

Effective Resistance to Potyvirus Infection Conferred by Expression of Antisense RNA in Transgenic Plants

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Received 21 October 1994. Accepted 8 May 1995.

Approximately 660 nt including the carboxy-terminal portion of the bean yellow mosaic potyvirus (BYMV) coat protein gene, complete 3' noncoding sequence and a short poly(A) tract were introduced to produce antisense RNA in transgenic *Nicotiana benthamiana*. Original (R_0) transformants were selfed, and homozygous second generation (R_2) populations challenged with infected sap, purified virus, or viral RNA. One transgenic line of 10 examined was extremely resistant to infection by mechanical inoculation of 100 $\mu\text{g/ml}$ BYMV or 50 $\mu\text{g/ml}$ BYMV RNA (the highest concentrations tested); no virus could be detected in inoculated leaves of this line. Nine other lines were systemically infected when inoculated with BYMV or BYMV RNA, with initial symptoms indistinguishable from those in nontransgenic plants. One of these lines subsequently developed reduced symptoms and then symptomless, virus-free leaves (complete recovery from BYMV infection), while other lines produced leaves with reduced symptoms and reduced virus titer compared to the controls (partial recovery). No transgenic lines had resistance to infection, nor recovery from symptom expression, following inoculation with pepper mottle or turnip mosaic potyviruses. Antisense RNA from 3' regulatory regions can confer multiple degrees of resistance to potyviruses, including extreme resistance to infection, presumably by interference in virus replication.

Additional keywords: pathogen-derived resistance.

Antisense (AS) RNA has been shown to be an effective means of regulation of gene expression in both prokaryotic (Inouye 1988; Simons 1988) and eukaryotic organisms (Krol et al. 1988a) when the gene to be regulated is expressed from chromosomal DNA. Control of replicating nucleic acids, such as RNA or DNA viruses, requires a greater repression of gene activity to be effective, or residual activity will be multiplied. Inhibition of replication has been reported for RNA coliphage SP (Coleman et al. 1985; Hirashima et al. 1986), polyomavirus (circular DNA; Ottavio et al. 1992), and tomato golden

mosaic geminivirus (Day et al. 1991); an AS RNA to the AL1 gene of tomato golden mosaic virus (single-stranded DNA) was able to protect against symptom production in a significant proportion of plants, and reduced levels of viral DNA in both symptomatic and symptomless infected plants (Day et al. 1991).

Attempts to use AS RNA in transgenic plants to inhibit replication of RNA plant viruses have met, however, with varying success. In some cases resistance has been observed only when very low levels of challenge inoculum are used, or with a slight delay in symptom production and reduced viral accumulation (Cuozzo et al. 1988; Rezaian et al. 1988; Powell et al. 1989; Kawchuk et al. 1991). In most cases AS RNA-mediated resistance was significantly less than protection conferred by coat protein (CP) expression (Cuozzo et al. 1988; Hemenway et al. 1988; Powell et al. 1989; Fang and Grumet 1993; Farinelli and Malnoë 1993). Huntley and Hall (1993a) showed that AS RNA to the brome mosaic virus (BMV) RNA 3 intercistronic region significantly reduced virus replication when coelectroporated into protoplasts with viral RNA transcripts. AS RNA targeted to the 3' noncoding region common to all BMV genomic RNAs, and containing the (–) strand promoter, also reduced replication in coelectroporated protoplasts (Huntley and Hall 1993b).

The most effective plant viral AS resistance reported to date was with potato leafroll luteovirus (PLRV). CP and AS PLRV constructs conferred similar levels of resistance in potato; PLRV titers were reduced at least 95% compared to nontransgenic controls, but virus replication continued for at least 8 weeks (Kawchuk et al. 1991). A tobacco etch potyvirus (TEV) AS RNA, consisting only of CP gene sequence, caused a delay in symptom production, reduced symptom severity, and reduced levels of viral replication (Lindbo and Dougherty 1992a, 1992b); this TEV AS RNA was found more effective than expression of the native TEV CP (Lindbo and Dougherty 1992a).

We introduced sequences complementary to the carboxy-terminal portion of the bean yellow mosaic potyvirus (BYMV) CP gene and complete 3' noncoding sequences into transgenic *Nicotiana benthamiana* plants. Homozygous (R_2) populations were challenged with BYMV and heterologous potyviruses to examine the efficacy and specificity of protection. We report here that BYMV AS RNA is capable of conferring a high level of resistance to BYMV infection (effective immunity), or resistance to continued replication and/or transport in infected plants (leading to recovery from

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BYMV infection). The extreme resistance observed with one out of 10 lines exceeds the degree of resistance observed with AS RNA to other viruses. A preliminary report has been presented (Hammond and Kamo 1993).

RESULTS

Transformant selection and analysis.

Selection for successful transformation with the BYMV AS construct was initially based on kanamycin resistance, followed by PCR analysis of leaf samples from tissue culture. Kan^r lines shown by PCR analysis to lack BYMV sequences were discarded as apparent chimeras or false positives. Rooted R₀ plantlets that retested positive by PCR, that had normal appearance, and that set adequate numbers of seed to allow the determination of segregation ratios were examined further. Most R₁ populations segregated approximately 3:1 for expressors: nonexpressors (by kanamycin resistance of seedlings, and by PCR), suggesting a single linked insertion of the T-DNA carrying the selectable marker and AS gene. Others segregated approximately 15:1, and thus apparently had two independent insertions of the T-DNA. Transgenic lines AS 30, AS 42, and AS 112 (segregating 3:1 in the R₁ generation) and AS 140 (15:1 at R₁) were selected for further characterization and subsequent virus challenge. Putative homozygous AS⁺ R₂ populations were identified in which each of 24 seedlings tested was PCR-positive for the AS gene. An R₂ population from a nonexpressing (AS⁻) R₁ plant of AS 30 was selected as an additional control. Six additional independent R₂ transgenic lines (AS 16, AS 31, AS 115, AS 139, AS 143, and AS 7.16) were challenged with sap extracts only.

