RNA-Mediated Virus Resistance in Transgenic Plants: Exploitation of a Cellular Pathway Possibly Involved in RNA Degradation

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Transgenic *Nicotiana tabacum* cv. Burley 49 plants were generated that express the 5′ untranslated region of the tobacco etch potyvirus (TEV) genome ligated to a mutated version of the TEV coat protein gene sequence that rendered it untranslatable. Eight different transgenic plant lines were analyzed for transgene expression and for resistance to TEV. Three different responses were noted when the transgenic plant lines were inoculated with TEV: 1) some were highly resistant, and no virus replication occurred; 2) some were susceptible but able to recover from systemic TEV infection; and 3) some were susceptible to TEV infection. Plant tissue displaying the recovery phenotype was analyzed for virus replication and transgene expression. Recovered tissue could not be infected with TEV and had steady-state transgene RNA levels which were five- to eightfold lower than those of unchallenged transgenic plant tissue. Nuclear runoff assays suggested a post-transcriptional reduction in specific RNA levels. The highly resistant and recovery phenotypes associated with TEV challenge inoculation and the reduction of steady-state RNA levels in recovered transgenic leaf tissue may be manifestations of a common mechanism.

Additional keywords: posttranscriptional gene regulation, RNA stability, tobacco etch virus, untranslatable RNA.

Sanford and Johnston (1985) proposed the concept of pathogen-derived resistance (PDR) as a convenient way to create resistance genes. They suggested that pathogen genes, when expressed by a potential host organism, could render that organism resistant to the pathogen. For example, a transgenic plant cell expressing either a functional or dysfunctional plant virus gene product may not support replication of that virus. The host-expressed virus-derived gene or gene product presumably disrupts the pathogen's normal replicative cycle, resulting in an aborted or attenuated infection of that transgenic organism. Herskowitz (1987) expanded on this concept in proposing a *trans* dominant negative mutation scheme to map cellular gene functions. In each model, the expressed exogenous (wild-type or mutant) gene product is predicted to interfere with the normal endogenous or viral gene product. Increasing the expression level of a transgene proposed to interfere with the virus should, up to a point, increase the effectiveness of that transgene gene product in arresting or interfering with virus replication.

There is a plethora of reports dealing with PDR to various plant viruses (see the reviews by Wilson [1993], Scholthof *et al.* [1993], and Fitchen and Beachy [1993]). However, the virus resistance phenotypes reported are often quite varied, and the underlying mechanisms of resistance are not well understood. In general, transgenic plants which express a particular plant virus coat protein (CP) or nonstructural gene can be resistant to that particular virus or a closely related virus. In many examples of PDR, there appears to be a correlation between the expression level of the transgene and the level of resistance. However, in an increasing number of examples, there is no apparent relationship between resistance and the steady-state accumulation of transgene transcript or encoded protein product.

Several examples of PDR to members of the potyvirus group of plant viruses have been reported (see the review by Lindbo *et al.* [1993a]). Potyviruses constitute a large family of aphid-transmitted plant viruses that are members of the picornavirus superfamily (Goldbach 1987). Potyviruses are flexuous rod-shaped viruses with a plus-sense, single-strand RNA genome, ~10,000 nucleotides (nt) in length, organized as a single large open reading frame. Translation of the potyvirus genome could produce a polyprotein of approximately 350 kDa. However, nine individual gene products are released from this polyprotein by the enzymatic activities of three proteinases contained in the polyprotein (for review see Riechman *et al.* [1992]). Tobacco etch virus (TEV) and potato virus Y (PVY) are two members of the potyvirus family.

We have reported that transgenic plants which express an untranslatable form (referred to as RC, for RNA control) of the TEV CP gene possess a high level of resistance (~30% of the lines) or are susceptible (~70% of the lines) to TEV infection (Lindbo and Dougherty 1992a,b). In this report, we describe a line of transgenic plants (referred to as 2RC lines) which express a similar untranslatable form of the TEV CP gene. Resistance in 2RC lines appears to occur at a higher frequency than resistance in RC lines and is displayed either as a highly resistant phenotype or as a "recovery" phenotype after inoculation with TEV. The resistance is virus-specific, complete, and effective in grafted plant studies. We have

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analyzed these 2RC transgenic plants and continue the development of a possible mechanism which may account for the observed resistance.

RESULTS

Putative R1-generation transgenic plants were initially screened for transgene expression (by RNA dot blot analysis) and for kanamycin resistance (data not shown). Transformed R1-generation plants were self-fertilized, and seed was collected. R2-generation plants were grown and screened for the kanamycin resistance phenotype. Plants which displayed 100% kanamycin resistance were selfed and selected for further study. The general growth characteristics of the transgenic 2RC lines were indistinguishable from those of untransformed Burley 49 plants in greenhouse studies.

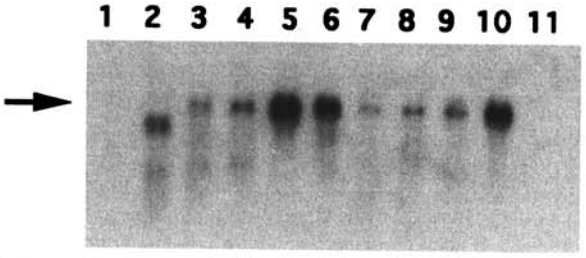

Fig. 1. Northern hybridization analysis of RNA from untransformed and 2RC transgenic tobacco plants. Total RNA was extracted, and 5 µg was denatured and electrophoretically separated in a 1.2% agarose gel containing 6% formaldehyde. The RNA was transferred to nitrocellulose and hybridized with a ^{32}P-labeled RNA probe specific for plus-sense transcript sequences of tobacco etch virus coat protein. After being washed, the filter was exposed to Kodak X-Omat X-ray film. A picture of the autoradiogram is presented. Lane 1, *Nicotiana tabacum* cv. Burley 49; lane 2, RC-7; lane 3, 2RC-1.8; lane 4, 2RC-3.3; lane 5, 2RC-4.4; lane 6, 2RC-5.2; lane 7, 2RC-6.13; lane 8, 2RC-8.10; lane 9, 2RC-8.11; lane 10, 2RC-8.13; lane 11, *N. tabacum* cv. Burley 49. The mobility of a molecular weight marker of ~1,200 nucleotides is indicated by the arrow on the left.

