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Trans-acting untranslated elements of groundnut rosette virus satellite RNA are involved in symptom production

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Isolates of groundnut rosette umbravirus (GRV) contain a satellite RNA (sat-RNA), about 900 nucleotides (nt) in length, different variants of which are responsible for the symptoms of different forms of rosette disease in groundnuts and, in the particular instance of sat-RNA YB3b, for the production of yellow blotch symptoms in *Nicotiana benthamiana*. Sat-RNA YB3b does not affect the accumulation of GRV genomic or subgenomic RNAs in infected plants. Replication of sat-RNA YB3b and induction of yellow blotch symptoms do not require the production of any sat-RNA-encoded proteins. Experiments with deletion mutants identified three functional untranslated elements in sat-RNA YB3b. One (designated R) comprises nt 47–281, is essential for sat-RNA replication and appears to be *cis*-acting. The other two (designated A and B) comprise nt 280–470 and 629–849, respectively, are both involved in yellow blotch symptom production and can act in *trans*. Element A contains the determinant that is unique to sat-RNA YB3b. The process of symptom induction by sat-RNA YB3b apparently involves a novel type of specific interaction of two untranslated RNA elements, which can complement each other, with a host factor or factors.

Introduction

Plant viruses cause various kinds of disease symptom in infected plants and the means by which they do so can involve the activity of one or more viral genes and/or the activation of diverse host responses. However, proteins may not be the only virus-specific inducers of symptoms in infected plants; some plant pathogenic RNAs may be directly involved in symptom production and disease development. Viroids, despite their small size and lack of any potential translation products, can induce severe symptoms in infected plants. Dispensable satellite RNAs (sat-RNAs) are associated with many plant RNA viruses. They range in size from 194 nucleotides (nt) to about 1500 nt, and are completely dependent on the helper virus for replication and systemic spread (for review see Roossinck *et al.*, 1992). The larger sat-RNAs appear to contain open reading frames (ORFs) for non-structural proteins. In some instances, the gene product is required for sat-RNA replication (e.g. sat-RNA of tomato black ring virus; Hemmer *et al.*, 1993), whereas in others it is not (e.g. sat-RNA of bamboo mosaic virus; Lin *et al.*, 1996). The smaller sat-RNAs (194–700 nt), including those of cucumber mosaic virus (CMV) and turnip crinkle virus (TCV), do not encode any functional ORFs (Roossinck *et al.*, 1992). However, despite the absence of gene products, they may have dramatic effects on the symptoms induced by their helper viruses, ranging from amelioration to severe exacerbation. It seems that this ability to modulate symptoms must be a property of the sequence or structure of the sat-RNA itself. Although most CMV sat-RNAs attenuate the symptoms induced by CMV in all host species tested, some satellite isolates intensify symptoms in certain hosts, for example inducing chlorosis in tomato and tobacco (Palukaitis, 1988) or necrosis in tomato (Takanami, 1981; Sleat *et al.*, 1994). Sequences in the sat-RNA that control the symptom intensification have been mapped to specific nucleotide residues, and it has been shown that a few nucleotide sequence changes can alter the host response (Palukaitis, 1988; Masuta & Takanami, 1989; Jaegle *et al.*, 1990; Sleat & Palukaitis, 1990; Sleat *et al.*, 1994; Zhang *et al.*, 1994). Furthermore, identical sat-RNAs can have different effects on symptoms when associated with different helper virus strains (Roossinck *et al.*, 1992). In the case of TCV and its sat-RNA, it has been shown that mutations in the RNA-dependent RNA polymerase gene of the helper virus alter the effect of the sat-RNA on symptom production (Collmer *et al.*, 1992; Oh *et al.*, 1995). Thus, sat-RNA-mediated symptom modulation involves interactions between sat-RNA, helper virus and host plant, although it is unclear whether sat-RNA sequences are directly

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involved in interaction with components of the host plant or whether they function through interaction with the helper virus.

To study the contribution of sat-RNA sequences to symptom production, we have used the satellite of groundnut rosette virus (GRV). Although this RNA plays an essential role in the aphid transmission of its helper virus and is therefore indispensable for the survival of GRV in nature (Murant, 1990), in all other respects it has the characteristics of a sat-RNA. GRV is a member of the genus *Umbravirus*, whose positive-sense RNA genome has recently been sequenced. GRV RNA contains four ORFs and the two ORFs nearest the 3′ end are probably expressed from subgenomic RNA (Taliansky *et al*., 1996). However, it is the sat-RNA that is primarily responsible for the symptoms of groundnut rosette disease. GRV isolates that lack the satellite induce no symptoms or only a transient mild mottle when inoculated to groundnut (Murant *et al*., 1988). Moreover, different variants of the sat-RNA are responsible for the different forms of rosette disease, such as green rosette and chlorotic rosette (Murant & Kumar, 1990). The sequences of 10 variants of GRV sat-RNA have been determined (Blok *et al*., 1994).

One particular isolate of GRV, known as YB, contains a sat-RNA variant that induces brilliant yellow blotch mosaic symptoms in certain experimental hosts, including *Nicotiana benthamiana* but not groundnut (Kumar *et al*., 1991), and provides a convenient experimental system for studying determinants of symptom production. Experiments in which the sat-RNA was removed from and restored to isolate YB, or in which it was exchanged with sat-RNAs from other GRV isolates, clearly showed that the YB sat-RNA carries the determinant(s) for the yellow blotch symptoms (Kumar *et al*., 1991). The symptoms produced were independent of the strain of helper virus, and indeed indistinguishable symptoms were produced when a quite different virus, pea enation mosaic enamovirus (PEMV), was substituted for GRV as the helper virus (Demler *et al*., 1996). In this paper, we show that two segments of the YB sat-RNA sequence, which can act in *trans*, are involved in the development of the yellow blotch symptoms.

