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Interference with Brome Mosaic Virus Replication in Transgenic Rice

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Rice protoplasts were transformed with four separate constructs derived from the genome of brome mosaic virus (BMV) and regenerated into callus and plant lines. Constructs used for transformation were designed to yield (i) an artificial, defective, interfering RNA derived from RNA-2, (ii) a sense tRNA-like structure corresponding to the 3′ end of RNA-2, (iii) an antisense sequence corresponding to the intercistronic region of BMV RNA-3, and (iv) RNA encoding the viral capsid protein. Protoplasts obtained from transgenic plants or callus lines showed up to 95% reduction in accumulation of progeny viral RNAs when inoculated with virion RNA. The addition of up to 20-fold the normal level of inoculum was required to overcome the induced resistance. The observed interference appeared to be mediated through viral RNAs rather than protein products, but was not proportional to detectable levels of messenger expression, suggesting the induction of a host defense mechanism.

Additional keywords: DI-RNA, replication, rice.

The development of novel strategies for engineering resistance to plant viral pathogens is a major focus of current biotechnology efforts, and will significantly impact world food production. One approach is to employ naturally occurring host resistance genes such as *Tm-1* or *Tm-2* in tomato, or *N* and *N′* in tobacco. However, since few such genes have thus far been isolated, alternative approaches are being sought for developing transgenic plants resistant to viral infection. Tactics based on the pathogen-derived resistance (PDR) concept (Hamilton 1980; Sanford and Johnston 1985) aim to disrupt the viral replicative cycle through the expression of viral sequences (or their protein products) and exploitation of these approaches has yielded considerable information about host-pathogen relationships (reviewed by Wilson 1993).

Brome mosaic virus (BMV) has proven to be a fruitful model system for testing novel methods of interference with virus replication. BMV is an icosahedral, positive-strand RNA virus with a broad host range, infecting both monocots and dicots worldwide (Ford et al. 1970). While many events in its life cycle remain to be elucidated, the tripartite genome has been well characterized and the *cis* and *trans* acting sequence elements required for replication extensively mapped (Pogue et al. 1994). This knowledge has proven to be especially valuable for PDR strategies as shown by previous reports of transient interference with BMV replication in barley

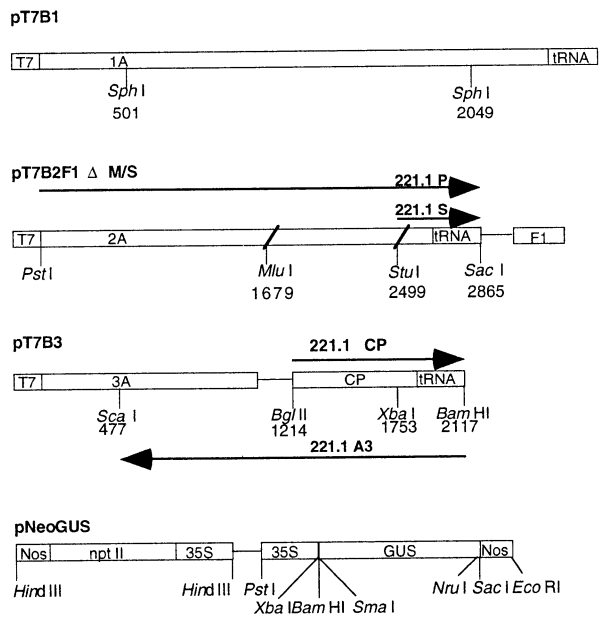

Fig. 1. Plasmids used for construction of rice transformation vectors. Sequences from plasmids pT7B2F1 Δ M/S, and pT7B3 were ligated into the plant transformation vectors pNeoGus or pBI221.1 as described in detail under Materials and Methods. Specific viral sequences used for cloning are indicated by arrows, and have the name of the resultant construct directly above. Restriction endonuclease sites utilized for cloning of viral sequences or for the preparation of random primed cDNA probes are shown for pT7B1, pT7B2F1 Δ M/S, and pT7B3, and are numbered with respect to the 5′ end of viral sequences. The nontranslated tRNA-like regions common to all genomic BMV RNAs are as indicated at their 3′ ends. In the construct pT7B2F1 Δ M/S, the region of pT7B2 between the *Mlu*I (1679) and *Stu*I (2499) has been deleted, and the position of the F1 origin used for the creation of the 5′ *Pst*I site is as indicated. The transformation vector pNeoGUS, created from a fusion of pBI221.1 and pCaMVNEO, has back-to-back CaMV 35S promoters for co-expression of the neomycin phosphotransferase II selection marker and GUS gene in plant cells, and restriction sites used for cloning of viral sequences and Southern blot analysis are as indicated.