Molecular genetic analysis of 2RC lines.

We first determined if the expected transgene transcript was accumulating in the different 2RC plant lines. Total RNA was extracted and analyzed in northern gel and slot blot hybridization studies. The expected 1,200-nt transgene transcript was readily detected in all 2RC transgenic plants (Fig. 1), and it was apparent that steady-state accumulation of transcript differed among transgenic plant lines (Table 1).

The difference in 2RC transcript accumulation could be due to the number of transgenes being transcribed in each line. Genomic DNA was digested with the restriction enzyme *Xba*I, *Eco*RI, *Hin*dIII, or *Bam*HI; separated by electrophoresis in an agarose gel; and analyzed by southern hybridization analysis. The results from an *Eco*RI digest of genomic DNA isolated from 2RC plants are presented in Figure 2. A determination of the exact copy number was difficult for most lines, and one copy to four copies were estimated to be present. There was no apparent correlation between transgene copy number and the steady-state accumulation of the 2RC transcript.

Response of 2RC lines to virus infection.

Transgenic 2RC plants were screened for virus resistance after mechanical inoculation (Table 1). Most inoculation series examined transgenic plants challenged with either TEV- or PVY-infected plant sap. After inoculation, plants were observed daily for the appearance of virus-induced symptoms. Three different TEV-specific responses were noted: 1) com-

Table 1. Molecular and phenotypic characterization of 2RC transgenic tobacco plants

Plant line[a]	Kanamycin resistance (%)	Transgene expression[b] (pg CP RNA/µg total RNA)	TEV phenotype (%)[c]		
			Susceptible phenotype[d]	Recovery phenotype[e]	Highly resistant phenotype[f]
Burley 49	0	0.8	100	...	...
RC-7	100	2.3	...	...	100
2RC-1.8	100	3.3	...	25	75
2RC-3.3	100	8.5	17	...	83
2RC-4.4	100	3.2	...	97	3
2RC-5.2	100	3.2	...	58	42
2RC-6.13	100	5.8	...	...	100
2RC-8.10	100	2.6	...	100	...
2RC-8.11	100	3.8	9	79	12
2RC-8.13	100	0.0	6[g]	...	94

[a] Plant line nomenclature is as in Figure 4. Burley 49 is the untransformed tobacco tissue, and the RC and 2RC lines are transgenic tobacco plants expressing untranslatable forms of the tobacco etch virus (TEV) coat protein (CP) gene that differ in the 5′ and 3′ untranslated sequences.

[b] Transgene expression level was estimated by slot blot hybridization analysis of total RNA preparations hybridized with TEV CP-specific probes.

[c] The TEV phenotypes are the symptomatic responses the various lines displayed after inoculation with TEV. The results are averages of three different experiments; 10 or 20 plants were inoculated in each experiment.

[d] In the susceptible phenotype, typical TEV-induced symptoms were identical to symptoms on TEV-infected untransformed Burley 49 tissue. Atypical symptoms appeared in line 2RC-8.13 (see note g).

[e] In the recovery phenotype, plants initially displayed typical systemic symptoms associated with TEV infection, but new leaves emerging 2–5 wk after inoculation displayed fewer symptoms. This trend continued until new leaf tissue was completely devoid of symptoms and virus.

[f] In the highly resistant phenotype, generally, no symptoms were observed. In some inoculation studies numerous (>100) small, chlorotic local lesions formed on a few uninoculated leaves. These lesions were approximately 0.5–1.0 mm in diameter, appeared in a temporal fashion for approximately 1 wk, and then were no longer observed. Infectious TEV was not detected in this tissue in back-inoculation of *Nicotiana tabacum* cv. Burley 21 or in enzyme-linked immunosorbent assay.

[g] Atypical symptoms appeared in uninoculated leaf tissue in the form of irregular chlorotic spots, 1–3 cm in diameter. There were one to four spots per leaf, but not all leaves had this symptom.

plete resistance and no detectable TEV, 2) the ability to "recover" from TEV infection, and 3) susceptibility (Table 1). All 2RC lines displayed some resistance to TEV; however, the response to TEV infection was uniform in only two transgenic lines. Line 2RC-6.13 was completely resistant to TEV, and typical TEV systemic symptoms were never observed. Line 2RC-8.10 consistently recovered from TEV infection. In these plants, an apparently normal TEV infection was initially established, and systemic symptoms developed. However, approximately 2–5 wk after inoculation, each emerging apical leaf displayed fewer virus-induced symptoms than the leaf before. In these leaves, virus-infected tissue was distinctly localized in chlorotic interveinal regions. Eventually new leaves emerged devoid of virus-induced symptoms. Infectious particles or virus-encoded proteins could not be detected by back-inoculation or double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (data not shown), suggesting that recovered tissue was virus-free. The recovered 2RC tissue was similar to recovered tissue described for transgenic lines (FL and ΔN-29) that accumulate different forms of the TEV CP (Lindbo *et al.* 1993b).

