

Strong host resistance targeted against a viral suppressor of the plant gene silencing defence mechanism

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The 2b protein encoded by cucumber mosaic cucumovirus (Cmv2b) acts as an important virulence determinant by suppressing post-transcriptional gene silencing (PTGS), a natural plant defence mechanism against viruses. We report here that the tomato aspermy cucumovirus 2b protein (Tav2b), when expressed from the unrelated tobacco mosaic tobamovirus (TMV) RNA genome, activates strong host resistance responses to TMV in tobacco which are typical of the gene-for-gene disease resistance mechanism. Domain swapping between Cmv2b, which does not elicit these responses, and Tav2b, revealed functional domains in Tav2b critical for triggering virus resistance and hypersensitive cell death. Furthermore, substitution of two amino acids from Tav2b by those found at the same positions in Cmv2b, Lys21→Val and Arg28→Ser, abolished the ability to induce hypersensitive cell death and virus resistance. However, in *Nicotiana benthamiana*, a species related to tobacco, Tav2b functions as a virulence determinant and suppresses PTGS. Thus, a viral suppressor of the host gene silencing defence mechanism is the target of another independent host resistance mechanism. Our results provide new insights into the complex molecular strategies employed by viruses and their hosts for defence, counter-defence and counter counter-defence.
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Introduction

Gene-for-gene disease resistance is an important plant defence mechanism against pathogens. It is induced when the host plant carrying a resistance (*R*) gene is challenged by a pathogen carrying a matching avirulence (*Avr*) gene (Keen, 1990). The specific interaction between a matching pair of *R*–*Avr* genes usually induces the hypersensitive response (HR) and results in the local containment of the invading pathogen at sites of attempted ingress. Features of an HR include the formation of necrotic lesions, an oxidative burst, alterations in cell wall structure, increases in endogenous salicylic acid levels and activation

of a complex array of defence-related genes, including genes encoding the pathogenesis-related (PR) proteins (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997; Yang *et al.*, 1997). In addition to these local responses, the uninfected portions of the plant also develop systemic acquired resistance (SAR), which is manifested by enhanced resistance to a subsequent challenge by the initial, or even unrelated, pathogens (Ryals *et al.*, 1996; Sticher *et al.*, 1997). More than a dozen *R* genes specific for bacteria, viruses, fungi or nematodes have been cloned from a variety of plant species, and it is striking to note that these *R* genes often encode structurally similar proteins, suggesting a high degree of mechanistic conservation among the pathways that plants use to trigger defence responses (Baker *et al.*, 1997; Hammond-Kosack and Jones, 1997).

The *Avr* genes of pathogens by definition encode, or result in the production of, signal molecules that can initiate an HR in the corresponding resistant plants. *Avr4* and *Avr9* from the extracellular growing fungal pathogen *Cladosporium fulvum* encode pre-proproteins that are processed into small secreted peptides that can elicit *R* gene-dependent defence responses even in the absence of pathogens (Knogge, 1996). Similarly, the bacterial *AvrD* locus of *Pseudomonas syringae* pv. *glycinea* encodes enzymes involved in the synthesis of exported syringolide elicitors, which when injected into the intercellular leaf spaces can elicit an HR in soybean cultivars that carry the *Rpg4* gene (Leach and White, 1996). However, for most of the bacterial *Avr* genes cloned, the *Avr* protein itself is the elicitor (Leach and White, 1996). These *Avr* genes generally encode hydrophilic proteins that lack signal sequences and do not induce an HR when injected into the leaves of plants with the matching *R* genes, unlike *Avr4*, *Avr9* or *AvrD* (described above). It has recently been demonstrated that recognition of several bacterial *Avr* gene products by their matching *R* gene products occurs inside plant cells (Gopalan *et al.*, 1996; Leister *et al.*, 1996; Scofield *et al.*, 1996; Tang *et al.*, 1996; Van den Ackerveken *et al.*, 1996). The import of the *Avr* proteins into plant cells may be through the type III secretory system encoded by the bacterial *Hrp* gene cluster required for HR induction and pathogenesis (Pirhonen *et al.*, 1996).

Plant viruses enter cells through existing wounds and replicate intracellularly. Therefore, it is likely that viral *Avr*–*R* recognition also occurs inside plant cells. Most plant viruses have small genomes that encode genes required for replication, movement and encapsidation. All of these three major types of viral genes, encoding the coat protein (Bendahmane *et al.*, 1995; Berzal-Herranz *et al.*, 1995; Taraporewala and Culver, 1996), RNA replicase (Meshi *et al.*, 1988; Kim and Palukaitis, 1997; Padgett *et al.*, 1997) or movement protein (Meshi *et al.*,

1989; Weber *et al.*, 1993) have been demonstrated to be avirulence determinants. The 126 kDa replicase protein (Padgett *et al.*, 1997; Abbink *et al.*, 1998) of tobacco mosaic virus (TMV) is the only viral Avr protein for which the cloning of a matching *R* gene, *N*, has been reported (Whitham *et al.*, 1994). The *N* gene product, predicted to be a cytoplasmic protein, belongs to the NBS-LRR family of resistance genes (Baker *et al.*, 1997).

It was reported recently that systemic leaves of nepovirus- and caulimovirus-infected plants exhibit a strong virus resistance, similar to post-transcriptional gene silencing (PTGS), thus providing evidence for a second type of natural defence mechanism against viruses in plants (Covey *et al.*, 1997; Ratcliff *et al.*, 1997). PTGS was first discovered in transgenic plants and involves the degradation of RNA species that are similar to the transcribed part of a silencer transgene. As a result, only a low level of the transgene RNA exists, even if it is transcribed at a high level (Depicker and Van Montagu, 1997). PTGS confers virus resistance in transgenic plants if the silencer transgene is derived from, or shares sequence homology with, the genome of a plant virus (Lindbo *et al.*, 1993; Guo and Garcia, 1997; Ruiz *et al.*, 1998).