Northern analysis.

Multiple bands hybridizing with a BYMV cDNA probe were observed in total RNA extracts of R₂ plants of AS 30⁺, AS 42, AS 112, and AS 140 (Fig. 1); no bands were detected in AS 30⁻ (data not shown). The major band observed corresponds to the approximately 1 kb transcript expected from the transgene and vector 3' sequences. The origin of the faster migrating bands is not clear. There were clearly higher levels of transcript in AS 140 and AS 42 than in AS 112 and AS 30⁺ (Fig. 1).

Southern analysis.

Only a single, unique insert was indicated for each R₂ transgenic line (AS 30⁺, AS 42, AS 112, and AS 140) by Southern analysis of genomic DNA digested with *Hind*III, which cuts only once in the T-DNA of pGA.BY6, adjacent to the CaMV 35S promoter (data not shown). No bands were detected from AS 30⁻.

Resistance to BYMV infection.

In initial experiments with inoculation of sap extracts, eight plants each of one or more R₂ populations of transgenic lines AS 30, AS 42, AS 112, and AS 140 plus nontransgenic controls were challenged with BYMV isolate GDD (BYMV-GDD). Symptoms as described in Table 1 were scored daily. In subsequent tests eight plants of one population from each transgenic line were inoculated with 100 µg/ml of BYMV isolate Ideal A (BYMV-Ideal A), and 10 plants each with 50 µg RNA of BYMV-GDD per ml. R₃ and R₄ generation plants

of these populations were also challenged with infected sap extracts of BYMV-Ideal A, BYMV-GDD, or BYMV-Scott.

Nontransgenic controls were infected in all experiments, and no recovery was ever observed in infected nontransgenic plants. All BYMV-GDD sap-inoculated plants showed vein-clearing from 7 to 8 days postinoculation (d.p.i.), mild mosaic from 10 to 11 d.p.i., and more severe mosaic from about 25 d.p.i. All plants inoculated with 100 µg/ml of purified BYMV-Ideal A were visibly infected by 8 dpi, with a similar disease progression (Figs. 2A, 3). Three of 10 RNA-inoculated plants became infected, with typical disease development. Infection of BYMV-Ideal A or BYMV-Scott sap-inoculated leaves was readily detected by ELISA ($A_{405} > 1.3$ OD; control noninoculated < 0.03 OD) despite absence of any visible symptoms. Symptoms and disease progression of BYMV-Ideal A, BYMV-GDD, and BYMV-Scott were similar.

AS 30⁻ plants were also infected in all experiments (including R₃ and R₄ plants), with similar symptom development and virus titer to the nontransgenic controls (Fig. 3A, E). One of 10 RNA-inoculated plants was infected, with symptoms and disease progression similar to the nontransgenic controls.

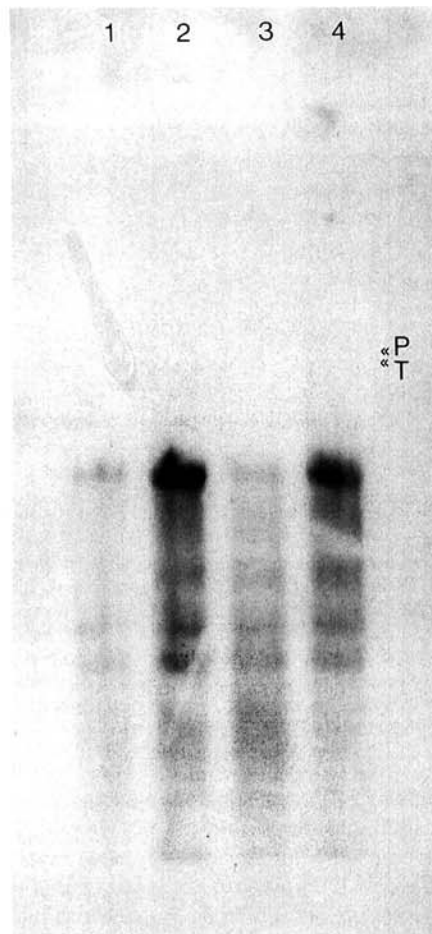


Fig. 1. Northern analysis of transgenic lines AS 30⁺ (lane 1), AS 42 (lane 2), AS 112 (lane 3), and AS 140 (lane 4). The major transcript in each lane corresponds to approx. 1 kb, including 660 nt of the AS construct plus 3' sequences from the pGA643 vector transcription termination region. The relative positions of potato virus X (P; 6,435 nt) and TMV (T; 6,395 nt) genomic RNAs are indicated (arrowheads).