Methods

■ **Virus culture.** The satellite-free GRV culture MC1 used in this work was derived by Murant & Kumar (1990) from a GRV isolate obtained from a Malawian groundnut plant showing symptoms of chlorotic rosette. MC1 was propagated in *N. benthamiana* by manual inoculation.

■ **Generation of mutant and chimeric GRV sat-RNAs.** Plasmids pYB3b and pMC3a, from which biologically active GRV sat-RNA can be transcribed, were described by Demler *et al*. (1996). Mutagenesis of the AUG initiation codons in pYB3b was done with a U-DNA mutagenesis kit (Boehringer) according to the manufacturer's protocol. The mutagenic primers were as follows (ORFs numbered as in Blok *et al*., 1994; see Fig. 3).

5′ CCAGGATCTCCATAAATCTCTCCT 3′ for pYB-ORF I^-
5′ CTGTACATACACTTGAGGCCGGTG 3′ for pYB-ORF III^-
5′ ATGTGGCATATATATATCAACCC 3′ for pYB-ORF IV^-
5′ CAAACTAGGCAATTCATATGC 3′ for pYB-ORF V^-

Deletion mutants of pYB3b (see Fig. 4) were constructed by cutting out the following fragments: for pYBΔ1, the *Eco*NI (nt 280)–*Bbs*I (nt 470) fragment; for pYBΔ2, the *Eco*O109I fragment (nt 625–794); for pYBΔ3, the *Eco*O109I (nt 625)–*Bgl*II (nt 845) fragment; for pYBΔ4, the *Eco*NI (nt 280)–*Bgl*II (nt 845) fragment; and for pYBΔ5, the *Pml*I (nt 46)–*Eco*NI (nt 280) fragment. Recessed 3′ termini were filled in with the Klenow fragment of DNA polymerase I and the plasmids were religated.

Chimeric sat-RNA plasmids pYB/MC and pMC/YB (see Fig. 6) were constructed by replacing the *Bbs*I (nt 470)–*Spe*I (nt 903) fragment of pYB3b with the corresponding fragment of pMC3a and vice versa. pMC(Δ1YB) was generated by replacing the *Eco*NI (nt 280)–*Bbs*I (nt 470) fragment of pMC3a with the corresponding fragment of pYB3a, and pYB(Δ1MC) was the converse construct.

All plasmids were multiplied in *Escherichia coli* DH5α and DNA was purified by standard procedures (Sambrook *et al*., 1989). The identity of the recombinant sat-RNA clones was confirmed by restriction analysis and DNA sequencing.

■ **Transcription.** All sat-RNA plasmids were linearized with *Spe*I and used as templates for *in vitro* transcription as described by Demler *et al*. (1996). Transcripts were left uncapped. Sat-RNAs obtained by transcription were named according to the plasmid from which they were derived: thus, YB-ORF I^- is the sat-RNA obtained by transcription from pYB-ORF I^-, etc.

■ **Plant inoculation.** *N. benthamiana* plants were manually inoculated with a mixture of total RNA from GRV MC1-infected plants (Blok *et al*., 1994) and sat-RNA transcripts, suspended in 0·1 M phosphate buffer, pH 7·4, containing 0·1% bentonite. Each plant received 5–10 μg transcript RNA. Each experiment was repeated two or three times with three replicate plants per treatment.

■ **RT–PCR amplification and sequencing.** To confirm the sequence of mutated sat-RNAs in progeny from infected plants, total RNA preparations were used for reverse transcription and PCR amplification (RT–PCR) as described by Blok *et al*. (1994). PCR products, as well as recombinant plasmids, were sequenced on an ABI model 373 Stretch DNA sequencer using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit.

■ **Dot-blot and Northern blot hybridization.** For dot-blot analysis, samples of total RNA extracted from *N. benthamiana* leaf tissue by the method of Blok *et al*. (1994) were spotted onto Hybond-N nylon membrane (Amersham) and immobilized by UV-crosslinking in a Stratalinker 2400 (Stratagen).

For Northern blot analysis, total RNA preparations were denatured with formaldehyde and formamide. Electrophoresis was in 1·2% agarose gels for analysis of GRV RNAs or in 2% agarose gels for analysis of sat-RNAs, as outlined in Sambrook *et al*. (1989). RNA was transferred to Hybond-N membrane by the capillary method with 20 × SSC (3 M sodium chloride and 0·3 M sodium citrate, pH 7·0) and immobilized by UV-crosslinking.

Hybridizations were done as described by Sambrook *et al*. (1989) with probes labelled with [^{32}P]dATP using a Random Primers DNA Labelling kit (Life Technologies). GRV-specific probes were prepared from the inserts of clones grmp and gr51 (Taliansky *et al*., 1996), which represent sequences towards the 3′ end of the GRV genome. Sat-RNA-specific probes were prepared from the complete insert of pYB3b or from the fragments representing nt 282–470 (Δ1), nt 629–845 (Δ3) or nt 47–280 (Δ5). Control experiments showed that hybridization of the full-sized

probe with equal amounts of transcript RNA from pYB3b and pMC3a gave signals of similar strength.