In support of PTGS as a natural plant defence against viruses, two proteins encoded by plant RNA viruses were identified as suppressors of PTGS in transgenic plants (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998). The HC-Pro encoded by potato virus Y blocks the maintenance of PTGS in tissues where silencing has already been established, whereas the 2b protein encoded by cucumber mosaic virus (Cmv2b) prevents the initiation of gene silencing at the growing points of the plants (Brigneti *et al.*, 1998). Both viral proteins have been shown to be important for virulence determination and systemic spread (Cronin *et al.*, 1995; Ding *et al.*, 1995). Thus, plant viruses possessing a PTGS suppressor function provide an active and effective strategy to combat innate host resistance mechanisms. This strategy is distinct from the one employed by many plant pathogens to overcome the gene-for-gene disease resistance mechanism, which is to accumulate mutations in *Avr* genes that can escape recognition by corresponding host *R* genes.

Here we report that a plant RNA virus-encoded suppressor of PTGS, Tav2b, is targeted as an *Avr*-like factor by a strong host resistance mechanism resembling the gene-for-gene disease resistance mechanism. Tav2b is encoded by tomato aspermy cucumovirus (TAV; Ding *et al.*, 1994; Shi *et al.*, 1997) and is a homologue of Cmv2b. Molecular dissection of Tav2b revealed functional domains that were critical for triggering virus resistance and hypersensitive cell death. The possibility that disarmed *Avr* genes, i.e. those capable of resistance activation without triggering cell death, represent a novel class of disease resistance genes is discussed.

Results

Induction of hypersensitive cell death, PR gene expression and virus resistance by Tav2b

Nicotiana tabacum cv. Samsun (nn) is susceptible to TAV, and TAV-infected plants show visible systemic symptoms of mild chlorotic mottle and ringspots (Ding *et al.*, 1996).

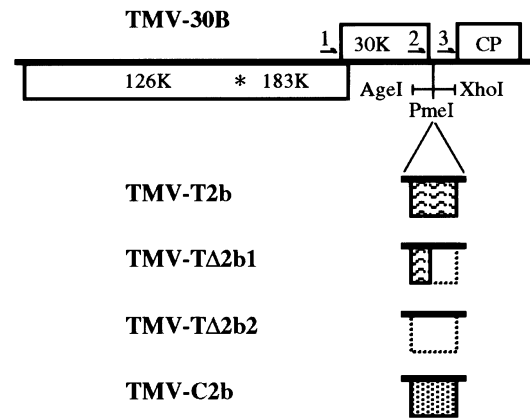


Fig. 1. Genomic organization of the TMV-30B vector and the inserted fragments from either TAV or CMV. Boxes represent ORFs encoded by TMV, TAV or CMV. The asterisk denotes the amber stop codon which can be read through to give the 183 kDa protein. The three sgRNA promoters are represented by arrows. Note that this vector is derived from the infectious cDNA clone of TMV-U1 except for the 3'-terminal sequence, including the coat protein (CP)-coding sequence plus its sgRNA promoter (arrow 3), which is from TMV-U5 (W.O.Dawson, personal communication). The 2b-coding sequences (or their mutants) from TAV (T2b) or CMV (C2b) were cloned at the *PmeI* site. TΔ2b1 and TΔ2b2 are derivatives of T2b with point substitutions that result in partial or complete disruption of the encoded ORF.

As this cultivar does not contain the *N* gene, it is also susceptible to infection by many TMV strains including U1 and U5 (Mathews, 1991). Systemically infected plants show characteristic leaf mosaic symptoms. The vector pTMV-30B (Figure 1) is similar to the TMV-based *in planta* expression vectors previously described (Donson *et al.*, 1991; Kumagai *et al.*, 1995), except that (i) expression of the inserted foreign gene is driven by the U1 coat protein (CP) subgenomic RNA (sgRNA) promoter (Figure 1, arrow 2) and (ii) the CP gene and its sgRNA promoter (Figure 1, arrow 3) were obtained from the U5 strain (W.O.Dawson, unpublished data). The recombinant viral RNA transcribed from pTMV-30B was named TMV-30B. This nomenclature system is used throughout the text. TMV-30B-infected Samsun plants displayed a milder systemic mosaic than did Samsun plants infected with the U1 strain of TMV (data not shown).

The coding sequences for Tav2b and Cmv2b were cloned downstream of the U1 sgRNA promoter of pTMV-30B to give pTMV-T2b and pTMV-C2b (Figure 1), respectively. Local necrotic lesions (Figure 2A, left), a morphological marker of an HR, appeared on the tobacco leaf inoculated with TMV-T2b ~3 days post-inoculation (d.p.i.) whereas the rest of the plant remained symptomless (Figure 2B, left) for as long as observations were made (5 weeks). In contrast, TMV-C2b did not induce necrotic lesions on the inoculated leaf (Figure 2A, right), and produced mild mosaic symptoms on the upper uninoculated systemic leaf (Figure 2B, right). These latter symptoms were similar to those caused by infection with TMV-30B (data not shown).

To determine the distribution pattern of TMV-T2b and TMV-C2b in the inoculated tobacco plants, total RNAs were extracted from the inoculated and systemic leaves and analysed by Northern blot hybridization using a probe specific to the genomic RNA of TMV (Figure 3A, right).