Table 1. Symptom scoring of plants infected with bean yellow mosaic virus (BYMV), pepper mottle virus (PepMoV) and turnip mosaic virus (TuMV). Symptom descriptions reflect the emerging or predominant symptoms on systemic leaves, except for local lesions of TuMV

Score	BYMV	PepMoV	TuMV
0	No symptoms	No symptoms	No symptoms
1	Vein clearing	Vein-clearing	Vein clearing
2	Interveinal chlorosis (IC) ± recovery at top	Systemic leafroll	Chlorotic or necrotic local lesions
3	Mild mosaic ± emerging IC	Reduced leaf distortion	Leafroll or distortion
4	Mosaic	Mottle and stunting	Systemic distortion, localized chlorotic or necrotic spotting
5	Severe mosaic	Strong chlorosis	Systemic veinal, stem or petiole necrosis
6	–	Severe chlorosis ± apical necrosis	Systemic petiole collapse or stem splitting
7	–	Stem necrosis	Stem necrosis
8	–	Death	Death

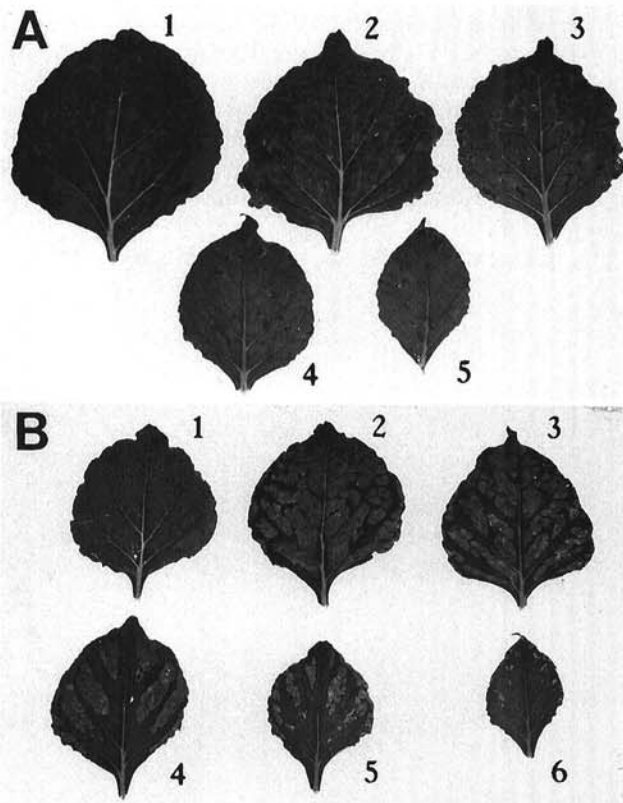


Fig. 2. Leaves of *Nicotiana benthamiana* inoculated with 100 µg/ml of bean yellow mosaic potyvirus (BYMV). **A**, a nontransgenic plant; Leaf 1, second systemically infected leaf; leaves 2 to 5, successively higher on the main stem. **B**, an AS 30⁺ plant showing partial recovery; 1, typical BYMV mosaic on second systemically infected leaf; 2, green vein banding; 3 to 6, leaves showing progressively reduced interveinal chlorosis and recovery towards top of plant. Compare to Figure 1 of Hammond and Kamo (1993) for complete recovery of AS 42 plants.

AS 30⁺ plants of three R₂ populations were all infected by sap inoculation and developed mosaic symptoms, but began to develop a novel symptom type beginning about 20 d.p.i. In the upper leaves chlorosis was reduced, initially with dark green vein-banding and chlorosis restricted to the interveinal area. All plants of the three populations developed this novel interveinal chlorosis by 27 d.p.i., and subsequent leaves produced up to at least 35 d.p.i. typically had further reduced areas of chlorosis and a greater proportion of darker green tissue. A similar course of disease occurred in plants inoculated with 100 µg/ml purified BYMV-Ideal A, except that the in-

terveinal chlorosis became apparent beginning at 17 d.p.i. (Figs. 2B, 3A) and reduced virus titer compared to nontransgenic controls was detected by ELISA in leaves showing interveinal chlorosis (Fig. 3E). None of 10 plants were infected by RNA inoculation. R₃ and R₄ plants exhibited partial recovery from BYMV-Ideal A, BYMV-GDD and BYMV-Scott as described for the R₂ plants.

AS 42 plants of three populations were uniformly infected with sap extracts, but showed interveinal chlorosis as described for AS 30⁺ above, starting at about 20 d.p.i. (8/8, 8/8, and 5/8 plants in the three populations). The progression of symptom recovery was more rapid than with AS 30⁺, with most plants producing entirely symptomless upper leaves by 21 to 25 d.p.i.; three plants of one population developed symptoms of green vein-banding without full progression to interveinal chlorosis and recovery. In plants inoculated with 100 µg/ml BYMV-Ideal A, systemic infection was followed by appearance of interveinal chlorosis starting at 13 d.p.i., and symptomless upper leaves from 18 to 21 d.p.i. (Fig. 3B; and see Figure 1 of Hammond and Kamo 1993) in which no virus was detectable by ELISA (Fig. 3F), bioassay or dot blot hybridization (results not shown). Virus titer in leaves showing interveinal chlorosis was reduced compared to the controls (Fig. 3F). None of 10 plants were infected after RNA inoculation. BYMV-Ideal A and BYMV-Scott were readily detected in asymptomatic inoculated leaves by ELISA (data not shown). R₃ and R₄ plants completely recovered from infection with BYMV-Ideal A, BYMV-GDD, and BYMV-Scott in a similar manner to that described for the R₂ plants.

AS 112: Seven of eight sap-inoculated plants were infected, with about a 1-day delay in symptom appearance and intensification compared to the controls. No recovery from symptoms nor reduction in virus titer was apparent. All eight plants inoculated with 100 µg/ml BYMV-Ideal A were infected, with a similar slight delay in symptom appearance, and no reduction in virus titer compared to the controls (Fig. 3C, G). One of 10 plants was infected by RNA inoculation, with typical symptom development. R₃ and R₄ plants were similarly susceptible to BYMV-Ideal A, BYMV-GDD, and BYMV-Scott. BYMV was readily detected by ELISA in symptomless inoculated leaves (data not shown) as well as systemically infected leaves.