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protoplasts (Marsh et al. 1991a, 1991b; Rao and Hall 1991; Huntley and Hall 1993a, 1993b). In the current study we have used the BMV model system to test strategies for engineered resistance to viral replication in transgenic rice using previous transient interference studies as guides. Although BMV is not currently considered a threat to contemporary rice varieties, replication has been detected (C. C. Huntley and T. C. Hall, unpublished observations) in protoplasts derived from elite cultivars and we have confirmed the report by Kahn and Dickerson (1957) of systemic BMV infection in older cultivars (data not shown). To test our PDR strategies in transgenic rice, we have generated rice plants and callus lines transformed with four constructs that appeared promising in our earlier studies. Protoplasts derived from these lines were inoculated with BMV RNA and assayed for viral progeny accumulation. High levels of interference with viral replication were observed in some transgenic lines, and our results suggest that the resistance phenotype was mediated by the expression of viral RNAs.

RESULTS

Molecular analysis of transformants.

Rice calli that developed from protoplasts electroporated with DNA constructs designed to express kanamycin resistance (Battraw and Hall 1990) and either the BMV coat protein mRNA (221.1 CP; Fig. 1) or the BMV RNA-2 3′ terminal 366 nucleotides (nt) (221.1 S; Fig. 1) were selected for their resistance to the antibiotic kanamycin. Transformation was confirmed by screening surviving calli for neomycin phosphotransferase (npt) activity (data not shown). Seven potential 221.1 CP and six potential 221.1 S callus lines were recovered and analyzed for the presence of the full-length chimeric viral coat protein gene or BMV RNA-2 3′ sequences by Southern blot hybridization (Fig. 2A and C, respectively). After digestion with restriction endonucleases *Pst*I and *Eco*RI to release the complete chimeric gene (35S promoter/viral sequence/Nos terminator), DNA from two callus lines (221.1 CP-6 and -7) were found to contain a 2.1-kb fragment that hybridized to a probe representing the full-length BMV coat protein cistron (Fig. 2A, lanes 6 and 7, respectively). This fragment co-migrated with that corresponding to the full-length chimeric coat protein gene (Fig. 2A, lane M). The presence of multiple bands in Figure 2A, lane 4, shows that callus line 221.1 CP-4 contains numerous rearranged copies of the chimeric BMV coat protein construct. Alternatively, the multiple band pattern observed in Figure 2A lane 4 (and some lanes in Figure 2B and D) could reflect CNG methylation of *Pst*I sites, resulting in only partial cleavage of the genomic DNA. However, the fact that genomic DNA analysis of some rice lines revealed only single bands (Fig. 2) makes this explanation less compelling.

RNA blot analysis of total RNA from calli electroporated with 221.1 CP revealed a transcript of approximately 1,100 nt in callus line 221.1 CP-7, and long exposure (not shown) revealed a faint signal of the same expected size from callus