The remaining transgenic plant lines displayed a mixture of responses in spite of the observation that all displayed kanamycin resistance. Inoculation of lines 2RC-1.8, 2RC-3.3, and 2RC-8.13 with TEV always resulted in a high proportion of plants that were completely resistant to TEV infection. However, in line 2RC-8.13 we consistently observed one or two plants per experiment which displayed pronounced chlorotic blotches on noninoculated leaves. Virus could be readily recovered from this tissue (data not shown). Lines 2RC-4.4, 2RC-5.2, and 2RC-8.11 predominately displayed a recovery phenotype when inoculated with TEV; however, some of the plants were susceptible, while others were completely resistant to TEV infection (Table 1).

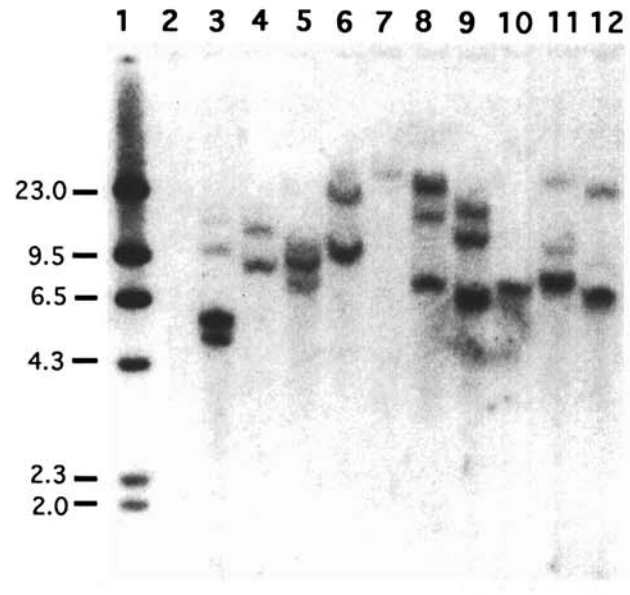

Fig. 2. Southern hybridization analysis of DNA extracted from transgenic and untransformed tobacco plants. Genomic DNA was extracted from leaf tissue and digested with the restriction endonuclease *Eco*RI. Restriction enzyme digest fragments were electrophoretically separated on a 1.0% agarose gel. DNA was transferred to nitrocellulose and hybridized with ^{32}P-labeled RNA specific for tobacco etch virus coat protein sequences. After being washed, the filter was exposed to Kodak X-Omat X-ray film. A picture of an autoradiogram is presented. Lane 1, molecular weight markers; lane 2, *Nicotiana tabacum* cv. Burley 49; lane 3, RC-7; lane 4, RC-9; lane 5, 2RC-1.8; lane 6, 2RC-3.3; lane 7, 2RC-4.4; lane 8, 2RC-5.2; lane 9, 2RC-6.13; lane 10, 2RC-8.10; lane 11, 2RC-8.11; lane 12, 2RC-8.13. Lengths (in kilobase pairs) of lambda DNA markers are indicated on the left.

We have also noted in 2RC-6.13 and in the highly resistant line RC-7 (Lindbo and Dougherty 1992b) the inconsistent formation (in less than 5% of the plants) of numerous faint, small, chlorotic lesions on noninoculated leaves of plants inoculated with TEV. These lesions (0.5–1.0 mm in diameter) could only be observed with backlighting, were often associated with small veins, and were transient, usually lasting about 1 wk. Attempts to detect TEV with DAS-ELISA or to recover infectious TEV in back-inoculation studies from tissue displaying this symptom were unsuccessful. Therefore, we still consider these plants to possess a highly resistant phenotype.

All transgenic plant lines tested displayed wild-type sensitivities to PVY and to cucumber mosaic virus. Typical necrotic local lesions formed when the 2RC lines were inoculated with tobacco mosaic virus (data not shown).

Grafting studies.

Grafting studies addressed three questions: How durable was the resistance? Did virus move through the highly resistant tissue? And could a highly resistant line induce resistance in a susceptible transgenic line through a translocatable signal? Scions and rootstocks of the highly resistant 2RC-6.13 transgenic line were grafted in a variety of combinations to susceptible untransformed Burley 49, RC-1.9, RC-9 (Lindbo and Dougherty 1992b), and plants which showed the recovery phenotype (RC-4.4 and FL-3.3). Inoculation of the susceptible portion of the grafted plant consistently resulted in TEV infection and typical symptom induction, while the 2RC-6.13 tissue remained free of TEV symptoms (Table 2). This result was consistent regardless of the type of graft made. In addition to visual monitoring of the plants, selected tissue was back-inoculated to Burley 21 tobacco plants. Virus could be readily recovered from all tissue displaying virus symptoms, but in only one instance was virus recovered from 2RC-6.13 tissue. (TEV was recovered from 2RC-6.13 tissue in back-inoculation studies from one grafted plant with a 2RC-6.13 scion on a 35S-4.3 rootstock.) TEV-encoded proteins could not be detected by DAS-ELISA of any 2RC-6.13 transgenic tissue used in these grafted plant studies (data not shown). The interstem graft studies demonstrated that TEV could move through 2RC-6.13 tissue; however, this tissue never displayed typical TEV symptoms. Grafting transgenic scions expressing a translatable (FL-3.3) or an untranslatable (RC-4.4) version of the TEV CP RNA, both of which recover from TEV infection, or a scion expressing an untranslatable RNA (RC-1.8) that was susceptible to TEV, onto 2RC-6.13 rootstock failed to convert the susceptible or recovery phenotype to the highly resistant phenotype.

Analysis of transgene RNA levels.