AS 140: Only one of eight plants inoculated with sap became infected, with chlorotic blotches appearing on a single leaf at 12 d.p.i., on a second leaf at 16 d.p.i., and mild mosaic on subsequent leaves from 18 dpi. None of the plants inoculated with 100 µg/ml BYMV-Ideal A showed any symptoms during a 28-day observation period, nor was any viral antigen

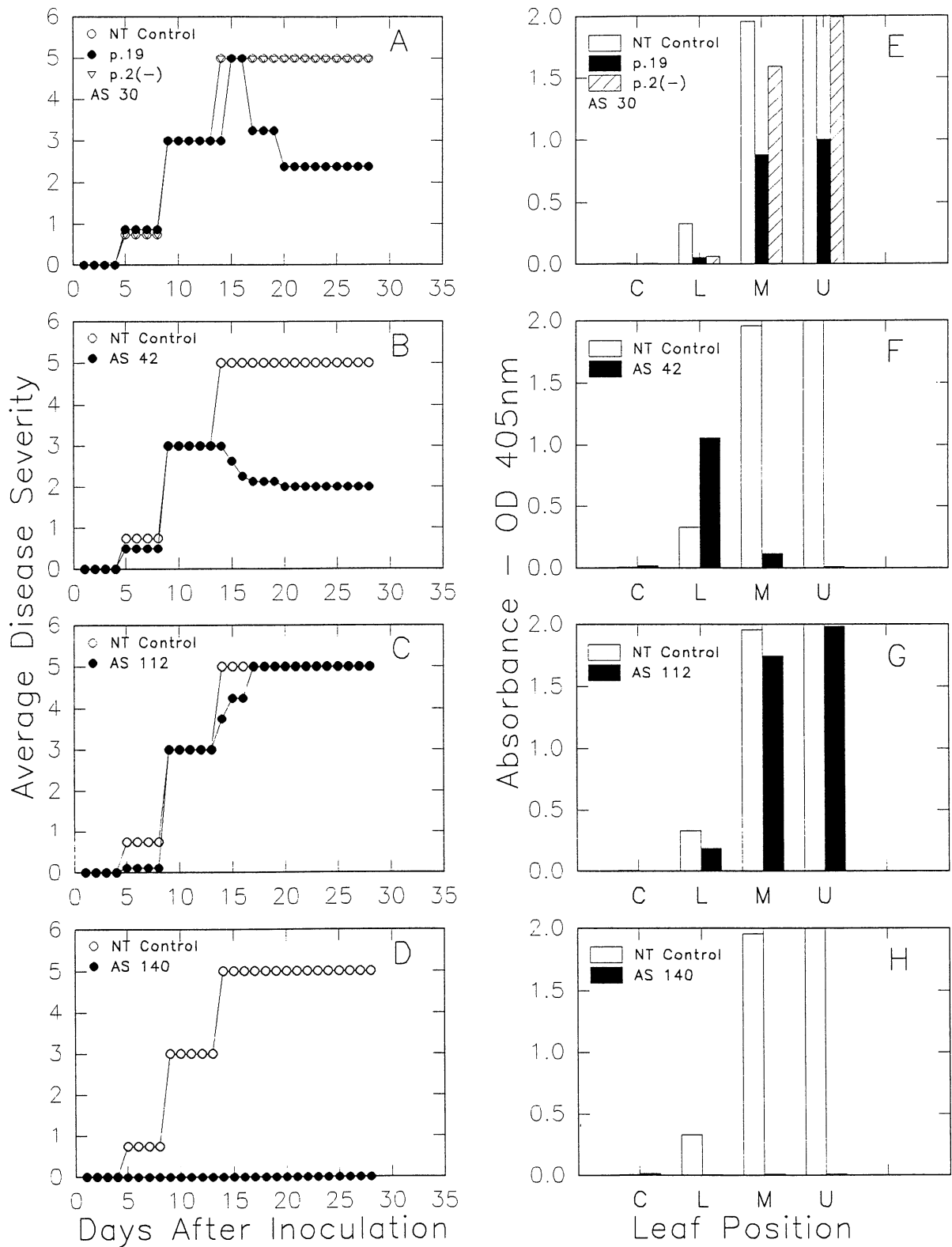


Fig. 3. Average disease severity of eight plants with time after inoculation with 100 µg/ml of bean yellow mosaic potyvirus (BYMV)-Ideal A (A–D), and relative ELISA values with MAb PTY 1 in uninoculated control (C), and lower (L), middle (M), and upper (U) leaves of selected inoculated plants (E–H). Note that disease severity scores are based on visually distinguishable symptoms (Table 1) and are not proportional. A, AS⁺ and AS⁻ populations of AS 30, showing partial recovery from symptoms in upper leaves of AS⁺ plants. B, AS 42 plants recovered from symptoms earlier, and completely in upper leaves. C, AS 112 plants showed a minor delay in symptom development. D, No plants of AS 140 were infected. E, Levels of BYMV in AS⁺ plants were reduced, and levels in AS⁻ plants were similar to controls. F, No BYMV was detectable in symptomless upper leaves of AS 42. G, Titer in AS 112 plants was similar to that in non-transgenic controls. H, No virus was detectable in symptomless AS 140 plants.

detected by ELISA (Fig. 3D, H), dot blot hybridization or bioassay from any of these plants (results not shown). No plants were infected following RNA inoculation or by other concentrations of sap or purified virus. No R₃ or R₄ plants were infected by BYMV-Ideal A, BYMV-GDD, or BYMV-Scott as determined by lack of symptoms, ELISA, and/or bioassay. No BYMV antigen was detectable in inoculated leaves tested 10 or 14 days after challenge with BYMV-Ideal A or BYMV-Scott (*A*₄₀₅ <0.05 OD; control noninoculated <0.04 OD).