Quantitative analysis of dot-blots was done by densitometry of the autoradiographic images, using a BioImage system (Millipore) and Visage version 4.2 software. The instrument was calibrated with a 21-step optical density standard wedge supplied by the manufacturer, and measurements were within this range. Dot-blots of total plant RNA extracts (four replicates of each) from two independent inoculation experiments were hybridized and autoradiographed together. The data presented are from a single autoradiographic exposure, although analysis of films obtained with different exposures revealed similar patterns of results.

Results

Sat-RNA YB3b does not affect the accumulation of GRV genomic or subgenomic RNAs

As observed by Demler *et al.* (1996), addition of sat-RNA MC3a to inocula containing GRV MC1 RNA did not alter the chlorotic mottle and leaf curling symptoms induced in *N. benthamiana*. In contrast, inocula containing sat-RNA YB3b induced brilliant yellow blotch symptoms (Fig. 1*a*) on all leaves above those that were inoculated, including leaves that were partially expanded at the time of inoculation, but not on the inoculated leaves themselves. These symptoms first appeared 10–12 days after inoculation and were similar to those induced by the GRV YB culture from which this sat-RNA was derived. Development of these relatively severe symptoms could be due to accumulation of YB3b sat-RNA to higher levels than sat-RNAs such as MC3a that do not affect symptoms, or to a YB3b-mediated increase in the amount of GRV MC1 RNA in the infected plants. However, Northern blot and dot-blot analyses did not reveal any obvious difference in levels of accumulation of YB3b and MC3a sat-RNAs in inoculated or upper uninoculated leaves of infected plants (Fig. 2; Table 1). Moreover, neither sat-RNA affected the accumulation or spread of GRV genomic RNA, or the accumulation of GRV subgenomic RNA (Fig. 2; Table 1). Thus, the effect of sat-RNA YB3b on symptoms seems not to be a consequence of alterations in the accumulation or spread of satellite or GRV genomic RNA.

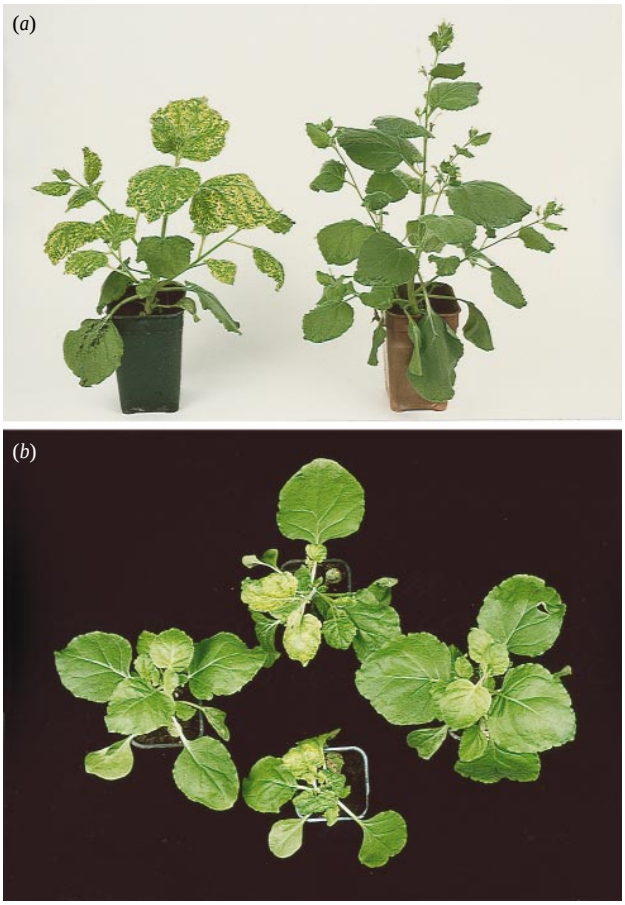

Fig. 1. (*a*) *N. benthamiana* plants showing (left) brilliant yellow blotch symptoms induced by infection with GRV and sat-RNA YB3b and (right) mild symptoms induced by infection with GRV and sat-RNA MC3a. (*b*) *N. benthamiana* plants infected with GRV together with (top) sat-RNA YB3b, (left) sat-RNA YBΔ1, (right) sat-RNA YBΔ2 or (bottom) both sat-RNA YBΔ1 and sat-RNA YBΔ2.

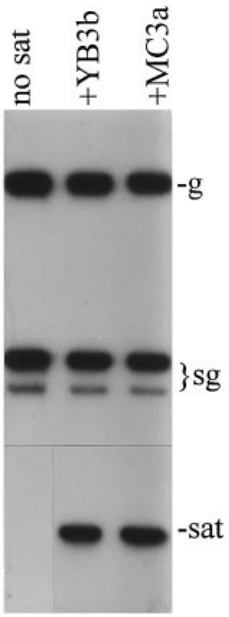

Fig. 2. Northern blot of RNA extracted from uninoculated, systemically infected leaves of *N. benthamiana* plants 7 days after inoculation with GRV alone or together with sat-RNA YB3b or sat-RNA MC3a. The upper portion of the blot was probed with a GRV-specific probe and the lower portion with a sat-RNA-specific probe. 'g' indicates the position of GRV genomic RNA, 'sg' that of GRV sub-genomic RNAs and 'sat' that of sat-RNA.

The potential ORFs of sat-RNA YB3b are functionally dispensable in *N. benthamiana*

Examination of the nucleotide sequences of different variants of GRV sat-RNA revealed the presence of up to five ORFs, of which sat-RNA YB3b contained four (ORFs I, III, IV and V; Blok *et al.*, 1994). Site-directed mutagenesis was used to

Table 1. Accumulation of viral and satellite RNA in *N. benthamiana* plants inoculated with GRV with or without sat-RNA

Data are means ± standard deviation from two independent experiments each with four replicates. The sets of data for GRV RNA and for sat-RNA are each internally comparable, but are not comparable with one another.

