 and 108 of RNA-2 (in parentheses) and caused a frameshift in the p2 coding sequence at the second codon. The frameshift resulted in termination of translation by tandem stop codon sequences at codon positions 7 and 8. The PCR fragment was digested with *EcoRI* and *BstBI* and cloned into the *EcoRI*-*BstBI* site of pRTLB2a to give pRTLB2aFS, which thus contains the 2-nt deletion (Fig. 1A). A *HindIII*-*KpnI* fragment (2,059 bp), corresponding to the 5' end of pRTLB2aFS (Fig. 1A), was cloned into the *HindIII*-*KpnI* site of the polycloning site of the plant transformation cloning vector pGA482 (Fig. 1B) to give pIH26. A *KpnI* fragment (1,869 bp) that corresponds to the 3' end of pRAB2a was then mobilized into the unique *KpnI* site of pIH26 to give pRAB2aFS. This construct differs from pRAB2a in only having a 2-nt deletion at positions 107 and 108 of RNA-2.

Agrobacterium-treated leaf disks were incubated in Murashige minimal organics medium (MS; Murashige and Skoog 1962; GIBCO BRL, Grand Island, NY) supplemented with 2 µg of N⁶benzylaminopurine per ml and 0.1 µg of α-naphthaleneacetic acid per ml with kanamycin (100 µg/ml) for selection and 150 µg of cefotaxime per ml to prevent growth of *Agrobacterium* and Phytigel (Sigma, St. Louis, MO) to a concentration of 0.1% (wt/vol). Shoots were excised and rooting was initiated on MS medium containing 100 µg of kanamycin and 150 µg of cefotaxime per ml. A total of five independent transformants was obtained, two of which (lines 2a.3 and 2a.4) were extensively used for the studies reported here. Five additional, independent transgenic plants were generated; these plants also showed partial or complete silencing of RNA-2, while complementing RNA-3 replication.

The construction of plasmids pT7T3B1, pT7T3B2 and pT7T3B3 for making RNA-1, RNA-2, and RNA-3 has been

described previously (Dreher et al. 1989). To make deletions of different lengths between the *EcoRV* (position 1440) and *StuI* (position 2502) sites of RNA-2, three PCR fragments were generated from pT7T3B2 by Pfu polymerase (Stratagene) with a forward primer (5'TGATTCGTACC ATCAAGCCC3') that included the *NcoI* site upstream of the *EcoRV* site on RNA-2 and the reverse primers (5'GAAGAG AGACGTAAACATATC3') (5'TTAATAAACTTCATTTCGAT CAC3'), and (5'ACTTACATACAGGATCGCTC3'). The PCR products were digested with *NcoI* to give fragments of 959, 1,159, and 1,359 bp, respectively, and cloned into the region between the *NcoI* (position 882) and *StuI* (position 2502) sites of pT7T3B2 to give deletions of 662, 462, and 262 bp, respectively, between the *EcoRV* and *StuI* sites (Fig. 5A). The construction of the deletion derivatives $\Delta 822$ (pRNA-2 M/S) and $\Delta 1062$ (pRNA-2 E/S) was described previously (Marsh et al. 1991). For construction of pT7T3B2, which was used to make RNA-2-specific riboprobes, a 1,125-bp fragment from a *KpnI* (position 1252) site to a *SacI* (position 2376) site of pT7T3B2 was cloned into the vector pT7T3 18U (Amersham Pharmacia Biotech, Piscataway, NJ) to give pT7T3B2 (C. C. Huntley and T. C. Hall, *unpublished results*). For construction of pT7T3B3SB, which was used to transcribe RNA-3-specific probes, a 262-bp fragment from pT7T3B3 (Dreher et al. 1989) was generated by cloning a *BglII* (position 1221) to *SacI* (position 1482) fragment from RNA-3 into a *BamHI*-*SacI*-digested pT7T3 18U vector (Amersham Pharmacia Biotech) to give pT7T3B3SB (C. C. Huntley and T. C. Hall, *unpublished*).

