Resistance to Cymbidium Ringspot Tombusvirus Infection in Transgenic *Nicotiana benthamiana* Plants Expressing a Full-Length Viral Replicase Gene

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***Nicotiana benthamiana* plants were transformed with a full-length replicase gene, corresponding to the first open reading frame of Cymbidium ringspot tombusvirus (CyRSV) RNA. Transgenic plants of one line out of five were highly resistant when challenged with CyRSV virions and immune when inoculated with viral RNA. Resistance was at the cellular level and was not due to inhibition of cell-to-cell spread, as shown experimentally by inoculation and resistance of protoplasts from these transgenic plants.**

Cymbidium ringspot virus (CyRSV), a member of the Tombusvirus group (Martelli *et al.* 1989), has icosahedral particles and a genome consisting of one messenger-sense RNA of 4,733 nucleotides (nt), coding for five proteins of 33, 92, 41, 22, and 19 kDa (Grieco *et al.* 1989) plus a putative protein of 4 kDa (Boyko and Karasev 1992). The genomic RNA functions as mRNA for the synthesis of the 33-kDa protein, and, by read through of an amber termination codon, a 92-kDa protein is produced. These two proteins are involved in the replication of viral RNA, since the read-through domain contains both the GDD motif of viral replicases (Kamer and Argos 1984) and an NTP-binding motif of nucleic acid helicases (Habili and Symons 1989); and mutants modified in this region are not viable (Dalmay *et al.* 1993). The 41-kDa coat protein is translated from a subgenomic RNA of 2.1 kb, and the 22-kDa protein, which is required for cell-to-cell movement, is translated from a second subgenomic RNA of 0.9 kb (Grieco *et al.* 1989). Putative 19- and 4-kDa proteins are not necessary for virus replication and movement (Dalmay *et al.* 1993).

Nicotiana benthamiana Domin. plants infected with CyRSV and grown at 21° C days and 16° C nights reacted with local lesions in 2–3 days followed in 3–4 days by systemic mosaic and leaf distortion, and, after an additional 5 days, by apical necrosis and death. Transgenic *N. benthamiana* plants expressing the coat protein (CP) gene showed resistance to infection only when virus concentration in the inoculum was very low (0.05 μg/ml), whereas no protection was observed in transgenic plants inoculated with virus concentrations of 0.5 and 5.0 μg/ml, or when the inoculum was *in vitro* synthesized genomic RNA (Rubino *et al.* 1993). Plants transformed with sequences derived from a satellite RNA that may be associated with CyRSV, were not protected from apical necrosis and death (Rubino *et al.* 1992). Conversely, protection from disease was obtained in engineered plants expressing defective interfering (DI) RNA sequences, much the same as observed in nontransformed plants inoculated with virus cultures containing DI RNA (Kollar *et al.* 1993). Since the first report of engineered virus resistance in tobacco plants expressing the CP gene of tobacco mosaic tobamovirus (TMV) (Powell Abel *et al.* 1986), CP-mediated resistance has been shown to be effective against several viruses (reviewed by Beachy *et al.* 1990). A different approach to obtain genetically engineered virus resistance was adopted by Golemboski *et al.* (1990), who showed that tobacco plants expressing the read-through portion of the putative TMV replicase, potentially coding for a protein of 54 kDa, were resistant to infection with TMV virions or viral RNA.

To determine whether a strategy involving the replicase gene for introducing resistance to viral infection could be effective also with tombusviruses, *N. benthamiana* plants were transformed with sequences derived from this gene. The behavior of plants expressing the full-length replicase gene of CyRSV is described in this paper.

RESULTS

Construction of CyRSV replicase gene expression vector and transformation of plants.

The replicase gene of CyRSV starts at position 161 in the genomic RNA and extends to a UGA stop codon at position 2,614 by read through of a leaky UAG stop codon at position 1,048 (Fig. 1). During preparation of the plant expression vector, the nontranslated sequence preceding the start codon was replaced by the leader sequence of TEV. This is known to be an efficient enhancer of heterologous reporter gene expression (Carrington and Freed 1990), which was also demonstrated in transgenic plants expressing the CyRSV CP gene (Rubino *et al.* 1993).