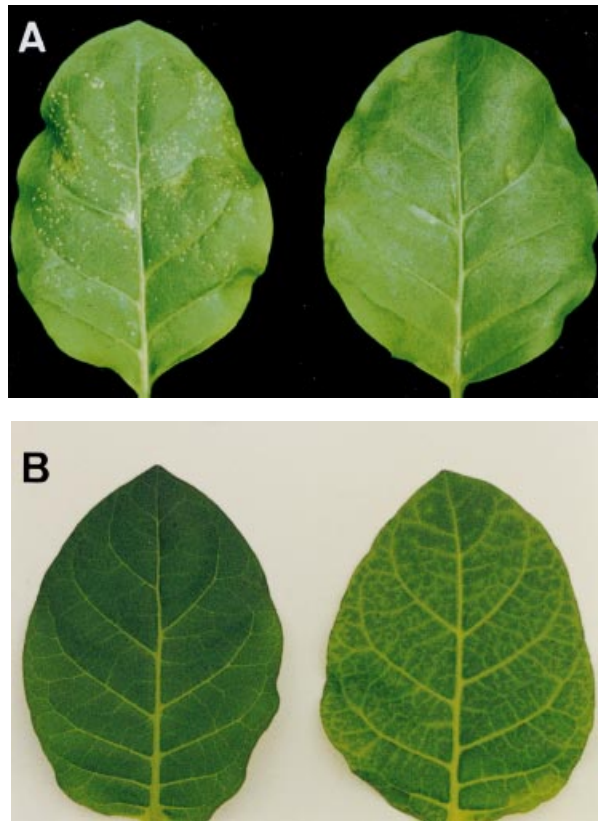


Fig. 2. The responses of the Samsun (nn) cultivar to infection by TMV-T2b or TMV-C2b. (A) The local hypersensitive response to TMV-T2b but not to TMV-C2b. (B) The systemic susceptibility to TMV-C2b but not to TMV-T2b. The tobacco plants were inoculated with either TMV-T2b (left leaf) or TMV-C2b (right leaf) and the inoculated (A) and upper uninoculated (B) leaves were photographed 7 days after inoculation.

Similar high levels of accumulation of genomic RNAs were detected in both the inoculated leaves (top panel) and the systemic leaves (middle panel) of the plants that were inoculated with either TMV-30B (lane TMV) or TMV-C2b (lane C2b). In comparison, a much lower level of TMV-T2b accumulated in the inoculated leaves (lane T2b, top panel) and no accumulation was detected in the systemic leaves (lane T2b, middle panel). The presence of the coding sequence for *Tav2b* or *Cmv2b* in the progeny viral RNAs extracted from the inoculated leaves was confirmed using sequence-specific probes (data not shown) and by sequencing the cDNA fragments obtained from RT-PCR. In addition, the genomic RNAs of TMV-C2b and TMV-T2b migrated more slowly than the corresponding genomic RNA of TMV-30B during denaturing agarose gel electrophoresis (Figure 3A, compare the TMV lane with the four lanes next to it). Thus, the expression of *Tav2b* induces hypersensitive cell death and strong virus resistance in the Samsun tobacco cultivar. In contrast, *Cmv2b* is inactive in this respect.

Does the challenge inoculation of TMV-T2b also lead to transcriptional induction of genes encoding the PR proteins—molecular markers associated with disease resistance responses? To test this, total RNAs extracted from the tobacco leaves, at different time points following TMV-T2b inoculation, were subjected to Northern blot analysis using probes specific for PR-1a mRNA or PR-5

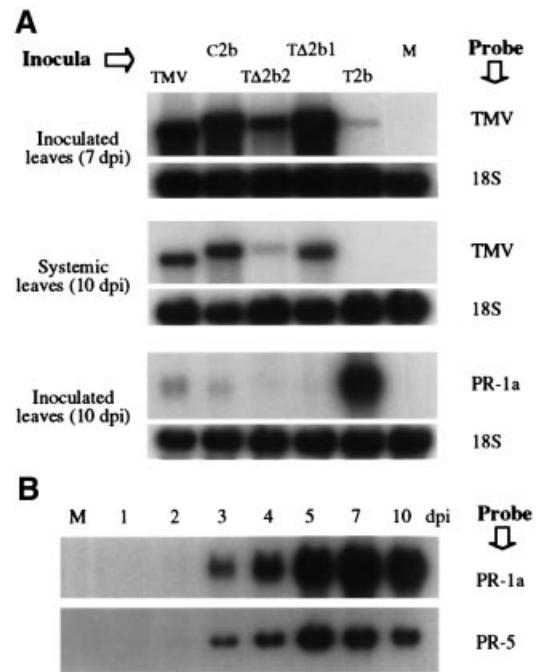


Fig. 3. The accumulation of viral and plant RNAs in tobacco plants. (A) The Samsun plants were inoculated with TMV-30B (TMV), TMV-C2b (C2b), TMV-T Δ 2b2 (T Δ 2b2), TMV-T Δ 2b1 (T Δ 2b1), TMV-T2b (T2b) or mock-inoculated with buffer alone (M). At different d.p.i. as indicated, total RNAs were extracted from either the inoculated leaves or systemic leaves and subjected to Northern blot analysis using 32 P-labelled DNA probes specific for TMV genomic RNA (TMV), PR-1a mRNA or the 18S RNA (as a loading control). (B) Time course of the accumulation of mRNA encoded by PR-1a and PR-5 in the Samsun plants inoculated with TMV-T2b. The time (d.p.i.) when total RNAs were extracted after inoculation is indicated above each lane.

mRNA. As shown in Figure 3B, the synthesis of both PR-1a (top panel) and PR-5 (lower panel) mRNAs was detectable at 3 d.p.i. and reached maximums of steady-state RNA at 5 (PR-1a) and 7 (PR-5) d.p.i. Thus, mRNA induction coincided with the appearance of macroscopic necrotic lesions. In contrast, the transcription of PR-1a mRNA (Figure 3A, lower panel) and PR-5 mRNA (data not shown) was not significantly induced in plants infected by TMV-30B (Figure 3A, lane TMV) or TMV-C2b (Figure 3A, lane C2b) even at 10 d.p.i. (or at any other earlier time points; data not shown). Thus, the challenge inoculation by TMV-T2b also led to the transcriptional induction of PR-1a and PR-5 genes. As summarized earlier (see Introduction), hypersensitive cell death, PR gene expression and strong virus resistance induced by *Tav2b* in the Samsun cultivar are the typical host responses associated with the gene-for-gene disease resistance mechanism. Thus, our results indicate that *Tav2b*, when expressed from the TMV genome, functions as an *Avr* gene.