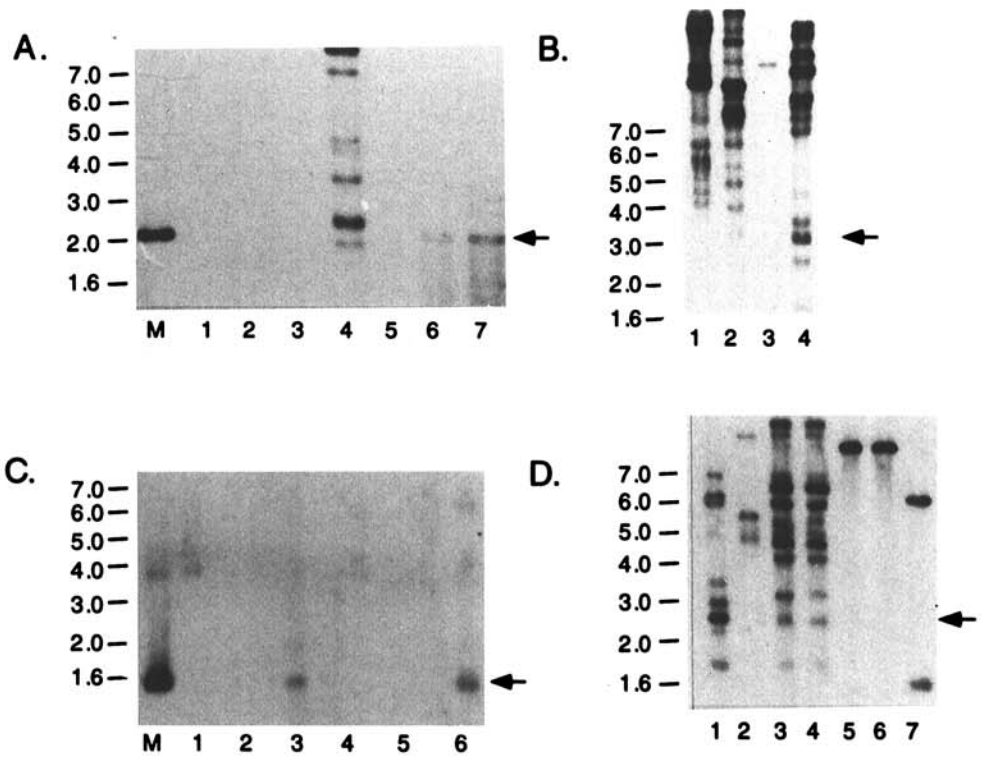

Fig. 2. Southern blot analysis of rice tissues transformed with brome mosaic virus sequences. Genomic DNA extracted from rice callus lines transformed with constructs to express viral sequences (**A,** 221.1 CP 1 to 7, lanes 1 to 7; **B,** 221.1 P 1 to 4, lanes 1 to 4; **C,** 221.1 S 1 to 6, lanes 1 to 6; **D,** 221.1 A3 1 to 7, lanes 1 to 7) was digested with *Eco*RI and *Pst*I to release the chimeric genes (35S promoter/viral sequence/nos terminator, see Figure 1), and fractionated by electrophoresis in 1% agarose. Random primed cDNA probes, as described under Materials and Methods, were used for detection of the transformed viral sequences. The position of end-labeled DNA size standards is shown to the left of each panel, and arrows showing the expected fragment size for each transformant are shown to the right. DNA from plasmids 221.1 CP and 221.1 S, digested with *Eco*RI and *Pst*I, served as additional size markers (M) in panels **A** and **C,** respectively.

line 221.1 CP-6 (Fig. 3A, lanes 7 and 6, respectively). No signal could be detected from other 221.1 CP–electroporated callus lines, including Southern positive callus line 221.1 CP-4 (Fig. 3A, lane 4), indicating that only two of the three transformed callus lines expressed coat protein mRNA at a detectable level. Western blot analysis of the transformed lines failed to detect BMV coat protein (data not shown). This indicated either that capsid mRNA was not translated or that the protein product was below the threshold level of detection.

Southern blot analysis of rice callus lines electroporated with 221.1 S showed that lines 221.1 S-3 and -6 (Fig. 2C, lanes 3 and 6, respectively) contained *PstI*/*Eco*RI fragments of the expected 1.5 kb size. Northern analysis confirmed that line 221.1 S-3 expressed an RNA of the expected 650 nt size (Fig. 3B, lane 3), but no RNA was detected in extracts from the other five callus lines.

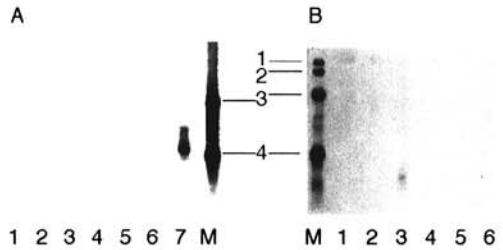

Fig. 3. Northern blot analysis of transformed rice calli. **A,** Total RNA extracted from rice callus transformed with 221.1 CP, lines 1 to 7 (lanes 1 to 7), encoding the brome mosaic virus (BMV) coat protein cistron (Fig. 1), was denatured and fractionated on a 1% agarose gel. The blot was probed with ^{32}P-labeled DNA prepared by random priming of a gel-purified 540-bp *BglII*/*Xba*I fragment from pT7B3 (Fig. 1) The autoradiograph shows a message of the expected size (approximately 1,100 nucleotides [nt]) in lane 7. Long exposure revealed a faint signal of the expected size in lane 6. BMV RNA (M) served as a size marker. **B,** Total RNA extracted from rice callus transformed with 221.1 S, lines 1 to 6 (lanes 1 to 6), encoding BMV RNA-2 3′ sequences (Fig. 1) was denatured and fractionated on a 1% agarose gel. The blot was probed with ^{32}P-labeled DNA that was prepared by random priming of a gel-purified pT7B2F1 Δ M/S *StuI*/*Sac*I fragment containing BMV RNA-2 3′ sequences (Fig. 1). The autoradiograph shows a message of approximately 650 nt in lane 3. BMV RNA (M) served as a size marker.

Rice plants were regenerated from protoplasts co-electroporated with pUC19 Bar, a construct conferring bialaphos resistance and a construct (221.1 P; Fig. 1) designed to express a pRNA-2 sequence (Pogue et al. 1990) previously shown in protoplast experiments to function as a defective interfering (DI) RNA (Marsh et al. 1991a). Southern blot analysis revealed that four of the rice plants regenerated contained single or multiple copies of the 221.1 P construct (Fig. 2B, lanes 1 to 4). Seven rice plants were regenerated from protoplasts co-electroporated with pUC19 Bar and the 221.1 A3 construct that was designed to express antisense RNA including the intercistronic region of BMV RNA-3 (Fig. 1). Southern analysis of the seven regenerated rice plants (Fig. 2D) revealed numerous integration events and the presence of two pairs of siblings (Fig. 2D, lanes 3, 4 and 5, 6) among the seven regenerated 221.1 A3 plants. Northern blot analysis (data not shown) failed to detect viral RNA expression from any of the 221.1 P or 221.1 A3 plant lines.