For construction of the chimeric RNA-3 containing an *EcoRI*-*EcoRV* (RE) fragment from pRAB2a, a 1.4-kb PCR fragment (Fig. 5C) was amplified with the forward primer (5'TTGCGCGCAACACAACATATACAAAACAA3') containing a *BssHII* site (**bold**) and a reverse primer (5'TCT AGGTGCAGCTCACCC3') downstream from an *EcoRV* site (Fig. 5C). This was cloned into the vector pCR-Script (Amp)-SK+ vector (Stratagene, La Jolla, CA) and contains a subsequence of pRAB2a from the *EcoRI* site in the TEV leader sequence and includes the *EcoRV* site on the coding region of p2 (corresponding to the region 104–1440 of RNA-2). The PCR fragment (1.4 kb) was released by digestion with *BssHII* and *EcoRV* and cloned into the region between the *BssHII* and *StuI* sites of pT7B3 (positions 1252–1751 of RNA-3; Marsh et al. 1988). RNA-3 containing the fragment ES (1440–2502 of RNA-2) was constructed by digesting the vector pT7B2 (Dreher et al. 1989) with *EcoRV* and *StuI* and purifying a 1,062-bp DNA fragment. This fragment was then cloned between the *BssHII* site, which was blunt-ended with Pfu polymerase (Stratagene), and the *StuI* site (position 1751 of RNA-3) of pT7B3. Chimeric RNA-3 containing the SH fragment from RNA-2 was constructed by digesting RNA-2 with *StuI* and *HindIII* and isolating a 163-bp fragment. This was then cloned between the *StuI* and *HindIII* sites (positions 1751 and 1917 on RNA-3, respectively) of pT7B3. The construction of chimeric RNA-3 containing the 3' end of RNA-2 (RNA-3/2t) has been described before (Duggal et al. 1992).

In vitro transcription, protoplast transfection, and time course analyses.

Plasmids pT7B1, pT7B2, and pT7B3 were linearized and transcribed with the T7 Megascript kit (Ambion, Austin, TX) with a final concentration of 0.9375 mM GTP and 6 mM cap

analog (m⁷GppG; New England Biolabs, Beverly, MA) to generate capped transcripts. The RNA-2 plasmids containing the deletion derivatives and the RNA-3 plasmids containing regions of RNA-2 (described above) were digested with *BamHI* and transcribed in vitro with the Ambion T7 Megascript kit to make the deletion derivatives used for the experiment.

Protoplast transfection in the presence of polyethylene glycol (PEG) was performed as described previously (Loesch-Fries and Hall 1980; Dreher et al. 1989). Plants transformed with pRAB2a were tested for p2 protein expression by transfection of approximately 200,000 protoplasts with RNAs 1+3; functional complementation was shown by replication of RNA-1 and RNA-3 in the absence of supplied RNA-2 (Fig. 2). The ability of plants transformed with pRAB2aFS to silence BMV replication was tested by transfecting approximately 200,000 protoplasts with virion RNA (Fig. 9). For time course analyses, approximately 1.6×10^6 protoplasts were transfected with 16 μ g of each RNA and divided into eight samples (1.5 ml each), one of which was taken for RNA extraction at each desired time point. RNA degradation was followed by RNA blot analysis over a 24-h period with RNA-2- or RNA-3-specific probes; initial (zero time) band intensities were designated as 100% and the degradation in Figure 3 is shown with reference to this value. For analysis of the replication of deletion derivatives (Fig. 5) in wt and transgenic plants, approximately 200,000 protoplasts were transfected with 2 μ g of RNA-1, RNA-2, and the deletion derivative.

RNA-gel blot analysis.