The full-length translatable *Tav2b* ORF is required for the *Avr* activity

Point mutations were introduced into the coding sequence of *Tav2b* in the plasmid pTMV-T2b to give pTMV-T Δ 2b1 and pTMV-T Δ 2b2 (Figure 1). The recombinant virus TMV-T Δ 2b2 was not expected to yield any *Tav2b* product in infected plants because the second codon (GCA) was converted to a stop codon (TAA). In pTMV-T Δ 2b1, codons









44 (CAA) and 46 (CGA) of *Tav2b* were changed to stop codons (TAA and TGA). Neither TMV-TΔ2b1 nor TMV-TΔ2b2 induced the formation of necrotic lesions in the inoculated leaves, and the inoculated plants developed systemic mosaic symptoms (data not shown) similar to those caused by TMV-C2b (Figure 2A, right). Both TMV-TΔ2b1 and TMV-TΔ2b2 accumulated to detectable levels in the inoculated and systemic leaves, as revealed by Northern blot hybridization (Figure 3A, top and middle panels, lanes TΔ2b1 and TΔ2b2). Progeny analysis by RT-PCR and sequencing showed that the mutations introduced in both TMV-TΔ2b1 and TMV-TΔ2b2 were stably maintained and no second site mutations were detected in the inserted TAV sequence. Furthermore, unlike TMV-T2b, infection by TMV-TΔ2b1 or TMV-TΔ2b2 did not lead to the transcriptional induction of PR-1a (Figure 3A, bottom panel, lanes TΔ2b1 and TΔ2b2) or PR-5 (data not shown) genes. Thus, the induction of hypersensitive cell death, PR gene expression and strong virus resistance in the Samsun cultivar by TMV-T2b correlates with the ability of TMV-T2b to encode the full-length translatable *Tav2b* open reading frame (ORF). This suggests that the encoded protein, *Tav2b*, is the active molecule and that the observed *Avr* activity is not related to the inserted TAV RNA sequence. This suggestion is also supported by the loss of *Avr* activity observed for a mutant form of *Tav2b*, T2bC (see below), which contained nucleotide substitutions introduced at positions different from those that occurred in TΔ2b1 and TΔ2b2. Furthermore, as TMV-TΔ2b1 infected the Samsun cultivar systemically, it suggests that the truncated *Tav2b* of 43 amino acids, predicted to be expressed *in vivo*, is insufficient to elicit the resistance responses.

Functional domains of the *Tav2b* protein

The above experiments indicated that *Tav2b*, when expressed *in cis* from the TMV vector, induced hypersensitive cell death and strong virus resistance in the Samsun (nn) tobacco cultivar. However, *Cmv2b* did not elicit these responses when similarly expressed from the TMV genome. *Cmv2b* and *Tav2b* are encoded by two different viral species from the same *Cucumovirus* genus (Ding *et al.*, 1994; Shi *et al.*, 1997). The predicted amino acid sequences of the two proteins are 24% identical (46.2% similarity), making them the least conserved pair among the known cucumoviral 2b proteins (Ding *et al.*, 1994). As *Tav2b* is only 95 amino acids long, results from deletion mutants, such as TMV-TΔ2b1, may not be informative for mapping its functional domains. Thus, *Tav2b*, as encoded by pTMV-T2b, was replaced progressively from either the N-terminus (pTMV-CT1, 2 and 3) or the C-terminus (pTMV-TC1, 2 and 3) with the equivalent regions of *Cmv2b*, according to the alignment of the known cucumoviral 2b proteins (Ding *et al.*, 1994). The structures of the resultant 2b chimeras are given in Table I. Regions (shown as numbers of amino acids) derived from *Tav2b* are depicted as open sections and those from *Cmv2b* as filled sections.

The results of the infectivity experiments in *N.tabacum* using the recombinant TMV transcripts from the six chimeric 2b constructs are shown in Figure 4 and summarized in Table I. CT1, CT2 and CT3 were derived by replacement of the N-terminal 91, 69 or 50 amino

Table I. Structures of the 2b chimeras and plant response

TMV-	Length (aa)	Structure (amino-carboxyl)	Plant response ^a
T2b	95		HR
TC1	102		HR
TC2	103		R
TC3	100		S
CT3	95		S
CT2	92		S
CT1	93		S
C2b	100		S

^aHR, necrotic lesions and virus multiplication in the inoculated leaves of *N.tabacum* cv. Samsun nn only; R, virus multiplication in the inoculated leaves only but with no visible necrotic lesions; S, susceptibility to systemic infection.