Analysis of viral replication in transformed rice protoplasts.

To evaluate the effect of rice transformation for the expression of the various BMV-derived RNAs, protoplasts were isolated from the various transformed rice callus lines. Following inoculation with 1 μg of virion-derived BMV RNA, the protoplasts were incubated for 24 h (see Materials and Methods). Protoplasts from calli transformed with 221.1 CP (BMV coat protein, lines 221.1 CP-4, -6, and -7) accumulated only 10 to 20% of the progeny RNA found in control protoplasts from calli transformed with pNeoGUS (Fig. 4A, compare lanes 4, 6, and 7 with lane 8). The pNeoGUS line was derived from rice protoplasts electroporated with a GUS construct and served as a source of control protoplasts that had undergone parallel treatment to the lines transformed with constructs designed to express BMV-derived sequences.

Interference with viral replication in rice protoplasts isolated from calli transformed with the 221.1 S construct corre-

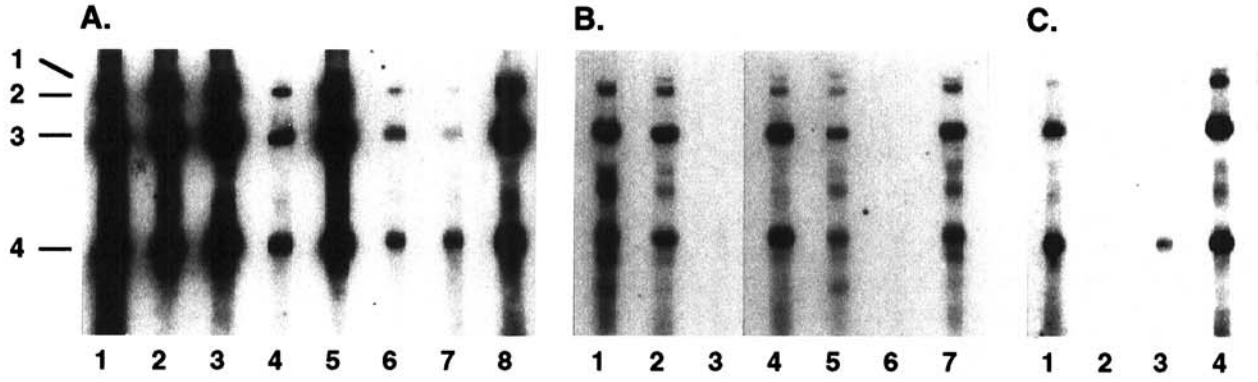

Fig. 4. Replication of genomic brome mosaic virus (BMV) RNAs in rice protoplasts transformed with viral sequences. Protoplasts (approximately 5 × 10^5) isolated from transformed or control callus lines were inoculated with 1 μg of virion RNA. Total nucleic acid was extracted 24 h postinoculation, denatured with glyoxal, separated in a 1% agarose gel, and electrophoretically transferred to nylon membrane. Each lane corresponds to RNA from approximately 1 × 10^5 protoplasts. The blot was probed with a mixture of ^{32}P-labeled DNA prepared by random priming of gel purified fragments from BMV RNA-1 (*SphI*/*Sph*I), RNA-2 (*StuI*/*Sac*I), and RNA-3 (*BglII*/*Xba*I). The positions of BMV RNAs 1 to 4 are shown on the left. Band intensities were determined using a Fujix BAS 2000 phosphorimager. Relative accumulation of progeny RNAs are the average of at least three independent experiments. **A,** Progeny viral RNA accumulation in protoplasts from callus lines 221.1 CP 1 to 7 (lanes 1 to 7, respectively) was compared with that in protoplasts transformed with pNeoGUS (lane 8). **B,** Progeny viral RNA accumulation in protoplasts from callus lines 221.1 S 1 to 6 (lanes 1 to 6, respectively) was compared with that in protoplasts transformed with pNeoGUS (lane 7). **C,** Viral RNA replication in protoplasts from callus lines 221.1 P-1 and -4, and 221.1 A3-2 (lanes 1 to 3, respectively) was compared with replication in protoplasts transformed with pNeoGUS (lane 4).

sponding to BMV RNA-2 3′ sequences was very pronounced, progeny accumulation being only 5% of that in pNeoGUS control protoplasts (Fig. 4B, compare lanes 3 and 6 with lane 7).

Replication of BMV in protoplasts derived from 221.1 P-1 and -4 (transformed with the DI-like pRNA construct) was 35 and 7%, respectively, of that observed in control protoplasts (Fig. 4C, compare lanes 1 and 2 with lane 4). Protoplasts derived from 221.1 A3-2 (antisense to BMV RNA-3) yielded 15% of the viral RNA accumulating in protoplasts from the control line (Fig. 4C, compare lanes 3 and 4). A summary of the characteristics and resistance to viral replication shown by the various transgenic lines tested is shown in Table 1.