Expression of CyRSV replicase in transgenic plants could not be verified serologically, because an antiserum to this protein is not yet available. Successful transformation was therefore assessed by polymerase chain reaction and Southern blot analysis of DNA extracts. Fourteen transformant lines were obtained, five of which, selected for further analysis,

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were designated 92KA1, 92KA11, 92KB1, 92KB8, and 92KB16. Northern blot analysis of RNA extracts showed the presence of genomic CyRSV RNA-related transcripts of about 3 kb, corresponding to the size of the replicase gene, the vector sequences, and poly(A) tail (Fig. 2). Lines 92KA1 and 92KB16 contained the least amount of viral transcript, which was barely visible in gels loaded with 10 μg of RNA but was detectable in gels loaded with 30 μg of RNA (Fig. 2, lanes 7 and 8).

Resistance of transgenic plants to CyRSV.

R_1 seedlings (from approximately 100–200 seeds per R_0 plant of each line) were screened on MS medium (Murashige and Skoog 1962) containing kanamycin for resistance to the antibiotic. Statistical analysis indicated that the NPT II selectable marker gene was inherited in the segregation ratio of 3:1 only in line 92KA1, i.e., indicating insertion at a single locus. The segregation ratio in seedlings of other lines was either not compatible with a Mendelian ratio and could not be interpreted (lines 92KA11 and 92KB16), or was more compatible with a segregation ratio of 2:1 (92KB1 and 92KB8), indicating a possible lethal homozygous condition for insertion of T-DNA into a region of the genome that prevents the expression of essential genes (Table 1). To clarify this point, further experiments with R_2 progeny seedlings are in progress.

Self-fertilized kanamycin-resistant R_1 progeny from the five transformed lines were transferred to soil and assayed for resistance to CyRSV when they had reached a three- to four-expanded-leaf stage. Groups of 18–36 plants transformed with the CyRSV replicase sequence or with vector only, or nontransformed plants were inoculated with purified virus or *in vitro* synthesized RNA and observed daily for the appearance of symptoms. All nontransformed plants and plants transformed with vector only became infected regardless of the inoculum composition and died within 10–15 days of inoculation. A variable number of transformed plants of each line also became infected, up to 100% for lines 92KB1 and