As only an untranslatable RNA is expressed in 2RC plants, transgene steady-state RNA levels were examined in reference to the resistance phenotype. Total RNA was extracted from uninfected leaf tissue of 30-cm-tall transgenic plants

and quantitated in slot blot hybridization studies (Table 1). Plants selected were then inoculated with TEV to confirm they possessed the resistant phenotype predominant for that line. No obvious correlation between 2RC RNA transcript accumulation and resistance to TEV was noted.

Steady-state levels of transgene RNA transcripts were also measured in various recovered transgenic plant leaf tissue of lines 2RC-4.4 and 2RC-8.11, because they usually displayed the recovery phenotype after infection with TEV. Total RNA was extracted from TEV-recovered transgenic plant tissue

Table 2. Summary of grafting experiments

Graft type[a]	Number of Plants[b]	Scion / Rootstock[c]	Result[d]
Cleft 	4	→B49 / B49	+ / +
	4	B49 / →B49	+ / +
	12	→B49 / 2RC-6 .13	+ / −
	3	B49 / →2RC-6.13	− / −
	3	→2RC-6.13 / B49	− / −
	12	2RC-6.13 / →B49	− / +
	3	→35S-4.3 / 2RC-6.13	+ / −
	12	→RC-9 / 2RC-6.13	+ / −
	12	2RC-6.13 / →RC-9	− / +
	12	→RC1.8 / 2RC-6.13	+ / −
	12	2RC-6.13 / →RC1.8	− / +
	3	→FL-3.3 / 2RC-6.13	+ / −
	3	→RC-4.4 / 2RC-6.13	+ / −
Interstem 	1	RC-1.8 / RC-9 / →RC-1.8	+ / + / +
	1	RC1.8 / 2RC-6.13 / →RC-1.8	− / − / − [e]
	1	→RC-9 / 2RC-6.13 / RC-9	+ / − / +
	1	RC-9 / 2RC-6.13 / →RC-9	+ / − / +
Approach 	1	→B49 | B49	+ | +
	4	→B49 | 2RC-6.13	+ | − [3]; − | − [1] [e]

[a] The type of graft made is presented as a schematic drawing. Cleft, interstem, and approach grafts were analyzed.
[b] Number of plants containing 2RC-6.13 tissue examined in seven separate grafting experiments over a 1-yr period.
[c] The scion/rootstock combination is presented. Two scions are grafted to a single rootstock in the interstem graft. The plant nomenclature used is as presented in Figure 4. The arrow indicates the part of the graft that was inoculated with plant sap (in a 1:10 dilution in buffer) from *Nicotiana tabacum* cv. Burley 21 infected with tobacco etch virus (TEV).
[d] The results indicate the part of the grafted plant that became infected with TEV and displayed symptoms. +, TEV present; −, no TEV detected.
[e] A systemic infection of the susceptible tissue was never established. This likely represents a missed inoculation rather than resistant germ plasm.

and from the equivalent leaf of an unchallenged transgenic plant of the same developmental age and analyzed by northern hybridization procedures. Laser densitometer scanning of autoradiograms was used in estimating the differences in steady-state transgene RNA levels. This analysis revealed that steady-state transgene RNA levels were reduced approximately five- to eightfold in TEV-recovered 2RC transgenic plant tissue (Fig. 3A and B).

Nuclear runoff experiments were performed to determine if this reduction in transgene RNA levels in recovered tissue was due to transcriptional down-regulation. Nuclei were isolated from 2RC-4.4 plant tissues which were unchallenged or had recovered from a TEV infection. The results of the analyses, presented in Figure 3C and D, revealed that 2RC transgene transcription rates were similar in nuclei from transgenic plants that were unchallenged or had recovered from TEV infection.

DISCUSSION

Responses of eight different 2RC transgenic tobacco lines to infection by TEV were characterized. Phenotypically, most 2RC transgenic plant lines responded to TEV challenge with an extreme resistance or a recovery phenotype. Highly resistant 2RC lines generally accumulated less transgene transcript than susceptible RC lines or FL or 2RC lines that displayed the recovery phenotype. Transgene transcript accumulation also decreased significantly in leaf tissue displaying the recovery phenotype compared to unchallenged plant tissue of the same line (Fig. 3A and B). However, the reduction in the steady-state level of transgene transcript did not appear to be a transcriptionally controlled event (Fig. 3C and D). A similar observation has been reported with transgenic plants expressing a translatable version of the TEV CP (FL and ΔN-29 lines) and showing the recovery phenotype (Lindbo *et al.*