Inoculum		Relative amount of RNA (IOD per sample*)							
		Inoculated leaves				Uninoculated leaves†			
		7 days p.i.		14 days p.i.		7 days p.i.		14 days p.i.	
Helper virus	Sat-RNA	GRV	Sat-RNA	GRV	Sat-RNA	GRV	Sat-RNA	GRV	Sat-RNA
GRV (MC1)	–	8·47 ± 0·85	–	11·20 ± 1·22	–	7·96 ± 0·65	–	11·25 ± 1·41	–
GRV (MC1)	YB3b	8·35 ± 0·96	6·86 ± 0·89	12·75 ± 0·9	12·35 ± 1·24	7·28 ± 0·98	7·93 ± 1·2	12·34 ± 1·29	16·20 ± 1·21
GRV (MC1)	MC3a	9·25 ± 1·11	9·03 ± 1·60	12·40 ± 1·17	13·30 ± 1·13	8·15 ± 1·11	8·84 ± 0·85	13·14 ± 1·22	17·75 ± 1·39

* Measured by densitometry of autoradiograms of dot-blots: IOD, integrated optical density; sample corresponds to total RNA extract from 50 mg leaf tissue.
† Second or third leaf above inoculated leaves; these leaves showed symptoms at 14 days p.i. but not at 7 days p.i.

replace the initiation codon of each ORF with another triplet, in such a way as not to change the coding capacity of the opposite RNA strand (Fig. 3*a*). Transcripts from the mutated plasmids were co-inoculated with GRV MC1 RNA to *N. benthamiana*. All four mutants (YB-ORF I$^-$, YB-ORF III$^-$, YB-ORF IV$^-$ and YB-ORF V$^-$) induced the same symptoms at the same time after inoculation as YB3b. Levels of accumulation and spread of sat-RNA or of GRV RNA, as determined by Northern blot analysis, were not affected by any of the mutations (Fig. 3*b*). The presence of the mutated initiation codons in the progeny of the ORF$^-$ mutants was confirmed by direct sequencing of RT–PCR products, generated with 5′- and 3′-terminal YB3b-specific primers (Blok *et al.*, 1994) from RNA isolated from the infected plants. These results show that none of the ORFs previously identified in sat-RNA YB3b is essential for sat-RNA replication or for production of the yellow blotch symptoms. ORFs III and IV contain internal AUG codons, which could conceivably be used in YB-ORF III$^-$ and YB-ORF IV$^-$ to produce truncated peptides of 44 and 98 amino acids, respectively, and we cannot rule out the possibility that one or both of these products is biologically active. However, a more likely interpretation is that the RNA sequence itself is involved in symptom production.

RNA elements involved in replication and symptom induction

To identify the elements in sat-RNA YB3b that are involved in production of yellow blotch symptoms, five deletion mutants of pYB3b were generated (Fig. 4*a*) and transcripts were co-inoculated to *N. benthamiana* with GRV MC1 RNA.

Mutant YBΔ4, with a large deletion of nt 282–794, was able to replicate but had no effect on symptoms. However, levels of accumulation of sat-RNA YBΔ4 were noticeably lower than those of sat-RNA YB3b (Fig. 4*b*). Thus, the failure of YBΔ4 to induce yellow blotch symptoms might indicate that some of the deleted sequences are required for symptom production, but might also be a consequence of the lower level of accumulation of the sat-RNA.

In contrast, mutants with deletions of nt 282–470 (YBΔ1), of nt 629–794 (YBΔ2) or of nt 629–845 (YBΔ3) replicated and spread in infected plants to levels comparable with those achieved by YB3b (Fig. 4*b*) but did not induce yellow blotch symptoms. These results show that none of the sequences involved in these three deletions, which include between them all four ORFs, are essential for sat-RNA replication or spread, and confirm the previous conclusion that all the ORFs are dispensable for these functions. It is also clear that at least some of the sequences deleted in each of these mutants are essential for production of yellow blotch symptoms and that their loss prevented symptom induction. However, yellow blotch symptoms were produced in plants inoculated with mixtures of YBΔ1 and YBΔ2 (Fig. 1*b*), or of YBΔ1 and YBΔ3, together with GRV MC1. Northern blot analysis of the progeny sat-RNA in plants mixedly infected with YBΔ1, YBΔ3 and GRV MC1 detected the presence of both deletion mutants but no sat-RNA molecules of larger size (Fig. 5*a*), suggesting that recombination between the deleted sat-RNAs had not occurred. Similar results were obtained in tests on plants mixedly infected with YBΔ1, YBΔ2 and GRV MC1. Thus, symptom production in these mixedly infected plants seems to involve complementation between the two deleted sat-RNAs, involving two elements of the RNA sequence (designated A and B; Fig. 6*b*) which are able to function in *trans*.