Six additional transgenic lines challenged with sap extracts only were all initially susceptible to infection. Most plants of each genotype developed reduced symptoms of interveinal chlorosis, and some plants produced symptomless upper leaves by 35 d.p.i. (Table 2). None recovered as quickly and completely as line AS 42, nor were resistant to infection like AS 140. BYMV infection and the absence of other potyviruses was confirmed by ELISA with monoclonal antibodies (MAbs; Table 3; and data not shown).

Challenge with heterologous potyviruses.

Eight plants of each R₂ transgenic line and nontransgenic controls were inoculated with 100 µg/ml of pepper mottle virus (PepMoV) or turnip mosaic virus (TuMV). ELISA with MAbs (Table 3) was used to confirm infection with the appropriate virus and to compare virus titers. Plants of all transgenic and control lines inoculated with either PepMoV or TuMV were uniformly infected. No delay in symptom appearance nor significant differences in symptom expression or virus titer were observed between different transformants and nontransgenic controls (Fig. 4, and data not shown).

Table 3. Reactions of the monoclonal antibodies with the potyvirus isolates used

Monoclonal antibody	Virus				
	BYMV-GDD ^a	BYMV-Ideal A	BYMV-Scott	PepMoV NC 165	TuMV Linc-2
PTY 1	+++	+++	+++	+++	+++
PTY 43	+++	-	-	-	-
PTY 24	+++	+++	+++	-	-
PTY 10	-	-	-	+++	-
PTY 30	+++	+++	+++	-	-
TBV 27C2H2	-	-	-	-	+++

^a BYMV = bean yellow mosaic virus; PepMoV = pepper mottle virus; TuMV = turnip mosaic virus.

Table 2. Challenge inoculation of additional transformants with bean yellow mosaic virus (BYMV)-Ideal A sap extracts^a

Genotype	1:5 BYMV sap	1:50 BYMV sap
Nontransgenic	8/8	10/10
AS 16	10/10, 10 IC ^b , 1 RAT ^c	9/10, 6 IC, 2 RAT
AS 31	10/10, 10 IC, 1 RAT	10/10, 4 IC
AS 115	10/10, 10 IC, 3 RAT	10/10, 10 IC, 2 RAT
AS 139	10/10, 6 IC, 2 RAT	9/10, 3 IC, 1 RAT
AS 143	10/10, 8 IC, 4 RAT	10/10, 7 IC, 1 RAT
AS 7.16	10/10, 10 IC	9/10, 5 IC

^a Ten plants were inoculated with each concentration, with five uninoculated controls. Some plants died from fungal infection; numbers of infected plants are presented as the proportion surviving to 35 dpi, with additional information about symptom amelioration.

^b IC = Interveinal Chlorosis;

^c RAT = Recovery from symptoms At Top of plant (only observed in plants previously developing interveinal chlorosis).

DISCUSSION

The extreme resistance of line AS 140 must be due to an interaction at an early stage of the infection process. No BYMV CP could be detected by ELISA even in the inoculated leaves of AS 140; the assay used will detect BYMV CP below 0.5% of the amount present in a typical infection. BYMV CP was readily detected in symptomless inoculated leaves of nontransgenic or other AS transgenic lines. Only a single "AS 140" plant became infected with BYMV in any of these experiments; none were infected by 100 µg/ml of purified virus. The genotype of the single infected plant cannot now be determined, but it is possible that plants of two genotypes were mixed; a single "AS 112" plant remained uninfected in the same experiment, whereas in other experiments all AS 112 plants became infected. Although line AS 140 segregated with an apparent ratio of 15:1 in the R₁ generation, suggesting two independent insertions of the transgene, Southern analysis of the R₂ population examined in detail indicated the presence of only a single copy. The effective immunity therefore appears to segregate with a single copy of the gene. No R₃ or R₄ AS 140 plants were infected, so expression of the integrated gene is apparently stable.

In other lines, virus titer in leaves displaying the novel interveinal chlorosis was markedly reduced. No virus was detectable from the symptomless upper leaves of AS 42; recovery in such plants is thus complete. The pattern of recovery and the novel symptom type are suggestive of the pattern of expression of the CaMV 35S promoter, with possibly combined resistance to replication and to transport (Hammond and Kamo 1993).

Inoculation with RNA yielded results equivalent to challenge with virus. When AS transformants were challenged with PepMoV or TuMV, no delay of symptom expression was observed, nor any apparent symptom moderation. The mechanisms of AS protection for potyviruses appear to be through direct RNA:RNA interaction; the lack of resistance to PepMoV or TuMV would be expected, as there is limited sequence similarity with BYMV at the nucleotide level.