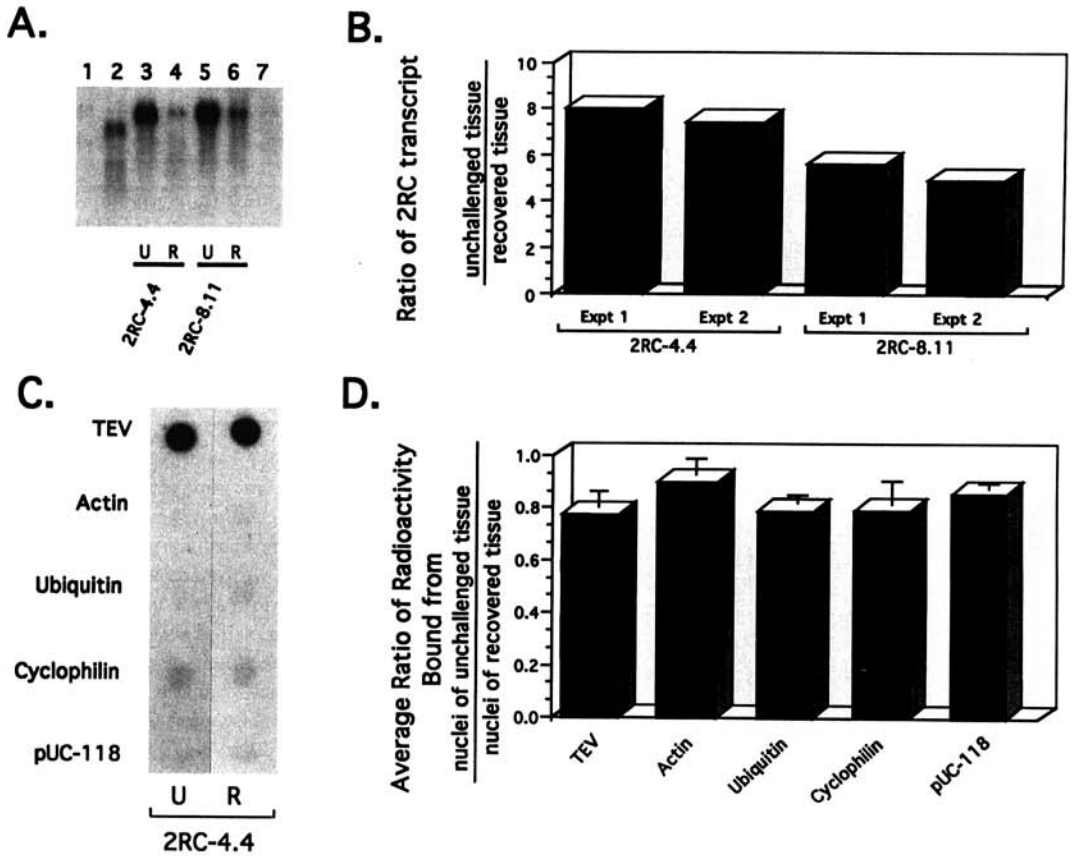

Fig. 3. Analysis of RNA synthesis and accumulation in transgenic tobacco plants displaying a recovery phenotype. **A,** Northern gel hybridization of total RNA extracted from leaf tissue of 2RC-4.4 and 2RC-8.11 transgenic plants. Plants were either unchallenged (U) or inoculated with tobacco etch virus (TEV) and permitted to recover (R). Leaf tissue of the same developmental stage was used as a tissue sample source. Total RNA was separated in agarose gels and transferred to nitrocellulose. A ^{32}P-labeled RNA specific for TEV coat protein sequences was used as a probe. Lanes 1 and 7, total RNA from *Nicotiana tabacum* cv. Burley 49; lane 2, total RNA from RC-7; lane 3, total RNA from an unchallenged 2RC-4.4 plant; lane 4, total RNA from a 2RC-4.4 plant which had recovered from a TEV infection; lane 5, total RNA from an unchallenged 2RC-8.11 plant; lane 6, total RNA from a 2RC-8.11 plant which had recovered from a TEV infection. **B,** The autoradiogram in **A** was scanned with a densitometer, and the ratio of signal of the 2RC transcript from unchallenged plants (i.e., lanes 3 and 5) was compared to the signal of the same germ plasm which had become infected and recovered from infection (lanes 4 and 6). Results are shown for two separate experiments for two lines, 2RC-4.4 and 2RC-8.11. **C,** Results from a nuclear runoff experiment. Nuclei were isolated from 2RC-4.4 transgenic plants which were unchallenged (U) or had recovered (R) from TEV infection. These nuclei were used in cell-free transcription reactions containing ^{32}P-CTP. [^{32}P]-labeled transcripts were hybridized with nitrocellulose filters containing 5 μg of linear plasmid DNA containing the following sequences: a cDNA of the TEV coat protein gene sequence, a cDNA of an *Arabidopsis* actin mRNA, a cDNA of a tomato ubiquitin mRNA, a cDNA of a tomato cyclophilin mRNA, and a plasmid DNA control (pUC-118). The filters were washed and exposed to Kodak X-ray film. **D,** Ratio of radiolabeled transcripts (in counts per minute) from unchallenged or recovered tissue bound to DNA on filters. Radiolabeled transcripts, from nuclei isolated from 2RC-4.4 plants which were unchallenged or which had recovered form TEV, were hybridized with filters containing five different DNA sequences. The experiment was repeated five times, and the average ratio of labeled transcripts from unchallenged plants to those from recovered plants is presented. One standard deviation is indicated by the error bars above the graphs.

1993b). Therefore, low or decreasing steady-state levels of transgene transcript appeared to correlate with resistance to TEV.

We previously speculated that the untranslatable RC RNA may associate with the minus-sense TEV RNA replicative intermediate and arrest replication (Lindbo and Dougherty 1992b). However, the biochemical analysis of transgene expression during recovery and the unlikely prospect that two RNAs, an ~1,200-nt 2RC plus-sense transcript and the 10,000-nt TEV minus-sense genomic RNA, would hybridize with each other would not appear to support such a mechanism. We suggest that the highly resistant and recovery phenotypes of the 2RC lines can be accommodated by the working model we have formulated to explain the recovery phenotype displayed by FL and ΔN-29 transgenic plants (Lindbo *et al.* 1993b). This model proposes that an inducible, cytoplasmic-based, cellular activity degrades specific RNA sequences. In transgenic plants displaying the recovery phenotype, this RNA degradation system is activated only after virus infection and by the additive level of transgene RNA and viral RNA present. In contrast, in highly resistant lines the activity may be fully induced by the transgene transcript. The failure of a rootstock from a highly resistant line to induce a scion from a susceptible line in grafting studies suggests the activity is a programmed cell response not induced by a diffusible signaling molecule, as is the case with systemically acquired resistance (Kuc 1982; Ward *et al.* 1991). Once the antiviral system is activated, it is absolute in its efficacy against TEV, yet it is not effective against the closely related virus PVY (Lindbo *et al.* 1993b and this paper).