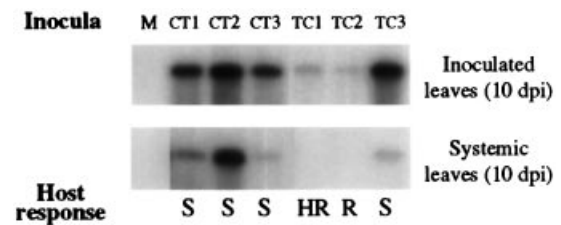


Fig. 4. Accumulation of the six TMV recombinants in the inoculated and systemic leaves of Samsun tobacco plants. A probe specific for the genomic RNA of TMV was used for the hybridization. The plant responses (as abbreviated in the footnote to Table I) to each of the recombinants are also indicated. For all six recombinants, the 2b-coding sequence in the progeny virus was obtained by RT-PCR followed by DNA sequencing and found to be identical to the respective inoculum. M, mock-inoculated; CT1, TMV-CT1; CT2, TMV-CT2; CT3, TMV-CT3; TC1, TMV-TC1; TC2, TMV-TC2; TC3, TMV-TC3.

acids of *Tav2b* with the equivalent regions of *Cmv2b* (Table I). The tobacco plants were susceptible to all of the three recombinant viruses (TMV-CT1, 2 and 3). None of these recombinants induced necrotic lesions (data not shown) and all accumulated in both the inoculated and systemic leaves (Figure 4, lanes CT1, CT2 and CT3). The infected plants also displayed systemic mild mosaic symptoms similar to those caused by TMV-C2b (Figure 2B, right). Thus, the absence of the *Tav2b* N-terminal 50 amino acids in a 2b chimera resulted in a loss of the avirulence function, indicating that this region is essential for the induction of virus resistance and hypersensitive cell death in this host.

Consistent with the above observation, the *Tav2b* N-terminal sequence of 69 amino acids was sufficient to confer virus resistance in cv. Samsun in the absence of its C-terminal 26 amino acids as shown by the data obtained from three TMV-TC recombinants. TC1, TC2 and TC3 were derived by replacing the C-terminal four, 26 or 45 amino acids of *Tav2b* with the equivalent regions

from Cmv2b (Table I). TMV-TC1 and TMV-TC2 induced strong virus resistance, as both accumulated to low levels in the inoculated leaves (Figure 4, lanes TC1 and TC2 of the upper panel), but were undetectable in the systemic leaves (lanes TC1 and TC2 of the lower panel). The challenged plants remained symptomless for as long as observations were made (5 weeks). Thus, the C-terminal 26 amino acids of Tav2b are not essential for activating virus resistance. In contrast, TC3, unlike TC1 or TC2, did not induce viral resistance because TMV-TC3 infected the tobacco plants systemically (Figure 4, bottom panel), as found for TMV-CT3 (see above). TC1 and TC2 are avirulent, but TC3 is not (Table I), suggesting that amino acids 50–69 of Tav2b play a key role in resistance activation. However, CT3 contained the same 20 amino acids from Tav2b as TC1 and TC2, but it failed to induce virus resistance (Figure 4). This indicates that this region of Tav2b is insufficient to activate virus resistance, unlike the more extended N-terminal region of 69 amino acids.

Although the Samsun cultivar is resistant to both TMV-TC1 and TMV-TC2, TMV-TC1 induced necrotic lesions in the inoculated leaves identical to those triggered by TMV-T2b. Under the same conditions, TMV-TC2 caused no visible hypersensitive cell death (Table I). This result, a local and symptomless infection by TMV-TC2, was consistent and reproducible in six independent experiments, involving a total of 37 plants. This indicates that the sequence encompassing amino acids 70–91 of Tav2b is required for triggering visible hypersensitive cell death. However, TMV-CT2 and TMV-CT3, both of which encode the same 22 amino acids from Tav2b, did not cause necrotic lesions (Table I), suggesting that this sequence in the absence of the N-terminal 69 amino acids of Tav2b is insufficient to trigger hypersensitive cell death.

Two amino acid substitutions abolished the avirulence activity of the Tav2b protein

To analyse further the avirulence determinant, Lys21 and Arg28, both positively charged and located within the N-terminal region identified above, which was shown to be critical for the avirulence function of Tav2b, were replaced with Val21 and Ser28 found at the same aligned positions of the virulent Cmv2b (Ding *et al.*, 1994). TMV-T2bC, expressing the resultant Tav2b mutant, did not elicit any of the disease resistance responses associated with wild-type Tav2b. Neither necrotic lesions nor transcriptional induction of PR-1a mRNA were observed in the inoculated Samsun leaves (data not shown). Furthermore, the TMV-T2bC-inoculated plants became systemically infected. Northern blot analysis showed that TMV-T2bC accumulated at levels similar to TMV-TΔ2b2 in both the inoculated and the systemic leaves of the infected plants (data not shown). RT-PCR and DNA sequencing showed that the introduced mutations were maintained in the progeny viral genome and no additional mutations were found in the Tav2b-coding sequence of TMV-T2bC. Thus, the two amino acid substitutions abolished the Avr activity of Tav2b in this tobacco cultivar.

Tav2b confers virulence and suppresses gene silencing in a species related to tobacco

The above data indicate that Tav2b triggers strong virus resistance in *N.tabacum* when it is expressed from the

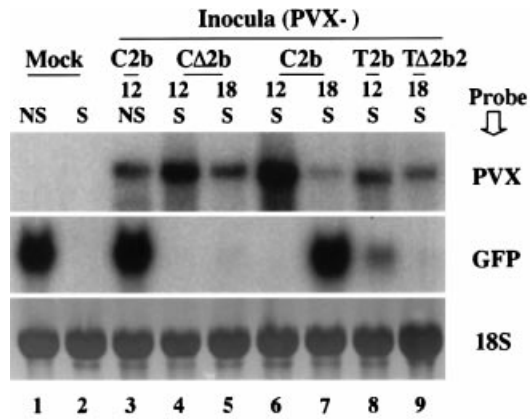


Fig. 5. Suppression of PTGS by Tav2b. Transgenic *N.benthamiana* seedlings (line 16c) that express high levels of GFP were infiltrated with a hypervirulent strain of *Agrobacterium tumefaciens* carrying a binary Ti plasmid vector into which a functional 35S–GFP cassette had been inserted (Brigneti *et al.*, 1998). PVX-C2b, PVX-CA2b, PVX-T2b or PVX-TΔ2b2 was then inoculated onto the silenced (S) as well as non-silenced (NS) *N.benthamiana* plants. Total RNAs extracted 12 or 18 d.p.i. (indicated on top of lanes) were subjected to Northern analysis using DNA probes specific for PVX genomic RNA or GFP RNA (Brigneti *et al.*, 1998). The filter was also stained with methylene blue to show the amount of RNA loaded (bottom panel).