The differences in performance between transgenic lines may be due to differences in levels or tissue specificity of AS RNA expression (Krol et al. 1988b). The level of AS transcript in AS 140 and AS 42 was clearly higher than in AS 30 and AS 112 (Fig. 1). Although extreme resistance to infection, (AS 140) and resistance to replication and/or transport (typified by AS 42) have been observed, these may be different levels of effectiveness of the same mechanism. The AS resistance and recovery that we have observed is phenotypically similar to that induced by untranslatable sense RNA of TEV (Lindbo and Dougherty 1992a, 1992b). It is possible that both arise from interference in rate-limiting steps in replication, but we differ from Dougherty's group in interpretation of the mechanism. Dougherty et al. (1994) have noted that increasing the level of transgene expression should increase the level of interference with virus replication, yet that steady-state levels of transgene transcript may be lower in lines with greater resistance. This has been ascribed to action of a sequence-specific host nuclease activity which may be induced by the additive effect of transgene RNA and viral RNA (requiring initial systemic infection; Lindbo et al. 1993), or even by high-level transcription of the transgene

(Dougherty et al. 1994). However, induction of a highly antiviral state through a sequence-specific nuclease (Lindbo et al. 1993) is not apparent in AS 30⁺ or other lines in which partial recovery was consistently observed; if sequence-specific nuclease activity is induced in such AS lines, then it is not very effective.

Alternative possibilities for an AS-RNA mediated mechanism of resistance to potyviruses include: (i) disruption of translation of the viral polyprotein, (ii) blocking the interaction of the viral polymerase with its recognition site, and (iii) formation and rapid degradation of dsRNA. Prevention of nuclear RNA export, or interference with mRNA processing (Inouye 1988) are not relevant to potyviral RNA, which replicates in the cytoplasm and is not processed (Reichmann et al. 1992). Our BYMV AS construct contains the complete 3' noncoding sequence as well as CP gene coding region (Fig. 5); we have now subdivided this effective construct into the partial coding region (potentially able to inhibit both translation, and polymerase procession), and the noncoding region (able to affect polymerase binding, but not translation) to distinguish between (i) and (ii). Contrary to the suggestion of Lindbo and Dougherty (1992b) that RNA:protein interactions interfere with RNA:RNA hybridization, we believe the 3' noncoding sequence is critical, and that RNA:RNA interaction precludes, or effectively competes with, the RNA:protein (viral replication complex) interaction. The importance of regulatory sequences for antisense targeting of viral infections has been previously demonstrated (Hirashima et al. 1986; Ottavio et al. 1992; Huntley and Hall 1993a, 1993b). Little resistance was observed with cucumber mosaic virus 5' and 3' AS constructs lacking 30 to 135 nt of the terminal sequences (Rezaian et al. 1988). Removal of the 3'-terminal 117 nucleotides (including the putative replicase binding site) abolished the low level of resistance conferred by a TMV AS construct (Powell et al. 1989); the 3' noncoding region was critical for AS resistance. Rapid dsRNA degradation (iii) may occur in AS RNA-expressing plants where both the AS RNA and the target RNA are expressed from the nuclear genome (Rothstein et al. 1987); however, viral dsRNA formed in the cytoplasm is so stable that it can be purified from air-dried leaves (R. L. Jordan, personal communication) and is useful for virus diagnosis (Morris et al. 1983).

Interference in production of the viral (-) strand may be an efficient strategy, as typically much lower levels of viral (-) strand RNA are produced than (+) strand; (-) strand production may be a rate-limiting step in replication (see also Huntley and Hall 1993b). Differences in the resistance phenotype between plants transformed with AS constructs of BYMV (extreme resistance or recovery: this work), zucchini yellow mosaic virus (ZYMV) (delay in symptoms and reduced titer: Fang and Grumet 1993), TEV (delayed and reduced symptoms and titer: Lindbo and Dougherty 1992a) and PVY (reduced symptoms and titer: Farinelli and Malnoë 1993; or no resistance: Smith et al. 1994), may in part be due to the structures of the constructs (Fig. 5). The BYMV and ZYMV constructs each contain the complete viral 3' noncoding region. The TEV (Lindbo and Dougherty 1992a) and PVY construct of Smith et al. (1994) contain only CP coding sequence, while the PVY^N AS of Farinelli and Malnoë (1993) lacks 128 3'-terminal nucleotides. Other differences may include the level and tissue specificity of AS RNA expression,