Deletion of nt 47–280 (sat-RNA YBΔ5) prevented detectable sat-RNA multiplication in inoculated or uninoculated leaves (Fig. 4*b*), implicating the deleted sequences in replication and/or cell-to-cell spread of the sat-RNA. Not surprisingly, inclusion of sat-RNA YBΔ5 in the inoculum did not alter the

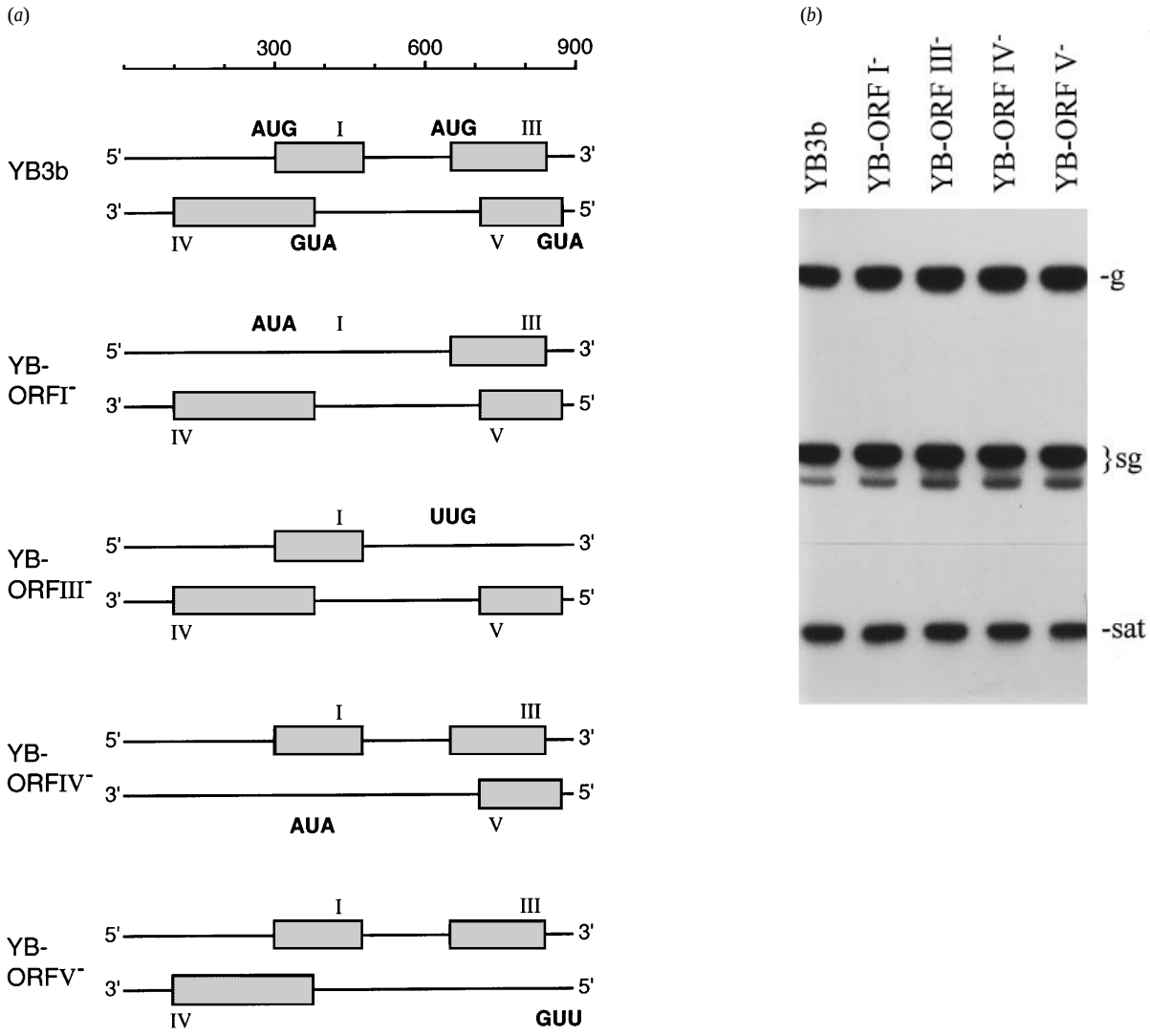

Fig. 3. (*a*) Diagram showing the positions and initiation codons of the ORFs in sat-RNA YB3b, and the codons substituted in mutants YB-ORF I⁻, YB-ORF III⁻, YB-ORF IV⁻ and YB-ORF V⁻. The scale is marked in nucleotides. (*b*) Northern blot of RNA extracted from uninoculated, systemically infected leaves of *N. benthamiana* plants infected with GRV together with (left to right) sat-RNAs YB3b, YB-ORF I⁻, YB-ORF III⁻, YB-ORF IV⁻ or YB-ORF V⁻. The upper portion of the blot was probed with a GRV-specific probe and the lower portion with a sat-RNA-specific probe. 'g' indicates the position of GRV genomic RNA, 'sg' that of GRV sub-genomic RNAs and 'sat' that of sat-RNA.

symptoms induced by GRV MC1 in *N. benthamiana*. Moreover, in plants mixedly infected with YBΔ1, YBΔ5 and GRV MC1 (Fig. 5*b*) or with YBΔ3, YBΔ5 and GRV MC1, replication of sat-RNA YBΔ1 or YBΔ3, respectively, but not of YBΔ5 was detected. Thus, replication of sat-RNA YBΔ5 could not be rescued by co-inoculation with another sat-RNA containing the deleted sequences, which are apparently required in *cis*.