genome of TMV. However, *N.benthamiana* plants were found to be fully susceptible to both TMV-C2b and TMV-T2b, although the latter induced a much more severe systemic necrosis on the infected plants (data not shown). Accumulation of TMV-T2b and TMV-C2b RNAs in both the inoculated and systemic leaves was confirmed by Northern blot hybridization (data not shown). Western blot analysis (Shi *et al.*, 1997) showed that Tav2b accumulated to similar levels in plants infected with TMV-T2b or TAV (data not shown). These results indicate that neither Tav2b nor Cmv2b activates virus resistance in *N.benthamiana*. Furthermore, Tav2b also suppressed PTGS of a green fluorescent protein (GFP) transgene in *N.benthamiana*. The systemic silencing of the GFP transgene in *N.benthamiana* was induced by agro-infiltration as described previously (Brigneti *et al.*, 1998) and, when silencing was complete, the silenced plants were inoculated with RNA transcripts from a potato virus X (PVX) vector encoding Tav2b (PVX-T2b). Twelve days after inoculation, the newly emerging leaves became green-fluorescent under UV illumination although at the same time PVX-C2b-infected plants were similar to the uninfected silenced plants and remained red-fluorescent (data not shown). Northern blot analysis (Figure 5) confirmed that there was a significant level of GFP RNA accumulation in the new green-fluorescent leaves of PVX-T2b-infected plants at 12 d.p.i. (lane 8). At this time, however, GFP RNA remained below the level of detection in the equivalent leaves from PVX-C2b-infected plants (lane 6) and in the uninfected silenced plants (lane 2). PVX-T2b was much more virulent in *N.benthamiana* than PVX-C2b. By 18 d.p.i., the plants infected with PVX-T2b were dead and severely dehydrated (thus no RNAs could be extracted). In contrast, new leaves continued to emerge from PVX-C2b-infected plants and these leaves contained high levels of GFP RNA (lane 7), comparable with the levels found in non-silenced plants (lanes 1 and 3). As found above for the Avr activity, rendering the

Tav2b sequence of PVX-T2b non-coding abolished PTGS suppressor activity (lane 9) and the hypervirulence phenotype, suggesting that the Tav2b protein is the active molecule.

Discussion

In this work, we examined the functions of *Tav2b* expressed from heterologous viral vectors in two related host species. We report that whilst *Tav2b* suppresses PTGS of a GFP transgene in *N.benthamiana*, it triggers a strong host resistance mechanism which resembles gene-for-gene disease resistance in a related species, *N.tabacum*. This finding provides the first example to show how plants may combat the virus-encoded suppressor of the plant gene silencing defence mechanism: by invoking another independent resistance mechanism. Thus, a further complexity in the molecular strategies employed by viruses and their hosts for defence and counter-defence is revealed.

A dual functionality for *Tav2b*

It has been found previously that replacing the 2b gene of CMV with *Tav2b* resulted in an interspecies hybrid virus (CMV-qt) significantly more virulent in several host species than either of the parental viruses (Ding *et al.*, 1996). In this work, we show that in *N.benthamiana*, *Tav2b* expressed from either TMV or PVX also specifies a highly virulent phenotype and suppresses PTGS of a GFP transgene. In addition, we show that PTGS suppression by *Tav2b* occurred several days earlier than that caused by *Cmv2b*. This difference between *Tav2b* and *Cmv2b* in PTGS suppression may explain why CMV-qt caused an unconventional synergism in the host species tested (Ding *et al.*, 1996).

In *N.tabacum* cv. Samsun (nn), however, *in cis* expression of *Tav2b* from the TMV genome induced strong disease resistance responses. These responses included the induction of a typical HR (formation of necrotic lesions and the transcriptional induction of PR mRNAs) and containment of the invading virus in the primary infected tissues. Mutational analysis showed that the induction of these responses was unrelated to the encoding RNA sequence but correlated with the full-length translatable *Tav2b* ORF, suggesting that the encoded *Tav2b* protein is the active molecule. However, *Cmv2b*, a homologue of *Tav2b*, was not active in HR induction and the tobacco plants inoculated with TMV-C2b became systemically infected. Furthermore, substituting two amino acids of *Tav2b* with those found at the same positions of *Cmv2b* abolished the activity of *Tav2b* in resistance activation. Thus, these data indicate that *Tav2b*, when expressed from the TMV genome, was recognized by the *N.tabacum* cultivar as a typical *Avr* gene, although it remains to be determined if *Tav2b* itself is the elicitor molecule. Accordingly, our findings predict that this host cultivar encodes a matching *R* gene, which may be absent in *N.benthamiana*, that can specifically recognize *Tav2b*. A TMV-T2b-susceptible cultivar of *N.tabacum* is needed for further investigation of the genetics of this resistance.

A number of bacterial and fungal *Avr* proteins appear to be involved in virulence functions in plant cultivars lacking the matching *R* genes (Kearney and Staskawicz, 1990; Ji *et al.*, 1998). It has recently been reported that

the bacterial *dspEF* locus of *Erwinia amylovora* is required for virulence in pear and apple, yet it acts as an *Avr* gene in soybean when expressed from *P.syringae* pv. glycinea race 4 (Bogdanove *et al.*, 1998). By using the PVX vector for *in planta* expression of fungal proteins, Lauge *et al.* (1998) successfully identified an *R* gene in tomato that targets a virulence protein of a fungal pathogen (*C.fulvum*). Thus, pathogen proteins displaying a virulence phenotype in one host can be recognized as *Avr* proteins in another. *Tav2b* expressed from the TMV genome is similar to these dually functional pathogen proteins because it displays virulent phenotypes in *N.benthamiana*, although it confers avirulence in the Samsun cultivar of *N.tabacum*.