Two results distinguished 2RC untranslatable lines from our RC untranslatable transgenic plant series (Lindbo and Dougherty 1992b). First, all of our 2RC lines expressed a resistant phenotype, whereas only 30% of our RC lines displayed virus resistance (Table 2 in Lindbo and Dougherty [1992b]). Second, we noted a number of 2RC lines which displayed 100% kanamycin resistance but varied in their response to TEV. Plants within these lines typically displayed a recovery or highly resistant response to inoculation with TEV.

What could account for the different responses within a line? Inoculation efficiencies among different plants can be ruled out. Plants displaying a highly resistant phenotype were not missed inoculations, as repeated attempts failed to infect them with TEV, though they were readily infected with other plant viruses. A possible explanation may involve the multi-transgene nature of our plants. All 2RC lines (with the exception of 2RC-4.4 and 2RC-8.10) have two or more copies of the TEV transgene, and these lines may be heterozygous for some of the transgenes. The heterozygous state of the transgene may result in sufficient expression of the neomycin phosphotransferase gene for the kanamycin resistance phenotype (i.e., 100% kanamycin resistance), but expression of the 2RC transgene may be insufficient to fully "preactivate" the resistance in lines which show a recovery or susceptible phenotype. Alternatively, the multigenic nature of the transgene may activate a host regulatory mechanism that represses transgene transcription. Depending on the site of transgene integrations, the host will vary in its ability to repress transgene expression, and variable levels of resistance may be observed as a result.

Why did a higher percentage of 2RC lines show a TEV resistance phenotype than the RC lines (Lindbo and Dougherty 1992b), and why did the expression of an untranslatable form of mRNA rather than a translatable version (i.e., FL or ΔN-29 transgenes) (Lindbo *et al.* 1993b) appear to elicit a resistance response more effectively? We suggest that both quantitative and qualitative characteristics of the expressed transcript will be important in optimizing development of resistant plants. mRNAs that are overexpressed may be deleterious to the cell, and a natural pathway may exist in which these RNAs are specifically eliminated. All of our transgene constructs contain the enhanced version of the cauliflower mosaic virus 35S promoter. There are few constitutive plant DNA–dependent RNA polymerase II promoters which direct transcription at this elevated level (Odell *et al.* 1985; Kay *et al.* 1987; Williamson *et al.* 1989). We speculate that Pol II transcription from this promoter sequence produces a level of transcript that saturates the ribosome pool, and RNA not compartmentalized on polysomes is sensed as being at an unacceptable elevated level and targeted for elimination. The system is sequence-specific; and, in targeting this sequence for elimination, it will also eliminate any exogenous viral RNAs containing this sequence. The 2RC transcript may be particularly effective in eliciting a resistance response because, in addition to the quantity of transcript produced, it possesses certain qualities which facilitate activation of the system. First, the RNA is untranslatable. Ribosomes do not translate the open reading frame and are not associated with the RNA for lengthy periods. As a result, the mRNA appears to be aberrant, and thereby more accessible to the cellular surveillance system than translatable mRNAs. Second, the 2RC transcript is a hybrid sequence containing two domains of TEV genomic RNA: the 5′ untranslated leader sequence (143 nt) and the CP sequence (792 nt). Therefore, we may have activated the cellular system to target two TEV genomic sequences. This may explain why 2RC transcripts appear to generate resistance more effectively than RC transcripts (Lindbo and Dougherty 1992b), which only contain the TEV CP gene sequence.

The results of our engineered, untranslatable mRNA-mediated virus resistance are similar to those for other transgenic plants, in particular selected examples of sense- or co-suppression of nuclear genes mediated posttranscriptionally (de Carvalho *et al.* 1992; Smith *et al.* 1990). Hobbs and colleagues (1990, 1993) carefully detailed the expression of an introduced *uidA* transgene and identified transgenic plants that were high or low expressors of β-glucuronidase activity. Genetically crossing a high and a low expressor always resulted in suppression of β-glucuronidase activity and the low-expressor phenotype. Interestingly, *uidA* genes introduced by particle bombardment were also suppressed in a low-expressor background, regardless of the promoter used to regulate the transiently expressed *uidA* gene. A cellular system that regulates mRNA by a posttranscriptional mechanism may explain these results. Recently, the expression of a chimeric transgene in tomato, composed of polygalacturonase and pectin esterase sequences, down-regulated the expression of the two endogenous gene sequences from which the transgene was derived (Seymour *et al.* 1993). This may be another example of how a chimeric gene provides multiple sequence targets for the RNA degradation system. Expressing an un-

translatable RNA which contains multiple (more than two) segments of a viral sequence should be an even more efficient elicitor of the resistance response.

Is the response of our 2RC transgenic plants to the untranslatable RNA and infecting viral RNA unique to plants and RNA plant viruses? A review of the literature suggests it is not. There appears to be a multitude of pathways via which specific RNAs are targeted and eliminated from a cell while others remain stable (see the reviews by Atwater *et al.* [1990] and Peltz *et al.* [1991]). However, the possible redirection of this system to generate virus-resistant cell lines has not been reported in other systems.