Roles of the two RNA elements required for induction of yellow blotch symptoms

Chimeric sat-RNAs were generated from plasmid clones containing different portions of the cDNA from pYB3b and pMC3a (Fig. 6*a*). When they were inoculated to *N. benthamiana* together with GRV MC1, all the chimeric sat-RNAs replicated to similar extents to the unmodified sat-RNAs (data not shown). However, sat-RNA YB/MC, consisting of nt 1–474 from YB3b and nt 471–903 from MC3a, produced yellow blotch symptoms at the same time as and with the same intensity as sat-RNA YB3b, whereas the converse construct, MC/YB, did not produce any yellow blotch symptoms (Fig. 6*a*). Thus, the key sequence difference that accounts for the difference in symptom induction between YB3b and MC3a is in the left half of the molecules, which contains only one of the two required elements, designated element A in Fig. 6(*b*). Indeed, sat-RNA MC(Δ1YB), in which element A in MC3a was replaced by the corresponding fragment from YB3b (Fig. 6*a*),

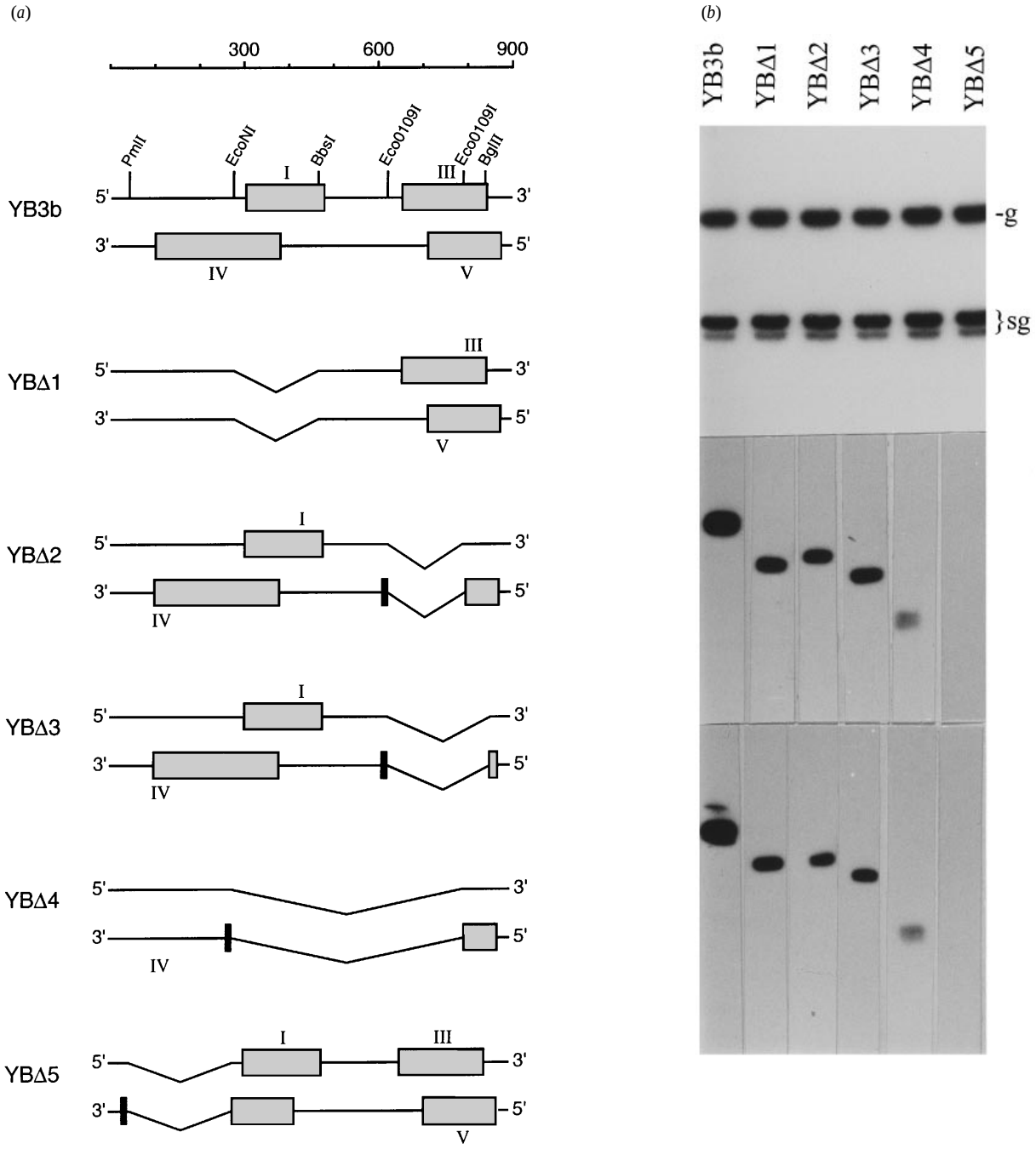

Fig. 4. (*a*) Diagram showing the positions of the ORFs and relevant restriction endonuclease sites in sat-RNA YB3b, and of the deletions and remaining ORFs and partial ORFs in mutants YBΔ1, YBΔ2, YBΔ3, YBΔ4 and YBΔ5. The black bars indicate the positions of termination codons that are in frame with truncated ORFs IV and V. The scale is marked in nucleotides. (*b*) Northern blot of RNA extracted from (top two panels) uninoculated, systemically infected leaves or (bottom panel) inoculated leaves of *N. benthamiana* plants infected with GRV together with (left to right) sat-RNAs YB3b, YBΔ1, YBΔ2, YBΔ3, YBΔ4 or YBΔ5. The top panel was probed with a GRV-specific probe and the bottom two panels with a sat-RNA-specific probe. 'g' indicates the position of GRV genomic RNA and 'sg' that of GRV sub-genomic RNAs.

induced yellow blotch symptoms identical to those of sat-RNA YB3b, whereas the converse construct, YB(Δ1MC), which consisted entirely of YB3b sequences apart from element A derived from MC3a (Fig. 6*a*), did not.