It is likely that many *Avr* gene products are virulence factors until a matching *R* gene evolves in a host cultivar. However, *Tav2b* is distinct from the known dually functional pathogen proteins as it clearly functions as a virulent determinant in the same Samsun cultivar when expressed from the CMV genome (Ding *et al.*, 1996). Samsun tobacco is also susceptible to wild-type TAV although systemically infected plants exhibit only mild chlorotic mottle and ringspot symptoms. In addition, no significant induction of PR genes was observed in TAV-infected Samsun plants (unpublished data). Therefore, *Tav2b* has dual functionality in the same tobacco cultivar. Depending on the viral genetic background (genome of the TMV vector or a cucumovirus) from which it is expressed, *Tav2b* can function as either a virulence or an avirulence determinant. This suggests that one may search in susceptible cultivars for *R*-like genes that potentially can recognize virulent pathogen proteins by *in planta* expression from heterologous viral vectors.

It is intriguing that *Tav2b* is recognized as an *Avr* gene in the host when expressed from the TMV genome, as it is unlikely that the induction of immunity by *Tav2b* requires interaction with a protein(s) encoded by TMV. This is because *Tav2b* expressed from the PVX vector also triggered the HR in the Samsun cultivars with (NN) or without (nn) the *N* gene (unpublished data). TMV and PVX are distinct plant RNA viruses of different viral taxonomy, and the encoded proteins of the two viruses have minimal sequence similarities (Matthews, 1991). One possibility that cannot be excluded is that the proposed *R* gene may require induction and/or activation during infection by certain virulent pathogens such as TMV or PVX before it is capable of recognizing *Tav2b*. The data from this work indicate that *Tav2b* has the potential to interact with both the gene silencing and the gene-for-gene disease resistance mechanisms in plants and that its N-terminal region of 69 amino acids is essential for virus resistance activation. We have shown recently that this N-terminal region is also critical for the PTGS suppressor activity of *Tav2b* (unpublished data), suggesting that the same or overlapping domains of *Tav2b* are involved in the interaction with these two virus resistance pathways. Thus, it may be possible that in CMV-qt- and TAV-infected tobacco plants, *Tav2b* is directed into PTGS suppression and prevented from being recognized by the putative *R* gene, possibly due to an interaction *in vivo* with another cucumovirus-encoded protein. This possibility is being examined as it may reveal yet another plant pathogen strategy for overcoming the host defence mechanisms.

A two-domain structure for *Tav2b*

There is a strong association between hypersensitive cell death and gene-for-gene disease resistance. However, recent studies have provided evidence that cell death is neither required nor sufficient for gene-for-gene resistance. For example, inhibition of cell death by incubation in very low oxygen (Mittler *et al.*, 1996) or in high humidity (Hammond-Kosack *et al.*, 1996) does not interfere with disease resistance. It has also been reported recently that an *Arabidopsis dnd1* mutant maintains the gene-for-gene resistance without hypersensitive cell death (Yu *et al.*, 1998). Moreover, *Arabidopsis ndr1* mutants were susceptible to previously avirulent *P.syringae*, despite retention of the HR phenotype (Century *et al.*, 1995). A single nucleotide mutation introduced into the *Avr* gene of a CMV strain avirulent to cowpea resulted in a systemic infection as well as a systemic HR (Kim and Palukaitis, 1997). In this study, we have found that TMV-TC2 induced strong viral resistance in the Samsun cultivar without visible hypersensitive cell death (Table I). Therefore, our results obtained through the molecular dissection of a viral *Avr* gene also support the view that cell death is not required for resistance.

Domain swapping between the avirulent *Tav2b* and its virulent homologue, *Cmv2b*, suggests, for the first time, that the *Avr* protein has a two-domain structure. The N-terminal region of 69 amino acids of *Tav2b* constitutes the first domain and is essential and sufficient for resistance activation. Thus, both TMV-TC1 and TMV-TC2, encoding a complete resistance domain, induced strong virus resistance in the challenged tobacco plants. Complete (e.g. TMV-CT1, 2 and 3) or partial (e.g. TMV-TC3) replacements or deletion (TMV-TΔ2b1) of this domain result in the loss of the resistance activation activity. The second domain, encompassing amino acids 70–91 of *Tav2b*, is referred to tentatively as the cell death domain because its absence in TMV-TC2 leads to a loss of visible cell death induction without any apparent effect on resistance activation. Distinct from the N-terminal resistance domain, which can act independently, the cell death domain is dependent on the presence of a functional resistance domain because both TMV-CT2 and TMV-CT3, which encode a complete cell death domain, fail to induce necrotic lesions in the inoculated leaves (Table I). Other evidence supporting this observation comes from the two amino acid substitutions, which rendered *Tav2b* inactive in triggering resistance and cell death. Both amino acid substitutions are located within the resistance domain but outside of the cell death domain. Alternatively, the N-terminal 69 amino acids of *Tav2b* may contain a basal activity which triggers virus resistance and cell death. This basal activity may be further enhanced or suppressed by its C-terminal domain, including that of *Cmv2b*, even though the complete *Cmv2b* protein is inactive, giving rise to the observed phenotypes of R, HR and S as listed in Table I. Nevertheless, it is interesting to note that these two functional domains correspond to the overlapping and non-overlapping regions of the cucumovirus 2b genes as defined previously, according to whether or not it overlaps with the 2a gene (Ding *et al.*, 1995).