Total nucleic acids were extracted from protoplasts by sodium dodecyl sulfate–phenol followed by ethanol precipitation and progeny RNA was analyzed by gel blot analysis as described previously (Loesch-Fries and Hall 1980; Dreher et al. 1989). For protoplast assays, half of the extracted samples were separated on a 10 mM sodium phosphate-buffered gel and transferred to a nylon membrane (Nytran; Schleicher and Schuell, Keene, NH) as described previously (Dreher et al. 1989). Riboprobes corresponding to the (+) and (–) strands of the 3' 200 bases were used to detect BMV progeny RNA as described previously (Dreher et al. 1989). For RNA-2 and RNA-3-specific riboprobes, pT7T3B2 and pT7T3B3SB were digested with *HindIII* and *EcoRI* and transcribed with T7 RNA polymerase and T3 RNA polymerases (Dreher et al. 1989) to make (+) and (–) strand probes, respectively. For time course studies, RNA gel blots were sequentially probed with the RNA-2- and RNA-3-specific probes. Gel blot exposures were done on a phosphorimager system (Fujix BAS 2000; Fuji Medical Systems USA, Stamford, CT) for quantitation of band intensities. For studies on the replication of chimeric RNA-3, the blots were probed with the RNA-3-specific probe.

Analysis of transgenic plants.

R₁ progeny of line 2a.4 were regenerated by selection on kanamycin containing MS media and then transferred to the greenhouse for further growth and analysis. Virus inoculation onto *N. benthamiana* plants was done as previously described (Rao and Grantham 1995). The plants were, however, not maintained in the dark prior to inoculation.

Approximately 200,000 protoplasts were made from each of the 14 R₁ plants prior to virus inoculation and transfected in

the presence of PEG (Loesch-Fries and Hall 1980; Dreher et al. 1989) with virion RNA or RNA-1 and RNA-3 (uncapped RNA-3 was used for this experiment) to test for their ability to replicate and the extent of suppression of RNA-2 (labeled as preinoculated, PI, in all Figure 7 lanes 1). Similarly, protoplasts were made from systemic leaves 8 to 10 dpi (10 dpi) or 20 to 25 dpi (20 dpi) and inoculated with virion RNA or RNA-1 and RNA-3. Total nucleic acids were extracted from these protoplasts, and half of the extracted sample was separated on a 10 mM sodium phosphate-buffered agarose gel and transferred to a nylon membrane and probed for the presence of BMV RNA with the 3' 200-nt sequence as probe (Dreher et al. 1989). The uninoculated protoplasts (marked S in Figure 7 lanes 3 and lanes 6) provide a control reference for any systemic virus present prior to inoculation. Total RNA was also extracted from inoculated leaves 20 dpi by the guanidine thiocyanate method (Huntley and Hall 1996) and analyzed for the presence and ratios of BMV RNAs (marked as I in Figure 7 lanes 2). Total RNA (2 µg) was loaded onto a 10 mM sodium phosphate-buffered agarose gel for separation and RNA analysis. Since (–) strands were not detected at this concentration of total RNA, 10 µg was loaded to enable their detection; this amount was also used for detection of (+)/(–) strands from class 2 plants (Fig. 7C, lane 2). The arrowheads in Figure 7 represent (–) strand transcripts that resulted from the use of uncapped RNA-3 transcripts (L. M. Iyer, *unpublished observations*). The absence of RNA-1 (–) strands reflects the lack of complementation by the transgene p2 protein. Note that when virion RNA is used there is no replication of RNA-3 or production of subgenomic RNA-4 (see Figure 7C, lanes 4 and lanes 7).

DNA gel blot analysis.

Total DNA was extracted from the *N. benthamiana* plants by the CTAB method (Taylor et al. 1993) and 10 µg of the extracted DNA was cleaved with *Xho*I, which cuts only once between the 35S promoter and the p2 coding region (Fig. 1A). DNA was separated on a 0.8% agarose gel and transferred by capillary blotting to a nylon membrane (Hybond N+; Amersham Pharmacia Biotech). The blot was then probed as described by Buchholz et al. (1998) with a *Hind*III-*Eco*RI fragment that spans the 35Sd promoter or a *Eco*RV-*Hind*III fragment that spans the 3' end of the p2 coding region (Fig. 1B).

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