Discussion

Although the influence of sat-RNA YB3b on the synthesis and accumulation of GRV-coded proteins was not investigated, the presence of the sat-RNA did not affect the accumulation or

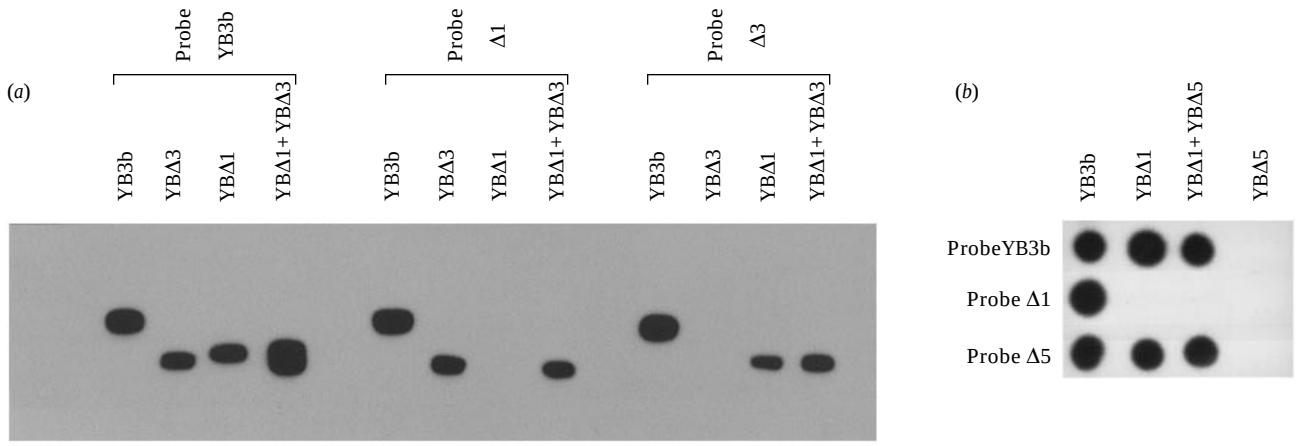

Fig. 5. (*a*) Northern blots of RNA extracted from uninoculated, systemically infected leaves of *N. benthamiana* plants infected with GRV together with sat-RNAs YB3b, YBΔ3 or YBΔ1, or both sat-RNAs YBΔ1 and YBΔ3. The left panel was probed with the complete insert of pYB3b, the middle panel with the Δ1 fragment (nt 281–470) and the right panel with the Δ3 fragment (nt 626–845). (*b*) Dot-blot of RNA extracted from uninoculated, systemically infected leaves of *N. benthamiana* plants infected with GRV together with sat-RNAs YB3b, YBΔ1 or YBΔ5, or both sat-RNAs YBΔ1 and YBΔ5. The top row was probed with the complete insert of pYB3b, the middle row with the Δ1 fragment (nt 281–470; specific for YBΔ5) and the bottom row with the Δ5 fragment (nt 47–280; specific for YBΔ1).

spread of GRV genomic or subgenomic RNA (Fig. 2; Table 1). Moreover, the observation (Demler *et al*., 1996) that similar yellow blotch symptoms are produced when YB3b is associated with PEMV as helper, instead of GRV, suggests that the sat-RNA induces these symptoms by directly interacting with the host plant rather than by modifying a function of the helper virus. No difference was observed between the accumulation and spread of YB3b and of MC3a, implying that accumulation of sat-RNA itself is not a primary determinant of symptom development.

Experiments with ORF$^-$ and deletion mutants suggested that the determinants of yellow blotch symptom production are not associated with any protein product of the YB3b sat-RNA, but with the sat-RNA itself. Deletion mutants YBΔ1, YBΔ2 and YBΔ3 all failed to induce yellow blotch symptoms when inoculated separately into *N. benthamiana*. However, when inocula contained both YBΔ1 and either YBΔ2 or YBΔ3, whose deletions do not overlap with that in YBΔ1, the ability to produce symptoms was restored. Thus, these deletions define two independent sequence elements (designated A and B; Fig. 6*b*) that are both involved in symptom production in *N. benthamiana* and that can act in *trans*. In a single experiment, groundnut plants infected with GRV MC1 together with either sat-RNA YBΔ1 or YBΔ3 did not develop symptoms of rosette, whereas plants infected with GRV MC1 and both deletion mutants developed mild but distinct chlorotic rosette (data not shown). This suggests that the same determinants are responsible for symptom production in groundnut. A synergistic effect of two independent sequence elements on symptom type has also been shown for beet necrotic yellow vein virus, where more severe symptoms are produced when both RNA-4 and RNA-5 are present in the inoculum although each on its own has no effect (Tamada *et al*., 1989). However, in this case it is not clear whether the RNAs themselves or their translation products are involved.

The experiments with deletion mutants also revealed an RNA element (designated R; Fig. 6*b*) essential for sat-RNA replication and defined by the deletion in YBΔ5. This element appears to be *cis*-acting, since replication of YBΔ5 was not rescued by co-inoculation with sat-RNAs containing element R. For all three elements, the actual determinant(s) of function may not correspond exactly with the sequence element defined by deletion, but may be only part of the element or may extend beyond it in one or both directions.