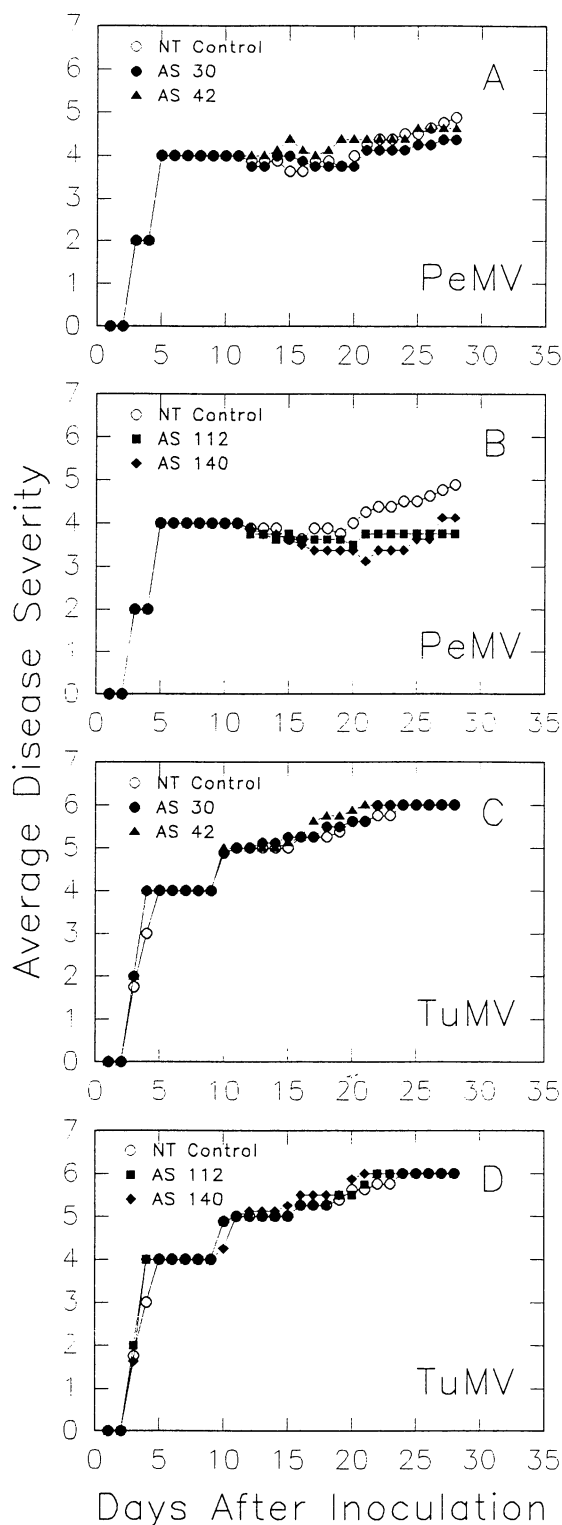


Fig. 4. Average disease severity of eight plants over time after inoculation with 100 µg/ml of pepper mottle virus (PepMoV; PeMV) (A,B) or turnip mosaic virus (TuMV) (C,D). Disease severity scores are based on visually distinguishable symptoms (Table 1) and are not proportional. For the sake of clarity results with an AS⁻ population of AS 30 are not shown. The apparent reduced symptom severity of PeMV-inoculated AS 112 and AS 140 (B) is partially due to the death of one plant each of the nontransgenic control, AS 30, and AS 42 (see text).

or differences between the potyviruses themselves. However, the extreme resistance of AS 140 and complete recovery of AS 42 plants from BYMV infection contrasts with the slight delay in symptom appearance in TEV and ZYMV AS transgenic plants. BYMV AS RNA was also effective against three biologically and/or serologically differentiated isolates, whereas the PVY^N construct (Farinelli and Malnoë 1993) conferred protection only to the homologous PVY^N strain, and not PVY^O.

We postulate that the resistance we have observed is due to BYMV AS RNA interfering in the production of (-) strand RNA, and predict that the noncoding region of our current effective AS construct will prove more effective than the CP region. We are also preparing a PVY AS construct similar to our original BYMV AS construct to examine the generality of the AS mechanism against potyviruses.

MATERIALS AND METHODS

Plasmid constructions.

Cloning of the BYMV isolate GDD 3' end has been described (Hammond and Hammond 1989). Plasmid pBY6 contains approximately 470 nt of the carboxy-terminal half of the CP gene, the complete 165 nt 3' noncoding region and 19 residues from the poly(A) tract (Hammond and Hammond 1989; Fig. 5). The insert of pBY6 was transferred as a *Hind*III/*Bam*HI fragment next to the cauliflower mosaic virus (CaMV) 35S promoter in the binary vector pGA643 (An et al. 1988), which was prepared by digestion with *Hind*III and *Bgl*II. The resulting plasmid, pGA.BY6, was then transformed directly into *Agrobacterium tumefaciens* strain 5922 (LBA4404 containing Ti helper plasmid PC2760; a gift of G. An) as described (An et al. 1988).

Plant transformation and analysis.

Cotyledons of *Nicotiana benthamiana* were cocultivated with *A. tumefaciens* essentially as described for leaf disks by Horsch et al. (1985). Selection of transformed shoots was in the presence of (per milliliter) 300 µg of kanamycin, 100 µg of cefotaxime, and 1 mg of carbenicillin. Shoots were rooted in the presence of the same concentrations of kanamycin and carbenicillin, and rooted plants transferred to Promix soil-less

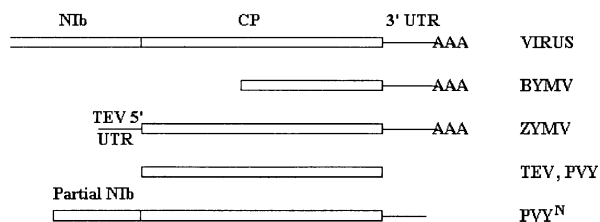


Fig. 5. Structure of the bean yellow mosaic potyvirus (BYMV) AS construct used in this study, compared to the 3' region of the potyviral genome, and AS constructs of zucchini yellow mosaic virus (ZYMV) (Fang and Grumet 1993), tobacco etch virus (TEV) (Lindbo and Dougherty 1992a, 1992b), PVY (Smith et al. 1994), and PVY^N (Farinelli and Malnoë 1993). Nlb, nuclear inclusion b; CP, coat protein; UTR, untranslated region. Note that the PVY^N construct contains only 212 of 330 nt of the complete PVY 3' UTR. In each case the viral sequence was inserted in AS orientation between the cauliflower mosaic virus 35S promoter, and transcription termination sequences from the T-DNA vector.

potting medium. Leaf tissue samples were taken during tissue culture, and again from rooted plants, and extracted for PCR analysis essentially as described (McGarvey and Kaper 1991). The upstream PCR primer was within the CaMV 35S promoter (JH 006, 5' CCTTCGCAAGACCCTTCC 3') and the reverse primer (JH 010, 5' GCAAAGCCAACATCCGC 3') from the CP sequence near the viral sense 5' end of pBY6 (Hammond and Hammond 1989). One cycle of denaturation at 94°C for 3 min was followed by 40 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, and one cycle of 72°C for 5 min. Plasmid pGA.BY6 was used as a positive control, and a DNA extract of healthy nontransgenic *N. benthamiana* as a negative control for all amplifications. The original transformants (R₀ generation) were allowed to self, and the R₁ progeny analyzed by PCR and by germination in the presence of 300 µg/ml kanamycin. Putative homozygous R₁ seedlings were selfed to obtain R₂ seed, and R₂ populations tested for homozygosity by PCR and/or kanamycin resistance. R₂ seed were also collected from some non-expressing R₁ segregants for use as additional negative controls.