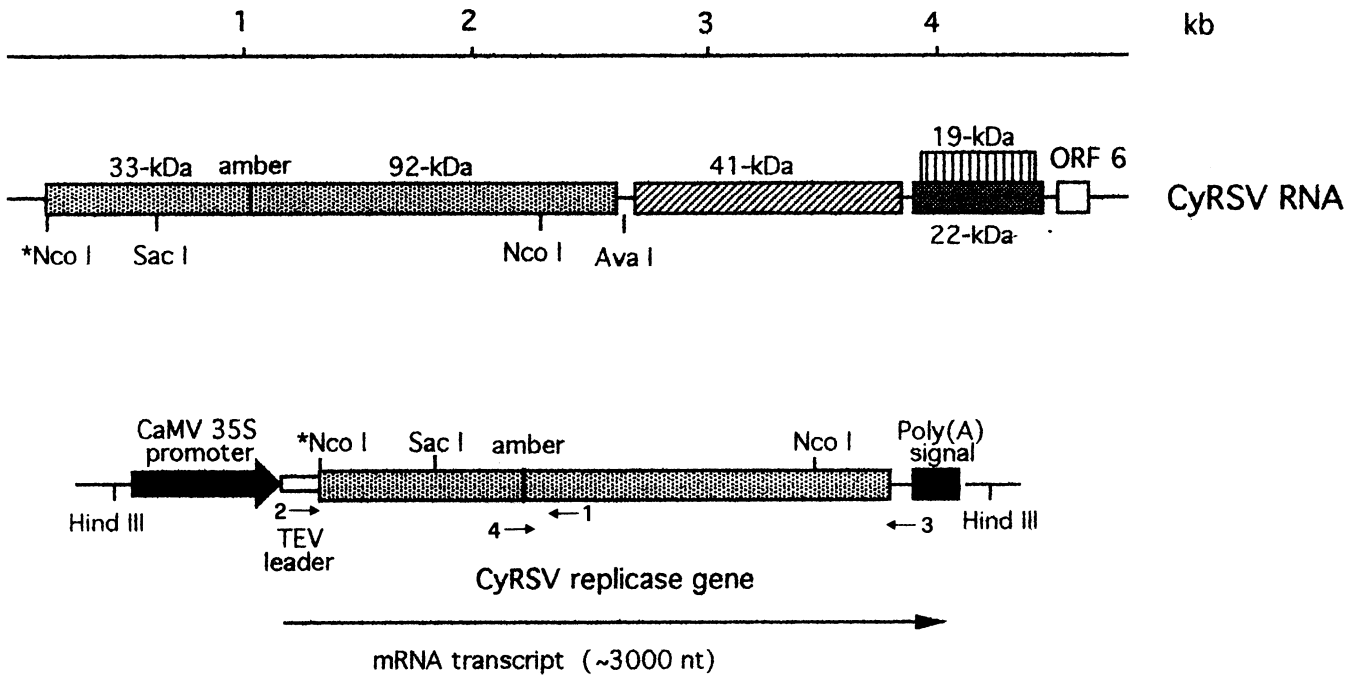

Fig. 1. Diagrammatic representation of CyRSV genomic RNA showing the positions of open reading frames and relevant restriction sites and insertion of replicase gene in the plant expression vector pRTL2. **NcoI* was constructed by site-directed mutagenesis of two nucleotides preceding the start codon of the 33-kDa protein. Small arrows indicate the position and direction of pairs of synthetic oligonucleotides used for polymerase chain reaction analysis of transgenic plants.

Table 1. Segregation of resistance to kanamycin in the R_1 progeny of self-fertilized transgenic *Nicotiana benthamiana* plants[a]

Plant line	No. of seeds sown	Germination (%)	Km^R[b]	Km^S[c]	χ^2[d] (3:1)	χ^2 (2:1)
92KA1	175	94	126	39	0.162	6.98
92KA11	173	82	82	60	22.53	5.1
92KB1	103	56	38	20	2.79	0.033
92KB8	160	76	86	36	1.32	0.8
92KB16	164	74	63	58	33.93	11.6

[a] Plantlets were checked after 1 mo for the presence of normal unbleached root systems.
[b] Km^R, kanamycin-resistant seedlings.
[c] Km^S, kanamycin-sensitive seedlings.
[d] A χ^2 of 3.84 is significant at the 0.05 probability level. This experiment was repeated once, yielding essentially the results shown.

92KB16 (Table 2). There was no significant difference in the timing of appearance of local and systemic symptoms in transgenic versus control plants. Northern blot analysis of systemically infected leaves from transgenic and control plants showed that the amount of viral RNA was similar in both groups, whereas symptomless plants did not contain any detectable viral RNA (Fig. 3). Northern blots of preparations from inoculated leaves had the same pattern (not shown). Transgenic plants that did not show symptoms even after the control plants had died continued to look healthy for several weeks after inoculation.

Line 92KA1 was noteworthy because a relatively small percentage of plants (25%, Table 2) showed symptoms when virus particles were used as inoculum, and none of them became infected when inoculated with *in vitro* transcribed viral RNA. Because of this unexpected and atypical result, this line was studied in more detail.

First, 28 92KA1 plants were inoculated with CyRSV RNA transcripts and screened for resistance. To eliminate the possibility that resistance could arise from somaclonal variation, seedlings were not screened for kanamycin resistance before inoculation, and seeds were sown directly in soil. Five plants showed typical disease symptoms at the same time and of the same severity as nontransformed control plants, whereas the other 23 did not show symptoms for several weeks after inoculation. No viral RNA could be detected in these plants, whereas it was present in the five symptomatic seedlings. The ratio of infected versus resistant plants approached the expected ratio 3:1 ($\chi^2 = 0.76$) for segregation of resistance to viral RNA as a single dominant trait.

In another experiment, 14 plants were assayed by enzyme-linked immunosorbent assay (ELISA) for the NPT II gene product before inoculation. These tests showed that three plants were negative for NPT II, and these were the only ones to be infected upon inoculation with CyRSV RNA transcripts. These data were interpreted as a confirmation that expression of NPT II and resistance to CyRSV RNA both cosegregate and in a 3:1 ratio ($\chi^2 = 0.1$).

The immune condition of line 92KA1 to RNA inoculation was also checked by inoculating 12 kanamycin-resistant plants with RNA extracted from virions. None of the transgenic plants became infected, in contrast to the control plants, all of which were infected (not shown).