To determine if the resistance conferred by the introduction of pathogen-derived sequences could be overcome, protoplasts from callus lines showing strong resistance to viral infection were challenged with various levels (0.1 to 30.0 μg) of viral RNA. Figure 5 reveals that resistance in all four transformed lines could be overcome by very high levels of inoculum, but that each was markedly resistant to viral RNA accumulation. In particular, callus lines 221.1 S-6 and 221.1 CP-6 required approximately 20-fold greater amounts of viral RNA inoculum to accumulate progeny to a level similar to that observed in protoplasts from control calli.

DISCUSSION

We previously reported that coinoculation of protoplasts with infectious viral transcripts and RNA corresponding to the 3′ 200 nt of RNA-2 led to interference by competition for viral or host factors required for (–) strand synthesis (Huntley and Hall 1993b). We now show that expression in transgenic cells of sequences corresponding to various regions of the BMV genome can yield substantial resistance to subsequent infection by viral RNAs. Presumably as a result of their more efficient capping, virion RNAs are typically severalfold more infectious than are transcript RNAs, yet some of the constructs described here yielded resistance levels that were overcome only in the presence of over 20-fold higher excess of virion RNA.

The production of RNA transcripts could not be detected from several of the transgenes in this study. However, the lack of an apparent correlation between expression levels of 221.1 S RNA and the level of interference observed was remarkable. Lindbo and Dougherty (1992b) have also reported a lack of clear relationship between the expression of a putative inhibitor and viral replication in transgenic plants. From their experiments with tobacco etch virus (TEV) replication in transgenic tobacco protoplasts, they suggested that subcellular localization of the noncoding transcripts may be more important than the absolute level of transcript.

Transgenic rice protoplasts expressing the BMV capsid RNA showed an 80 to 90% reduction in progeny virus accumulation. Although coat protein messenger RNA was detected in two of the three lines confirmed by genomic DNA blots to be transformed, the absence of detectable coat protein indicates that either the message was not well translated or that coat protein was unstable in the absence of viral infection, and suggests that the observed resistance was mediated through viral RNA rather than through the coat protein. However, there have been other recent reports of engineered resistance to plant viral infection by expression of viral cistrons that fail to produce detectable levels of protein. Kawchuk et al. (1990, 1991) observed strong resistance to potato leafroll leuteovirus in potatoes transformed with coat protein cDNAs. However, no coat protein was detected despite significant mRNA production. In addition, Lindbo and Dougherty (1992a, 1992b) have described experiments showing that transformation of tobacco with nontranslatable, but translation initiation competent, and antisense TEV capsid protein genes gave resistance to TEV infection that was dramatically more effective than were translatable coat protein sequences. Similar results have been reported for plants transformed with the potato virus Y coat protein gene (Lawson et al. 1990; van der Vlugt et al. 1992) and tomato spotted wilt virus nucleocapsid (N) gene sequences (de Haan et al. 1992). Such interpretations differ markedly from those for experiments describing engineered resistance to tobacco mosaic virus (TMV), in which coat protein was implicated as the bioactive agent (Powell et al. 1990).

The conclusion reached above, that a viral protein product was not likely to be the bioactive agent conferring resistance to BMV, is further supported by the results of interference studies in cell lines transformed with the 221.1 A3 antisense and 221.1 P DI-like constructs. Neither construct was de-

Table 1. Induced resistance to brome mosaic virus RNA infection of rice protoplasts

Construct	Transgenic callus line	Gene copy number	RNA expressed	Replication (% of wt)
Coat protein	221.1 CP-4	7	No	20
Coat protein	221.1 CP-6	1	Trace	20
Coat protein	221.1 CP-7	1	Yes	10
3′ sense	221.1 S-3	1	Yes	5
3′ sense	221.1 S-6	1	No	5
DI (pRNA)	221.1 P-1	>10	No	35
DI (pRNA)	221.1 P-4	>10	No	7
Antisense	221.1 A3-2	3	No	15

[a] Replication was based on an average of three independent trials using 1 μg of virion RNA / 5×10^5 protoplasts.