Northern analysis.

Total RNA was prepared according to Lichtenstein and Draper (1985), and 3 µg of total RNA per sample separated in glyoxal agarose gels as described (Ogden and Adams 1987). Samples of potato virus X (PVX) and TMV genomic RNAs (2 µg each) were electrophoresed in separate lanes as size standards. The lanes containing PVX and TMV marker RNAs were excised and stained in acridine orange prior to passive blotting of the transgenic plant RNA to Nytran membrane (Schleicher & Schuell, Inc., Keene, NH).

Southern analysis.

Genomic DNA of the R₂ populations of AS 30-, AS 30+, AS 42, AS 112, and AS 140 used for virus challenge experiments was extracted as described (Deblaeere et al. 1987), and 10 µg of each digested with *Hind*III prior to separation in a 0.8% agarose gel and passive transfer to Nytran membrane. Plasmids pGAoCP (Hammond and Kamo 1993) digested with *Hind*III, *Pst*I, or *Hind*III plus *Pst*I, and pGA643 digested with *Hind*III plus *Pst*I and separately with *Sal*I, were used as size markers; fragments of pGAoCP of approximately 12.8, 3.6, and 1.04 kb that hybridize with the BYMV probe were obtained from the digestion of pGAoCP.

Hybridization and detection.

For both Northern and Southern blots, a nonradioactive digoxigenin-labeled probe was prepared from the *Pst*I insert of clone pBY6 using the Genius kit (Boehringer Mannheim, Indianapolis, IN), following the manufacturer's instructions. Detection was using Lumiphos 530 (Boehringer), and exposure to Hyperfilm (Amersham, Arlington Heights, IL).

Virus and RNA purification, inoculation, and assay.

Turnip mosaic virus (TuMV) isolate Linc-2 (Hammond and Chastagner 1988; deposited at the American Type Culture Collection, Rockville, MD, as ATCC PV-389), pepper mottle virus (PepMoV) isolate NC 165, BYMV-GDD (ATCC PV-368) and BYMV isolate Ideal A, and RNA of BYMV-GDD were purified as described (Hammond and Lawson 1988).

Initial challenge inoculations were with BYMV-infected sap extracts (approximately 1:20 in 1% K₂HPO₄) and additional challenges with sap extracts at 1:5 and 1:50. Purified virus was diluted to 100, 20, 10, 2 or 1 µg/ml, and either Celite added to the virus solution, or leaves to be inoculated dusted with Celite or bentonite; 50 µl of diluted virus was used to inoculate each of three expanded leaves of plants at the 8- to 10-leaf stage. RNA was diluted in sterile water to 50 µg/ml, and 50 µl inoculated to a single bentonite-dusted leaf per plant using a sterile glass rod. Four to 10 plants of each population were inoculated for each treatment, including non-transgenic controls. Noninoculated plants of each transformant and nontransgenic plants served as negative controls.

Plants were observed daily for symptom development for 28 days after inoculation, and periodically afterwards. BYMV, PepMoV, and TuMV symptom severity was expressed as the average score of the inoculated plants on the scales shown in Table 1. Plants were scored on the predominant or emerging symptom type. Upper leaves were then tested by ELISA essentially as described (Jordan and Hammond 1991) with monoclonal antibodies PTY 1, PTY 10, PTY 43 (Jordan and Hammond 1991), and TBV 27C2H2 (Hsu et al. 1988) as appropriate to confirm the diagnosis of symptom status and the identity of the causal virus (Table 3). Additional ELISA tests were performed on leaves from lower, middle, and upper positions on the main stem of selected plants. Extracts of some upper leaves were bioassayed by inoculation to non-transgenic *N. benthamiana*. In some experiments BYMV was also detected by dot blot hybridization with a riboprobe transcribed from clone pBYr5 (Hammond and Hammond 1989) subcloned into pSP65. Clone pBYr5 does not cross-hybridize to the CP gene or 3' noncoding region, being derived from an upstream portion of the genome (J. Hammond, unpublished).

ELISA with MAbs PTY 1 and PTY 24 was also used to examine virus production in inoculated leaves, and leaves produced following inoculation, of non-transgenic *N. benthamiana*, and of AS 42, AS 112, and AS 140 plants challenged with BYMV Ideal A and BYMV-Scott (ATCC PV 684; Jones and Diachun 1977). Two inoculated leaves and one upper leaf were harvested 14 d.p.i. from each of two nontransgenic and five AS 140 plants (BYMV Ideal A and BYMV-Scott; experiment 1); and 10 d.p.i. (BYMV Ideal A) or 14 d.p.i. (BYMV-Scott) from AS 42, AS 112, and AS 140 plants (experiment 2).

ACKNOWLEDGMENTS

We would like to thank G. An for providing the binary vector and *Agrobacterium* strain, and R. L. Jordan and H-T. Hsu for monoclonal antibodies; Jeanne Williams, Alice Chen, Phuong Bui, and Marc Bauduy for technical assistance; and R. Grumet, G. M. Hellmann, R. L. Jordan, R. H. Lawson, and R. A. Owens for critical review of the manuscript.

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