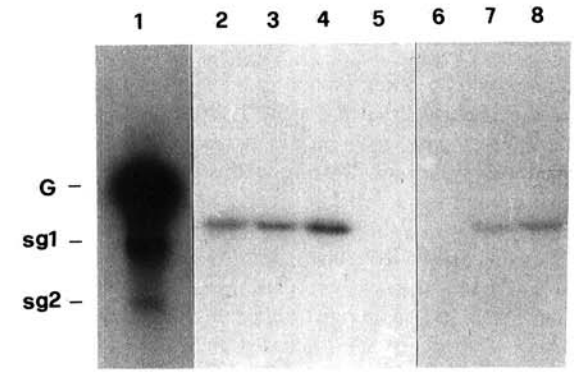

Fig. 2. Northern blot analysis of total RNA extracts from uninoculated *Nicotiana benthamiana* plants transformed with CyRSV replicase gene (lanes 2–4, lines 92KA11, 92KB1, 92KB8; lanes 7, 8, lines 92KA1 and 92KB16) or with vector pGA482 only (lanes 5 and 6). Lane 1 contained RNA (20 ng) from a CyRSV-infected nontransformed plant. The autoradiogram was first exposed overnight to mark the position of genomic (G) and subgenomic RNAs (sg1 and sg2) (4.7, 2.1, and 0.9 kb, respectively) and reexposed for 72 hr. Lanes 2–5 were loaded with 10 μg, and lanes 6–8 with 30 μg of total RNA in two separate gels. Hybridization was with nick-translated full-length CyRSV clone G11.

Table 2. Percentage infection of transgenic *Nicotiana benthamiana* plants 15 days after inoculation with CyRSV[a]

Plant line	Virus (5 μg/ml)	RNA (75 μg/ml)
92KA1	25	0
92KA11	90	85
92KB1	100	100
92KB8	65	75
92KB16	50	100

[a] Control plants were groups of nontransformed plants, or plants transformed with the vector only, and showed 100% infection in all cases.

Inoculation of protoplasts.

The above results clearly show that individual plants of line 92KA1 exhibit a different type of response to inoculation depending on the type of inoculum, i.e., whole virus particles versus viral RNA. A protoplast system was used to investigate whether the same plant would behave differentially when exposed to either type of inoculum.

Protoplasts were prepared from four 92KA1 plants, two of which were ELISA-positive and two ELISA-negative for NPT II, and from a single nontransformed plant. Protoplasts from each plant were divided into two equal aliquots, which were inoculated with whole virions or viral RNA. Northern blot analysis of RNA extracts obtained 24 hr after inoculation showed that one of the two plants expressing NPT II yielded protoplasts resistant to both virions and RNA, and the other was susceptible only to virus, whereas the two NPT II–negative plants and the nontransformed control plant became infected both by virions and RNA (Fig. 4). The amount of viral RNA detected in the extract from protoplasts susceptible to inoculation only with virus was very low; however, viral replication was confirmed by the use of a riboprobe, which detected minus-strand viral RNA (not shown). It was not determined whether the low level of viral RNA depended on a reduced number of infected protoplasts or on a reduced replication rate in infected cells.

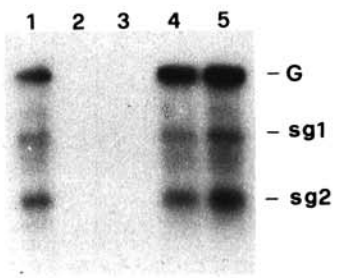

Fig. 3. Northern blot analysis of total RNA extracts from 92KA1 transgenic (lanes 2–4) and nontransgenic (lanes 1 and 5) plants inoculated with CyRSV *in vitro* transcripts (lanes 1 and 2) or virions (lanes 3–5). Plants of lanes 2 and 3 were symptomless. Hybridization was with a nick-translated clone, pCyR-7.

In another experiment, protoplasts were isolated from three kanamycin-resistant transgenic plants and from one nontransgenic plant. Protoplasts from each plant were again divided into two aliquots, which were inoculated with *in vitro* CyRSV RNA transcripts or RNA extracted from virus particles. No replication was detected following inoculation with either type of viral RNA (Fig. 5). Taken together, these results were interpreted as an indication that the resistance of line 92KA1 to inoculation with CyRSV is at the single-cell level and does not depend on inhibition of cell-to-cell movement.