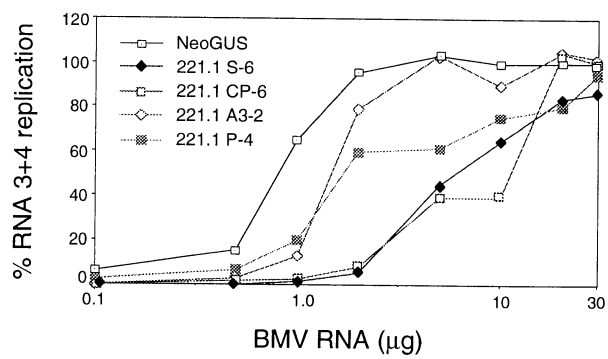

Fig. 5. Effect of inoculum concentration on accumulation of progeny brome mosaic virus (BMV) RNAs in transformed protoplasts. Rice protoplast samples from callus lines pNeoGUS, 221.1 S-6, 221.1 CP-6, 221.1 A3-2, and 221.1 P-4 were inoculated with 0.1, 0.5, 1, 2, 5, 10, 20, or 30 μg of BMV virion RNA. The RNA gel blot was probed with ^{32}P-labeled DNA prepared by random priming of a gel-purified *BglII*/*XbaI* fragment from pT7B3. The relative accumulation of progeny BMV RNAs-3 and -4 was measured as described for Figure 4, with progeny levels in pNeoGUS transformed protoplasts inoculated with 30 μg of virion RNA being designated as 100%.

signed to yield virally encoded proteins, yet protoplasts from lines 221.1 P-1 and -4 and 221.1 A3-2 show strong interference with viral replication (Fig. 4, panel C). It is not clear how this interference is accomplished, but hybridization to the virus strand of opposite polarity is one possibility. In this model, the RNA duplex formed would presumably block viral or host-associated functions such as replication or translation. However, it is unclear why helicase activity associated with these enzymatic functions should not simply separate the inhibitor from target strands. Another possibility is that duplex RNA is targeted for rapid degradation. Given the strong potential for secondary structure in plant viral RNAs, this would help to account for the effective interference with viral replication we observed in callus lines with little or no detectable mRNA expression from inserted viral sequences. Alternatively, a more global system for host-antiviral defense may be induced. Evidence for such a system has recently come to light in potato, as Kohm et al. (1993) have demonstrated that potato virus X coat protein induces an antiviral resistance mechanism in potato (Rx genotype) that is effective against the unrelated cucumber mosaic virus. Perhaps even more cogent are the recent observations by Mueller et al. (1995) on homology-dependent gene silencing and virus resistance in tobacco plants transformed with the potato virus X RNA polymerase gene.

That mechanisms for generalized resistance may be widespread is suggested by the work of Ward et al. (1991) and others who have demonstrated a systemic acquired resistance in tobacco that strongly reduces infection by TMV, and further by the hypersensitive response observed in a wide variety of plant hosts in reaction to infection by diverse viruses. The manner in which they are triggered is at present largely unknown, but one possibility is raised by the work of Huntley and Hall (1993a) whose results suggested that some, but not all, viral sequences could serve as templates for an endogenous host-encoded RNA-dependent RNA-polymerase that probably results in the formation of double-stranded RNAs (dsRNAs). Such molecules may serve as a elicitors of host defenses analogous to those described in animal cells, where dsRNA elicits a latent ribonuclease activity (Hovanessian, 1993). This possibility is supported by data from the present study, in which Southern positive transgenes had low or no apparent expression of mRNAs and yet were strongly resistant to infection by viral RNAs. Here, the absence of detectable transgene message might indicate the activation of a host defense system. Although a discrete antiviral pathway has yet to be identified in plant cells, it is clear that pre-existing defense mechanisms hold great promise for future plans to engineer resistance to plant viral infections. Unlocking the secrets that underlie the success of current resistance strategies may be the key to developing those plans.

MATERIALS AND METHODS

Chemicals and enzymes.

Restriction endonucleases and RNase free-DNase were obtained from Boehringer-Mannheim (Indianapolis, IN), New England Biolabs (NEB, Beverley, MA), and Bethesda Research Laboratories (BRL, now Gibco, Grand Island, NY). T4 DNA ligase was from NEB. Calf intestinal phosphatase (CIP) was procured from Boehringer-Mannheim. Cellulase "Onozuka" RS and pectolyase Y-23 were purchased from Yakult Honsha and Kikkoman (Toyko), respectively.

Plasmid constructs.

The construction of plasmids pT7B1, pT7B2, and pT7B3, bearing cDNA clones from which infectious full-length wild-type BMV RNAs-1, -2 and -3 can be transcribed in vitro, was described by Dreher et al. (1989). The construction of pT7B2F1 Δ M/S was described by Marsh et al. (1991a).

The 1.7-kb *Hin*dIII fragment from pCaMVNEO (Fromm et al. 1986) containing a chimeric npt-II gene (CaMV 35S promoter/npt-II coding sequence/nos 3′) was cloned into the *Hin*dIII site of pBI221.1 (Jefferson et al. 1987) that contains a chimeric GUS gene (CaMV 35S promoter/GUS coding sequence/nos 3′) to create pNeoGUS, in which CaMV 35S promoter sequences are back to back (Fig. 1). A vector for the expression of BMV coat protein was created by inserting the 900 base pair (bp) *Bgl*II/*Bam*HI fragment from pT7B3 (Fig. 1) into the *Bam*HI site of pNeoGUS treated with CIP; the GUS coding sequence was deleted by subsequent restriction with *Sma*I and *Nru*I followed by blunt-end ligation to create 221.1 CP (coat protein). To create a vector for the expression of the BMV (–) strand promoter element from RNA-2, pNeoGUS was digested with *Sma*I and *Sac*I to remove the GUS coding sequence and a 366-bp *Stu*I/*Sac*I fragment from pT7B2 ligated into its place (Fig. 1). This plasmid expresses a positive "Sense" orientation RNA corresponding to the 3′ end of BMV RNA-2, designated 221.1 S.