We speculate that the cellular activity induced in our transgenic plants represents just one of a number of pathways involved in RNA degradation in eukaryotic cells. Plants may share these pathways and possess unique ones as well. Studies of fungal, nematode, and mammalian cells have examined the instability of untranslatable RNA. Stop codons introduced into the coding region of triosephosphate isomerase and β-globin (Daar and Maquat 1988; Lim *et al.* 1993) or Rous sarcoma virus *gag* (Barker and Beemon 1991) mRNA sequences in various mammalian cell lines decreased the stability of these specific RNA species. In *Saccharomyces cerevisiae* and the nematode *Caenorhabditis elegans,* the rapid degradation of untranslatable mRNAs has been quantified, and genetic analysis of the system has begun. The translatability of the RNA appears to be a key trigger to the system, as stability of RNA can be reinstated if the gene coding for the RNA containing the premature stop codon is introduced into a yeast strain providing a suppressor tRNA. Additionally, *cis*-acting sequences in the untranslatable RNA have been identified which are important in RNA stability and instability. The yeast *UPF-1* and *UPF-3* genes have been implicated in the control of the phenotype, and both appear to be dispensable for growth. It has been proposed that the products of these yeast genes are normally involved in the elimination of unspliced or improperly spliced mRNA species (Leeds *et al.* 1991, 1992; Peltz *et al.* 1993).

In the *C. elegans* system, six genes, termed *smg-1* through *smg-6,* have been associated with the instability of mRNAs containing premature stop codons. Deletion of any one of the six genes eliminates the RNA degradation system. Pulak and Anderson (1993) suggested that truncated gene products derived from mRNAs containing premature stop codons may be lethal to the organism and that an "mRNA surveillance system" exists to eliminate transcripts which code for these truncated proteins.

In summary, we generated transgenic plants with an extreme resistance to TEV. We do not subscribe to the hypothesis that the transgene product interacts directly with the virus. Instead, the resistance is more likely mediated by a cellular pathway involved in the targeted elimination of unspliced or aberrant mRNAs. Transgenic plants displaying the recovery phenotype likely manifest the resistance using a similar mechanism. Such a system appears to be common in most eukaryotic cells, and exploitation of this cellular process may be an effective way to program cells to eliminate or diminish the level of particular RNAs in the cell. These RNAs may be exogenous or viral in nature; and, as we have demonstrated, exploitation of this system may be an effective way to generate virus-resistant plants. Alternatively, endogenous or cellular RNAs may also be targeted, and activation of this RNA degradation system may be an obvious and effective way to generate plants defective in a particular phenotype.

MATERIALS AND METHODS

Construction/generation of transgenic plants.

Construction of the untranslatable (2RC) TEV CP gene sequence was previously described (Lindbo and Dougherty 1992a, b). TEV CP gene constructs were inserted between the TEV 5′ genomic untranslated sequence (UTS) and the 3′ tumor-morphology-large UTS. Pertinent features of the transgene construct, its distinction from the RC and FL transgenes, and transgenic plant nomenclature are presented in Figure 4. Transgenic *Nicotiana tabacum* cv. Burley 49 plants were generated by *Agrobacterium tumefaciens*–mediated leaf disk transformation procedures (Lindbo and Dougherty 1992a, b).

Kanamycin resistance analysis.

To screen transgenic seeds for kanamycin resistance, seeds were germinated on agar plates containing kanamycin sulfate (Lindbo and Dougherty 1992b). Generally 100–200 seeds per transgenic seed sample were analyzed.

Analysis of RNA in transgenic plants.

Total RNA was isolated from transgenic plants by LiCl precipitation (Verwoerd *et al.* 1989). Denaturing RNA gels and northern gel blotting were described by Lindbo and Dougherty (1992a, b). Northern blots were hybridized with strand-specific [^{32}P]-labeled RNA probes generated from SP6/T7-based cell-free transcription reactions of a plasmid containing a cDNA copy of the TEV CP gene (Lindbo and Dougherty 1992a, b). The amount of radioactivity in the 1,200-nt RNA band was estimated by densitometric analysis of exposed X-ray films with a Zeineh soft laser scanning densitometer (model SL-DNA, Biomed Instruments Inc., Fullerton, CA).

Analysis of genomic DNA in transgenic plants.

Plant genomic DNA was extracted as described by Rogers and Bendich (1988). Genomic DNA was digested with a restriction enzyme that cut the DNA at a single site within the transferred DNA. Southern blotting procedures were performed as described by Sambrook *et al.* (1989). Southern blots were probed with α-^{32}P dCTP-labeled TEV CP DNA fragments. CP DNA probes were synthesized by the random prime method of Feinberg and Vogelstein (1984); a random prime extension labeling kit (DuPont) was used.

Plant inoculation experiments.

Plants were mechanically inoculated with a 1:10 dilution of virus-infected plant sap (Lindbo and Dougherty 1992a). Typically, the plants were observed daily for 45 days.

ELISA.

DAS-ELISA (Converse and Martin 1990) was used to detect TEV CP in plant extracts.

Plant grafting experiments.

Most grafting experiments used a cleft graft. Rootstocks were prepared by removing the shoot of the rootstock above

at least two healthy basal leaves. When recovered plant tissue was used as a rootstock, the shoot was removed above at least two asymptomatic (fully recovered) leaves. A vertical cut, 3–4 cm long, was then made in the center of the stem. Scions were prepared by removing leaves larger than 4 cm in length and trimming the base of the scion to a wedge. The cambia of stock and scion were aligned along the lengths of the cuts, secured with paraffin film (Parafilm), and covered with a polyethylene bag for 7 days. Rootstocks or scions were typically inoculated 10–14 days later. In total, 152 grafted plants containing the highly resistant 2RC-6.13 transgenic plant tissue were examined in seven different studies during 1992 and 1993.

Nuclear runoff assays.