DISCUSSION

The results of the present investigation show that at least one out of five transgenic *N. benthamiana* lines expressing the replicase gene from CyRSV was resistant to infection. Plants of the other four transgenic lines were either moderately resistant or completely susceptible (92KB1). Whether a positive correlation exists between the level of 92-kDa transcript and resistance could not be determined. In fact, highly resistant line 92KA1 produced a relatively low level of 92-kDa gene transcripts, as did the moderately resistant line 92KB16. This situation is not uncommon and is a hallmark of RNA-mediated resistance, at least in part. It was also detected in plants transformed with the CP gene of some potyviruses (Beachy *et al.* 1990), tomato spotted wilt virus (Gielen *et al.* 1991), or the amino-terminal portion of the PVX replicase gene (Braun and Hemenway 1992). Evaluation of the protein product of the 92-kDa gene in conferring resistance to transgenic plants must await production of a suitable antiserum.

Intertransformant variability is common among plants transformed via *Agrobacterium* and is often attributed to transgene copy number, position in the plant genome, unexpected molecular modifications of transgenes, or a number of experimental causes (for review see Hobbs *et al.* 1990). Further analysis of the copy number of the CyRSV 92-kDa gene in the different transgenic lines and the degree of methylation may help in understanding the variable degree of resistance exhibited by our transgenic lines.

Resistance to virus infection of transgenic plants expressing a partial or full-length replicase gene is now documented for a number of viruses. The Golemboski *et al.* (1990) report, showing that tobacco plants transformed with TMV 54-kDa gene were resistant to infection with TMV virions or RNA, was confirmed by other experimental work showing this type of resistance to be effective in 1) tobacco plants expressing the amino-terminal portion or full-length replicase gene of potato virus X (Braun and Hemenway 1992), or mutated forms of this gene (Longstaff *et al.* 1993), or a defective replicase gene of cucumber mosaic cucumovirus (Anderson *et al.* 1992); and 2) in *N. benthamiana* plants expressing the carboxy-terminal portion of the replicase of pea early browning tobravirus (MacFarlane and Davies 1992).

The mechanism(s) underlying replicase-mediated resistance are not yet clear. It was suggested (Longstaff *et al.* 1993) that replicase-mediated resistance may result from a dominant negative effect of mutant forms of the gene, even when it was claimed that the unmodified full-length gene of PVX replicase effectively conferred resistance (Braun and Hemenway 1992). This gene was derived from a cDNA clone possessing poor infectivity, and therefore it was suspected to be at least partially inactivated (Longstaff *et al.* 1993). This may not be the case for the full-length CyRSV replicase gene used in the present work, since it was derived from a full-length cDNA clone of CyRSV genomic RNA, which produces *in vitro* transcripts of the same specific infectivity as RNA extracted from virus particles (Dalmay *et al.* 1993). Another mechanism that disrupts the normal CyRSV host-plant relationship must be proposed. According to Sanford and Johnston (1985), an excessive amount of a pathogen gene strongly interferes with essential steps in pathogen replication. It was also shown that transformation of plants with an endogenous gene results in the suppression of normal gene expression (cosuppression; Napoli *et al.* 1990). The two phenomena are similar in that if a gene is expressed from more than one source, there may be a suppression effect. At this stage, however, it is not clear how the suppression mechanism works at the molecular level against CyRSV replication.

In all cases of replicase-mediated resistance against viruses, it has been shown that resistance is effective when transgenic plants are challenged either with whole virions or viral RNA, contrary to CP-mediated resistance, which, in most cases, is ineffective against viral RNA (Beachy *et al.* 1990). The behavior of line 92KA1, expressing CyRSV replicase gene, is unusual in this respect, because plants of this

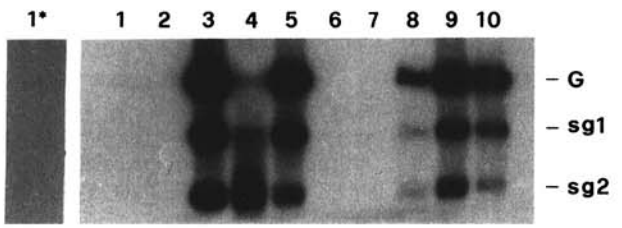

Fig. 4. Northern blot analysis of RNA extracts from protoplasts from two plants positive (lanes 1, 6, and 2, 7) or two plants negative (lanes 3, 8, and 4, 9) for expression of NPT II, and one nontransformed plant (lanes 5 and 10). Protoplasts were inoculated with CyRSV particles (lanes 1–5) or *in vitro* transcribed RNA (lanes 6–10). Lane 1* is an overexposed print of lane 1 to better visualize a faint band of genomic RNA barely visible in the autoradiogram. G, sg1, and sg2 indicate the positions of CyRSV genomic and subgenomic RNAs. Hybridization was with a nick-translated clone, pCyR-7.