It will be of practical importance to determine if the concept of the *Tav2b* two-domain structure also applies to *Avr* genes encoded by any other viral, bacterial and

fungal pathogens. Constitutive expression of an *Avr* gene in a cultivar that contains the matching *R* gene should generate constitutive broad-spectrum disease resistance. However, this type of resistance cannot be utilized readily because the specific *Avr*–*R* interaction also leads to immediate activation of hypersensitive cell death (Culver and Dawson, 1991; Gopalan *et al.*, 1996; Leister *et al.*, 1996; Scofield *et al.*, 1996; Tang *et al.*, 1996; Van den Ackerveken *et al.*, 1996; Gilbert *et al.*, 1998). This problem, of a constitutive HR phenotype associated with the transgenic plants, could probably be circumvented by removal of the cell death domain from the *Avr* gene before it is incorporated as a transgene.

Materials and methods

Plasmid constructs

DNA manipulations and cloning were carried out using standard procedures (Sambrook *et al.*, 1989) unless otherwise described. DNA inserts in all constructs were sequenced in two orientations prior to use.

pTMV-T2b, *pTMV-TΔ2b1*, *pTMV-TΔ2b2* and *pTMV-C2b*. Plasmid pTMV-30B (Figure 1, a gift from Professor W.O. Dawson, University of Florida, FL) is a plant gene expression vector based on TMV. The coding sequence of TAV ORF 2b (nucleotides 2447–2734 of RNA 2) was PCR amplified from pQCD2qt (Ding *et al.*, 1996) using the *Pfu* DNA polymerase (Stratagene) and cloned at the *PmeI* site of pTMV-30B to yield pTMV-T2b. pTMV-TΔ2b1 was generated from pTMV-T2b by PCR mutagenesis as previously described (Ding *et al.*, 1995). Two nucleotide substitutions (both C→T) were introduced at positions equivalent to nucleotides 2576 and 2582 of TAV RNA 2 (Moriones *et al.*, 1991) and converted both codons 44 (CAA) and 46 (CGA) of ORF 2b to stop codons (TAA and TGA). pTMV-TΔ2b2 contained the same two nucleotide mutations as in pQCD2qt2 (Ding *et al.*, 1996) that changed the second codon of ORF 2b to a stop codon. The coding sequence of CMV ORF 2b was obtained from pSK2b (Ding *et al.*, 1994) as a *Bam*HI–*Asp*718 fragment, which was end-filled and cloned into the *PmeI* site of pTMV-30B to give pTMV-C2b.

pTMV-TC1, *pTMV-TC2*, *pTMV-TC3*, *pTMV-CT1*, *pTMV-CT2* and *pTMV-CT3*. The previously described mutagenesis protocol (Ding *et al.*, 1996) that involves three separate PCRs was used with template pQCD2 (Ding *et al.*, 1995) or pQCD2qt (Ding *et al.*, 1996) to generate six chimeric DNA fragments (Table I). The final PCR fragments were cloned at the *PmeI* site of pTMV-30B to give the six plasmid constructs listed.

pTMV-T2bC. Nucleotide substitutions, AA→GT (equivalent to nucleotides 2508–2509 of TAV RNA 2) and CGA→TCT (nucleotides 2529–2531 of TAV RNA 2) were introduced by PCR into the coding sequence of *Tav2b* as encoded by pTMV-T2b. The resultant plasmid was called pTMV-T2bC.

pPVX-T2b and *pPVX-TΔ2b2*. The inserted TAV sequence in pTMV-T2b and pTMV-TΔ2b2 (Figure 1) was obtained as an *AgeI*–*XhoI* fragment and cloned in the PVX vector pP2C2S to yield pPVX-T2b and pPVX-TΔ2b2. pPVX-C2b (pTXMV-2b) and pPVX-CΔ2b (pTXMV-2bΔ) have been described previously (Brigneti *et al.*, 1998).

In vitro transcription, plant infection and Northern blot analysis

Plasmid pTMV-30B and its derivatives were linearized by *PstI* whereas pPVX derivatives were linearized by *SpeI* before they were transcribed *in vitro* in the presence of cap analogue using T7 RNA polymerase (New England Biolabs). *Nicotiana tabacum* cv. Samsun (nn) plants were grown in Conviron growth chambers (22°C constant, 75% humidity and 16 h photoperiod). At ~5 weeks, the youngest fully expanded leaves of the plants were dusted with carborundum and inoculated with the capped RNA transcripts (transcribed from 1 μg of plasmid DNA template/leaf). At various times after inoculation (as indicated in the text), leaves were excised and immediately frozen in liquid nitrogen before total plant RNAs were extracted and analysed by Northern blot analysis as previously described (Ding *et al.*, 1995).

The following DNA fragments were labelled with [α -³²P]dCTP by random priming as described (Sambrook *et al.*, 1989). The *SphI*–*StuI* fragment from pTMV-30B corresponds to nucleotides 445–1675 of the

TMV genome (Goelet *et al.*, 1982). The probes specific for the mRNA of PR-1a (Cornelissen *et al.*, 1987) and PR-5 (Cornelissen *et al.*, 1986) and for the 18S rRNA (Venkateswarlu and Nazar, 1991) were all obtained by PCR according to the published sequences and confirmed by sequencing. For Northern analyses, equal amounts of the total RNAs (10 µg) were used for all samples and the probe specific for 18S rRNA was employed to monitor RNA loading. Induction and suppression of PTGS of the GFP transgene as well as GFP imaging in whole plants were carried out as described previously (Brigneti *et al.*, 1998).

Virus progeny RNA analysis

The viral progeny RNAs were recovered from plants inoculated with each of the recombinant TMV transcripts and analysed by RT-PCR and DNA sequencing. The cucumovirus 2b coding sequences cloned at the *PmeI* site of pTMV-30B were first amplified by RT-PCR using a pair of primers flanking the *PmeI* site of pTMV-30B. The amplified fragments were then purified from agarose gels and either sequenced directly using the same pair of primers or cloned into a plasmid vector before sequencing.

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