Previous studies have shown that artificial parasitic RNAs (pRNAs) derived from BMV were effective at reducing BMV accumulation when coinoculated to barley protoplasts (Marsh et al. 1991a). To transfer the cDNA of one such mutant into pBI221.1, site-directed mutagenesis was used to create a *Pst*I restriction site at the 5′ end of the viral sequences in pT7B2F1 Δ M/S, essentially following the procedure described by Pogue et al. (1990). pT7B2F1 Δ M/S is a deletion of the BMV RNA-2 transcriptional clone pT7B2, between bases 1680 (*Mlu*I) and 2502 (*Stu*I) (Fig. 1). Viral sequences were isolated by restriction with *Pst*I followed by digestion with mung bean nuclease (leaving a single nontemplated G at the 5′ end), then released by restriction with *Sac*I (Fig. 1). 221.1 P (Parasitic RNA) was made by restriction of pBI221.1 with *Xba*I followed by mung bean nuclease treatment to produce blunt ends and restriction with *Sac*I before ligation of the viral sequences from pT7B2F1 Δ M/S. To create a vector for the expression of RNA antisense to BMV RNA-3, a *Sca*I/*Bam*HI fragment was isolated from pT7B3 (Fig. 1), and ligated with *Bam*HI/*Nru*I digested pBI221.1 to create 221.1 A3 (Antisense RNA-3). A vector for the expression of bialaphos resistance (bar) was created by isolating a *Sca*I/*Sna*BI fragment from PGSFR280 (Plant Genetic Systems, Gent, Belgium) that contains a chimeric bar gene (CaMV double 35S promoter/bar coding sequence/T7 3′ terminator). This fragment was ligated into the *Hin*dIII site of pUC19 (NEB), previously digested with mung bean nuclease to make blunt ends, creating pUC19 Bar.

Rice transformation and regeneration.

Transformation of rice protoplasts and subsequent growth of rice callus followed the protocol described by Battraw and Hall (1992). Briefly, immature embryos from rice (*Oryza sa-*

tiva cv. Taipei 309) were placed on LS2.5 media (Linsmaier and Skoog 1965) to initiate callus formation and subcultured for 2 months before initiation of cell suspension cultures in liquid AA media (Muller and Grafe 1978). Four-month-old suspension cultures were digested with cellulase and pectolyase to release protoplasts, and 2×10^6 cells were electroporated with 50 µg of either 221.1 CP or 221.1 S for transformation. Protoplasts electroporated with either pNeoGUS or no DNA served as controls. Electroporated protoplasts were plated on filters using a nurse culture technique similar to that used by Rhodes et al. (1988). Seven days after protoplast isolation, filters were moved to selection media containing 100 mg of kanamycin sulfate (Sigma Chemical Co., St. Louis, MO) per liter and maintained for approximately 30 days. Protoplast-derived calli surviving selection were transferred to LS2.5 media and grown with monthly subcultures of friable callus to proliferate the transformed tissues in an undifferentiated state. Rice callus transformed by either 221.1 A3 or 221.1 P was obtained by similar methods, except that protoplasts were co-electroporated with a mixture of 100 µg of construct DNA and 25 µg of pUC19 Bar DNA. Transformants were selected on the basis of resistance to 1 mg bialaphos (Meiji Seika Kaisha, Ltd., Tokyo) per liter and subsequently regenerated into rice plants following the procedure of Battraw and Hall (1992). Plants 221.1 P-1 and -4 and 221.1 A3-2 produced fertile seed from which immature embryos were isolated and subsequently used to initiate transformed callus lines as described above.

Southern analysis of putative transformants.

Total DNA was isolated from ground rice callus by precipitation with cetyltrimethylammonium bromide as described by Taylor et al. (1993). A 1C value of 0.86 pg (Arumuganathan and Earle 1991) was used for copy number reconstruction. DNA concentrations were determined by measuring the enhancement of fluorescence seen when bisbenzimidazole (Hoechst 33258) binds to DNA (Labarca and Paigen 1980). Calf thymus DNA quantified by the spectrophotometric method was used as the standard for the fluorescence assay. Restriction endonucleases *Pst*I and *Eco*RI were used to cleave sample DNA according to the manufacturer's instructions, and a 1-kb DNA ladder was used as a size standard. After separation of DNA (1 µg per lane) by electrophoresis on a 1% agarose gel, the DNA was transferred to nitrocellulose membrane (Schleicher and Schuell, Keene, NH) by capillary blotting (Maniatis et al. 1982). High molecular weight DNA was nicked using a Stratagene UV Crosslinker 1800. Membranes bearing the 221.1 CP and 221.1 A3 transformed samples were hybridized to a ^{32}P-labeled (5×10^6 cpm per ml) 540-bp *Bgl*II/*Xba*I fragment of pT7B3 (Fig. 1) as described by Maniatis et al. (1982). For detection of 221.1 S and 221.1 P sequences, membranes were probed with a random primed *Stu*I/*Sac*I 360-bp fragment of pT7B2F1 Δ M/S (Fig. 1).