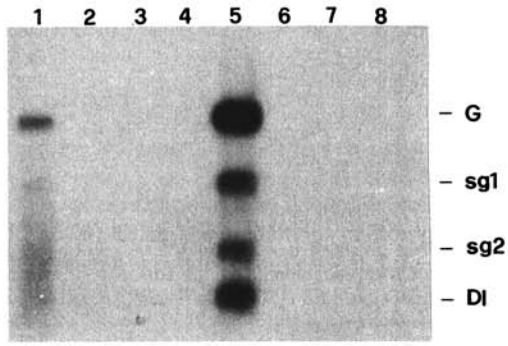

Fig. 5. Northern blot analysis of RNA extracts from protoplasts from three transgenic (lanes 2, 6; 3, 7; 4, 8) and one nontransgenic (lanes 1 and 5) plant. Protoplasts were inoculated with CyRSV RNA *in vitro* transcribed (lanes 1–4) or extracted from virus particles (lanes 5–8). DI indicates a DI RNA that replicates after inoculation with uncloned CyRSV RNA; G, sg1, and sg2 are CyRSV genomic and subgenomic RNAs. Hybridization was with a nick- translated clone, pCyR-7.

line display absolute resistance when challenged with viral RNA and susceptibility, albeit limited, to inoculation with virions. The different susceptibility is not due to genetic segregation, as shown by experiments with protoplasts from one plant, which were immune to RNA inoculation but susceptible to inoculation with virus particles. These results suggest that the coat protein may have a role at the onset of viral replication, perhaps in the very early stages of translation of inoculum virus. At this stage, the capsid may protect genomic RNA from interference by the replicase gene expressed constitutively by the transgenic host. Once the viral replicative cycle begins, there is little, if any, obstacle to disease development in infected plants. However, replication is reduced at the beginning of infection, as shown by the protoplast experiments.

Replicase-mediated resistance to Tombusvirus infections may be a useful tool for a better understanding of viral replication and of the mechanisms underlying genetically engineered cross-protection. Experiments are now under way to analyze the response to infection of plants transformed with a defective CyRSV replicase gene, or two portions of the gene coding for the 33-kDa protein and the read-through portion, respectively.

MATERIALS AND METHODS

Clone construction.

A full-length cDNA clone of CyRSV genomic RNA (G11; Dalmay *et al.* 1993) was first digested with *Cla*I, which cuts at a position upstream of the 5′ end of the CyRSV sequence (Burgyan *et al.* 1991), made blunt-ended with Klenow enzyme, and then cut with *Nco*I (position 2,308). The resulting 2,340-nt fragment, bearing the truncated replicase gene (nt 161 to 2,614), was eluted from a low-melting-point agarose gel, and cloned into the phagemid vector pTL7SN (Oh and Carrington 1989). It was then digested with *Eco*RI, made blunt-ended, and digested with *Nco*I. The CyRSV sequence was subjected to site-directed mutagenesis (Kunkel *et al.* 1987) using oligonucleotide 5′-CAA ATA AGC Acc ATG GAG AC-3′ (mismatched bases in lowercase letters; *Nco*I site underlined), homologous to nt 149–168 in the CyRSV RNA sequence that contains the start codon for the 33- and 92-kDa proteins. The mutation was verified by sequencing (Sanger *et al.* 1977).

The 92-kDa protein gene was inserted in two steps into the vector pRTL2, which contains the cauliflower mosaic virus (CaMV) 35S promoter and polyadenylation signal, and the 5′ leader sequence of tobacco etch virus (TEV) (Carrington *et al.* 1990). First, an *Nco*I-*Sac*I fragment of 564 bp was inserted into the vector, digested identically, followed by the addition of a fragment of 1,941 bp obtained by digestion of a full-length CyRSV clone G11 with *Sac*I, and *Ava*I which cuts 52 bp downstream of the stop codon of the 92-kDa protein (Fig. 1). The expression cassette was excised from the recombinant plasmid with *Hin*dIII and inserted into the binary vector pGA482 (An 1986), which was mobilized into *Agrobacterium tumefaciens* LBA4404 by the triparental mating procedure (Ditta *et al.* 1980).

Plant transformation.