Isolation of nuclei from transgenic plant tissue and *in vitro* labeling of runoff transcripts were as described by Cox and Goldberg (1988) except DEPC was omitted from the extraction buffer. Transcripts from nuclei from recovered or unchallenged transgenic plant tissue were labeled with α-^{32}P CTP (3,000 μCi/mmol). Labeled transcripts were isolated by the following modification of the protocol of Cox and Goldberg (1988): after DNase and proteinase K treatment of the *in vitro* labeling reaction, the reaction mix was extracted with phenol/chloroform (1:1). Transcripts were precipitated twice with 0.4 volumes of 5 M ammonium acetate and 2.5 volumes of ethanol. The final pellet was resuspended in 200–300 μl of double-distilled H_2O. The amount of ^{32}P-CTP incorporated per labeling reaction was estimated by precipitation of labeled RNA onto DE81 filters (Sambrook *et al.* 1989) and counting the sample in a liquid scintillation counter.

Labeled runoff transcripts were hybridized to specific DNA sequences bound to nitrocellulose filters. Duplicate nitrocellulose filter dot blots were prepared, and 5-μg samples of linearized plasmid DNAs were spotted onto them. Plasmid DNAs spotted contained sequences corresponding to 1) a cDNA copy of the TEV CP gene (pTRC-RC1), 2) an actin gene from *Arabidopsis* (pACT-4), 3) a ubiquitin gene from tomato, and 4) a cyclophilin gene from tomato. The ubiquitin and cyclophilin sequences were inserted into pUC-118 as 250- and 480-bp products, respectively, generated by polymerase chain reaction. Additionally, pUC-118 plasmid DNA was spotted. Following prehybridization (Sambrook *et al.* 1989), one filter was hybridized with labeled runoff transcripts from nuclei from unchallenged tissue, and the other filter with labeled transcripts from nuclei of recovered transgenic plant tissue. In five separate experiments, 1×10^6 to 1×10^7 cpm/ml of hybridization solution were used on each filter. Blots were hybridized overnight at 45–55° C, then washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 5 min, and washed two times in 0.2× SSC at 45–50° C for 60 min. Washed nitrocellulose filters were air-dried and exposed to Kodak X-Omat X-ray film with an intensifying screen. After autoradiography, the nitrocellulose dot blots were excised with a cork borer, and the radiolabeled RNAs bound to the individual

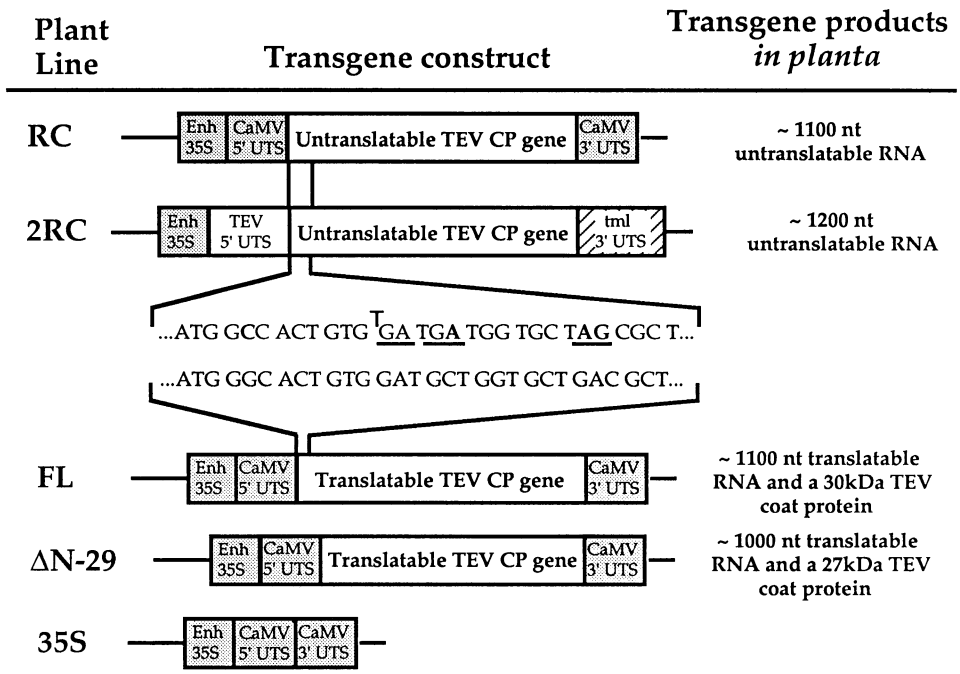

Fig. 4. Schematic drawing of transgene constructs expressing tobacco etch virus (TEV) coat protein (CP) sequences. The 2RC gene and four other transgenes discussed in the text are shown. The 2RC and RC lines (RC = RNA control) express a TEV CP gene sequence that has been rendered untranslatable by the introduction of three stop codons (underlined), four codons downstream of the ATG initiation codon. The nucleotide sequence comparison of the untranslatable and translatable version of the TEV CP transgene is presented. The nucleotides in bold represent site-directed mutations made to generate the stop codons. The next in-frame initiation codon is located 47 codons downstream. The transgenes contained in plants expressing the full length (FL) version of the TEV CP as well as an amino-terminally truncated version (ΔN-29) are also presented. The stippled boxes reveal nucleotide sequences derived from cauliflower mosaic virus (CaMV) DNA and include an enhanced 35S promoter (Enh 35S) sequence and 5′ and 3′ untranslated sequences (UTSs) derived from the 35S mRNA. The unshaded boxes represent TEV sequences derived from the 5′ genomic untranslated sequence (5′ UTS) or the CP open reading frame. The diagonally ruled box in 2RC constructs represents sequences derived from the 3′ UTS of the tumor-morphology-large (tml) gene of *Agrobacterium tumefaciens*.

DNA samples were quantitated in a liquid scintillation counter.

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