Thus, the process of symptom induction by YB3b seems to involve a novel type of interaction between two untranslated RNA elements, which can complement each other. Experiments with chimeric sat-RNAs suggested that, although both elements A and B of the sat-RNA are essential for yellow blotch symptom development, element A is the YB3b-specific element. Element A comprises nt 282–470 in the sequence of clone yb3b reported by Blok *et al*. (1994). In this region, the sequence of clone yb3b differs from that of clone mc3a at 14 positions, but nine of these differences are also found in clones derived from other GRV isolates that do not exhibit the yellow blotch phenotype. Tests with the programs FOLDRNA and SQUIGGLES did not reveal any remarkable secondary structure features in this region nor any obvious differences between the secondary structures predicted for YB3b and MC3a. In contrast, element B is not specific to sat-RNA YB3b. Sat-RNA YB/MC, in which element B was derived from MC3a, induced yellow blotch symptoms, as did a similar construct in which the right half of the sat-RNA was derived from clone nm3c (Blok *et al*., 1994) (unpublished data). Element B comprises nt

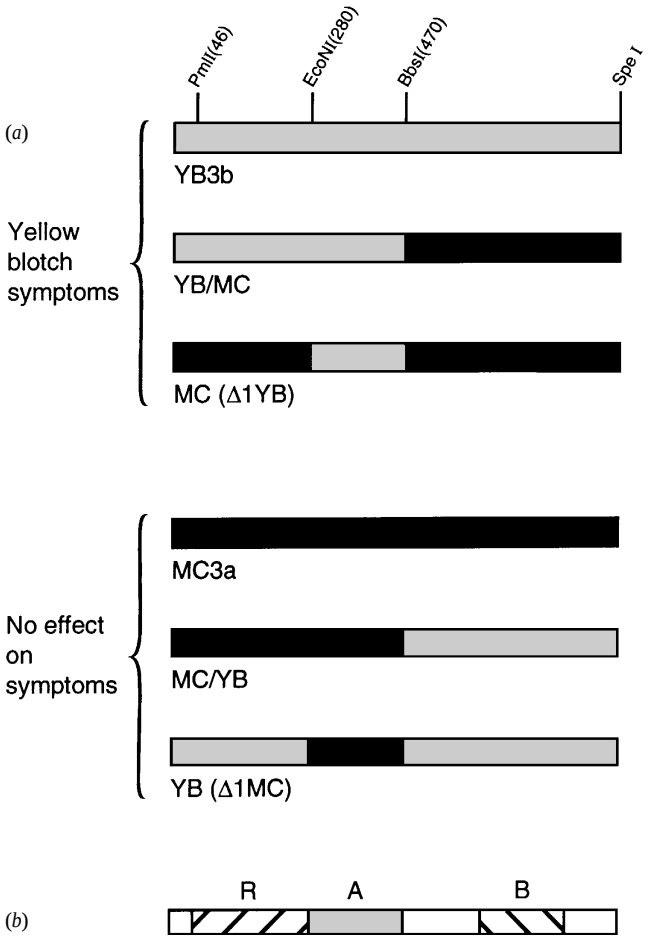

Fig. 6. (*a*) Diagram illustrating the construction of chimeric sat-RNAs YB/MC, MC/YB, MC(Δ1YB) and YB(Δ1MC). The positions of relevant restriction endonuclease sites are shown on the top line. Sequences derived from sat-RNA YB3b are lightly shaded and those from sat-RNA MC3a are black. (*b*) Diagram showing the positions in sat-RNA YB3b of the domains, designated A and B, involved in production of yellow blotch symptoms in *N. benthamiana* and of the domain, designated R, essential for sat-RNA replication.

629–849 in the sequence of clone yb3b, which differs from the sequences of clones mc3a and nm3c at 11 and 21 positions, respectively, in this region (Blok *et al.*, 1994).

Of the plant species tested by Kumar *et al.* (1991), yellow blotch symptoms were induced only in *N. benthamiana* and *N. occidentalis* and particularly not in another systemic host, *N. clevelandii*. We suggest, therefore, that sat-RNA YB3b carries a specific determinant (associated with element A) and a second determinant (associated with element B) common to several GRV sat-RNA variants, one of which is host-specific. These determinants are able to interact with some host factor or factors, leading to changes in host metabolism and thence to symptom development. The induction of yellow mosaic symptoms by the Y sat-RNA of cucumber mosaic virus is also specific to only some *Nicotiana* species, and the ability of the plant to respond seems to depend on the presence of a single incompletely dominant gene (Masuta *et al.*, 1993). However, neither the nature of the hypothetical host gene product nor the mechanism of the interaction are known.

There is growing evidence that certain RNA molecules can fold into specific three-dimensional shapes and generate active sites to fulfil particular biological functions (Bass & Cech, 1984; Cech, 1993). RNAs containing motifs able to bind small molecule ligands have been isolated by *in vitro* selection experiments (Sassanfar & Szostak, 1993), and peptide bond formation may be catalysed by the large ribosomal RNA (Noller *et al.*, 1992). RNAs that act as enzymes possessing RNA-cleaving activity (ribozymes) have been identified (for review see Edgington, 1992), and one obvious possibility is that the YB3b sat-RNA has ribozyme activity, although we could not find obvious similarity to the sequence of known ribozymes. Untranslated RNAs have also been shown to act as tumour suppressors in mammalian cells (Hao *et al.*, 1993; Rastinejad *et al.*, 1993) and as regulators of animal cell growth and differentiation (Hollander *et al.*, 1996). The term 'ribo-regulators' has been coined for RNAs involved in control of growth and differentiation (Rastinejad *et al.*, 1993), and several mechanisms have been proposed for their action, such as antisense activity on specific transcript RNAs, modulation of cellular kinases, perturbation of translation or effects on RNA metabolism (Rastinejad *et al.*, 1993; Crespi *et al.*, 1994). It is possible one of these mechanisms could be involved in the interaction of sat-RNA YB3b with its *N. benthamiana* host that leads to development of the brilliant yellow blotch symptoms.

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