N. benthamiana leaf disks were transformed with the CyRSV replicase gene or vector pGA482 alone using procedures described by Horsch *et al.* (1985) and Carrington and Freed (1990). Shoots were regenerated in 3–4 wk on MS medium (Murashige and Skoog 1962) containing kanamycin (0.1 mg/ml), carbenicillin (0.5 mg/ml), benzylaminopurine (1 μg/ml), naphthaleneacetic acid (0.01 μg/ml), and phytagar (Gibco) (0.8%), and then transferred to the same medium without hormones. After 3–4 wk, rooted plantlets were transferred to sterilized soil in Magenta boxes (Sigma, St. Louis, MO), then to pots. Five transformed plants were self-fertilized, and seed (R_1) was collected. Seeds were germinated directly in soil or on MS medium containing kanamycin (0.1 mg/ml), from which only unbleached seedlings were transferred to soil. Seedlings grown directly in soil were screened by ELISA for the presence of neomycin phosphotransferase II (selectable marker for kanamycin resistance) using an NPT II ELISA system following the manufacturer's (5 prime→3 prime Inc., West Chester, PA) instructions.

Growth and inoculation of plants.

Transformed and nontransformed *N. benthamiana* plants were maintained in growth chambers with 14-hr, 21° C light and 10-hr, 16° C dark cycles and 70% relative humidity. Satellite- and DI RNA-free virus was purified from plants inoculated with *in vitro* transcripts from full-length cDNA clone G11 (Dalmay *et al.* 1993) and used to inoculate plants at a concentration of 5 μg/ml in 0.02 M sodium acetate buffer, pH 5.5, containing 1% Celite. Infectious CyRSV RNA was obtained from 2 μg of CyRSV clone G11, linearized with *Sma*I, using T7 RNA polymerase and a T7 transcription kit according to the manufacturer's (Boehringer Mannheim) instructions, or was extracted from purified virus particles of an uncloned virus isolate (Gallitelli *et al.* 1985). For inoculation, RNA was diluted with an equal volume of buffer containing 1% Celite and 1% bentonite (Heaton *et al.* 1989) to a final concentration of approximately 75 μg/ml, and was applied to three leaves (10 μl/leaf) with a sterile glass spatula.

Nucleic acid extraction and analysis.

Total RNA extract preparations were obtained as in Dalmay *et al.* (1993) and fractionated with LiCl (Diaz-Ruiz and Kaper 1978). Material insoluble in 2 M LiCl was collected by centrifugation and resuspended in water. Viral RNA-related sequences were detected by Northern blot hybridization after electrophoresis in formaldehyde-permeated gels (Sambrook *et al.* 1989). DNA was prepared from leaves and PCR performed as previously described (Rubino *et al.* 1992). Two pairs of oligonucleotides were used to prime the synthesis of two overlapping molecules of approximately 1.0 kbp (oligos 1 and 2) and 1.5 kbp (oligos 3 and 4) in template DNA from transgenic plants. Oligos 1 and 3 were complementary to nt 1,129–1,149 and 2,640–2,660, respectively, of the CyRSV sequence; oligos 2 and 4 were homologous to the last 20 nt of the TEV leader and to nt 1,019–1,039 of the CyRSV sequence, respectively. Samples were analyzed by gel electrophoresis and Southern hybridization. Probing of blots was done with ^{32}P-labeled nick-translated full-length clone G11, or clone pCyR-7, which represents the 3′-1.0 kb of CyRSV RNA (Russo *et al.* 1988). Viral minus-strand RNA was detected with a ^{32}P-labeled T7-riboprobe prepared from clone G11 digested with *Sma*I. Filters were exposed to X-ray film for 48–72 hr.

Protoplast preparation and infection.

Protoplasts were prepared from leaf tissue and transfected as previously described (Nagy and Maliga 1976; Dalmay *et al.* 1993). Approximately 0.5×10^6 protoplasts were inoculated with virus particles (10 μg), or *in vitro* transcripts of genomic cDNA (2 μg), or RNA from virus particles (2 μg) and incubated for 24 hr. They were disrupted in glycine buffer containing 2% sodium dodecyl sulfate and 1% sodium lauroylsarcosine (White and Kaper 1989), and extracted sequentially with equal volumes of phenol, phenol/chloroform, and chloroform. Aqueous extracts were precipitated with ethanol and dissolved in 10 μl of sterile water. Northern blot analysis was performed as described above.

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