Northern blot analysis.

To examine protoplast derived rice callus for expression of viral sequence RNAs, total rice RNA was isolated. Callus (2 g) was ground with mortar and pestle to a fine powder in liquid nitrogen, then added to 42 µl of β-mercaptoethanol and 6 ml of solution D (4 M guanidine isothiocyanate [BRL], 25 mM Na citrate, 0.5% sarcosyl, pH 7.0) and vortexed well. To this was added 400 µl of 3M NaOAc (pH 5.0), 6 ml of Tris-EDTA–saturated phenol (pH 8.0) and 1.2 ml of chloroform/isoamyl (24:1). This mixture was vortexed, left on ice for 15 min, and spun at 8,000 rpm in a Beckman JA-14 rotor for 15 min at 4°C, after which the aqueous phase was removed, added to an equal volume of isopropanol, and precipitated for 60 min at –20°C. After centrifugation at 8,000 rpm for 15 min at 4°C, the pellet was washed in 70% ethanol and resuspended in 500 µl of diethylpyrocarbonate (DEPC)–treated H_2O before the addition of 55 µl of 10× DNase buffer and 2 µl RNase free-DNase (40 units per liter), then incubated at room temperature for 30 min. The solution was successively extracted with phenol-chloroform, then chloroform, and the RNA collected by the addition of 1/10 volume of 3 M NaOAc and 2.5 volumes of ethanol, followed by precipitation at –20°C overnight. The pellet was resuspended in 500 µl DEPC-treated H_2O, quantified by spectrophotometer, and stored at –80°C.

Total RNA (10 µg) isolated from callus was glyoxalated and electrophoresed on a 1% agarose gel using 10 mM sodium phosphate buffer (pH 7.0) (Dreher et al. 1989). RNAs were then transferred electrophoretically onto a nylon membrane (Nytran, Schleicher and Schuell). Membranes were hybridized with probes identical to those used for Southern blot analysis (Fig. 1, and see above) as appropriate for the source of the callus. Hybridization was performed at 55°C in 50% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 1% sodium dodecyl sulfate (SDS), and 0.1 mg of denatured salmon sperm DNA per ml. The blot was washed once in 2× SSC (1× SSC is 0.15 mM NaCl plus 0.015 M sodium citrate) for 30 min at 65°C, followed by 0.2× SSC, 0.1% SDS for 30 min at 65°C.

Protoplast replication assays.

Prior to protoplast preparation, rice callus lines were passaged 2 to 3 times at intervals of 3 weeks, rather than monthly, to provide rapid growth conditions. Rapidly growing callus was found to be essential for obtaining protoplasts that were uniformly small and without large vacuoles. An enzyme solution (25 ml) containing 1% (wt/vol) cellulase RS, 0.1% (wt/vol) pectolyase Y23, 0.5% (wt/vol) MES and 13% (wt/vol) mannitol in CPW salts pH 5.8 (CPW-13M: Frearson et al. 1973) was added to 1 to 2 g of callus and incubated without agitation for 3 to 4 h at 28°C. Protoplasts were isolated as in Loesch-Fries and Hall (1980) using CPW-13M throughout for resuspension, and 5×10^5 cells inoculated (Dreher et al. 1989) with virion RNA (1 µg unless otherwise noted). Following transfection, protoplasts were incubated at room temperature for 24 h. Total RNA was extracted with SDS and phenol/chloroform and then ethanol precipitated (Loesch-Fries and Hall 1980).

Analysis of progeny viral RNA.

Replication of viral RNAs in protoplasts was analyzed by Northern blot analysis as described by Dreher et al. (1989). DNA probes used for the detection of BMV progeny RNAs were ^{32}P-labeled by random priming (Boehringer-Mannheim). Probes for the detection of RNAs-2 and -3 were as described above under Southern blotting procedures, and that for RNA-1 was produced by random priming of a 1,548-bp *Sph*I fragment derived from pT7B1 (Fig. 1). Northern blots were

autoradiographed with pre-flashed film (Laskey and Mills 1977) and relative band intensities were determined using a Fujix BAS 2000 phosphorimager. Relative accumulation of progeny RNAs are the average of at least three independent experiments.

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