The Chlorosis-Induction Domain of the Satellite RNA of Cucumber Mosaic Virus: Identifying Sequences That Affect Accumulation and the Degree of Chlorosis

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A number of satellite RNAs of cucumber mosaic virus (CMV) can induce chlorosis in either tobacco or tomato. A region containing the chlorosis domain was delimited, and one nucleotide position (153) regulating chlorosis induction as well as the nucleotide position (149) controlling the host specificity for chlorosis were identified. A cDNA clone of the B5-sat RNA was modified by site-directed mutagenesis to identify other nucleotides affecting chlorosis itself, as well as the extent of chlorosis and the helper virus-strain specificity for chlorosis. Four nucleotides conserved in chlorosis-inducing satellite RNAs (positions 127, 148, 149, 158) as well as two nucleotides within the chlorosis-induction domain that vary among chlorosis-inducing satellite RNAs (170, 171) were altered. Only substitutions at nucleotide positions 127 and 171 did not affect expression of the chlorosis phenotype. Alteration of position 148 affected satellite RNA accumulation. The pleiotropic effects of various sequences within the chlorosis-induction domain are discussed relative to particular sequence contexts in different chlorosis-inducing RNAs.

Additional keywords: cucumovirus, pathogenicity.

Some satellite RNAs of cucumber mosaic virus (CMV) induce yellow or white chlorosis in tobacco or tomato when supported by various strains of CMV (Takanami 1981; Gonsalves *et al.* 1982; Garcia-Arenal *et al.* 1987; Palukaitis 1988). The extent of chlorosis depends on specific sequences present in some strains of helper virus, and also on specific sequences present in several satellite RNAs (Palukaitis 1988; Kurath and Palukaitis 1989a; Sleat and Palukaitis 1990, 1992). Mapping of satellite RNA sequences controlling the expression of chlorosis has been achieved to a limited extent in two satellite RNAs: the Y-sat RNA, which induces chlorosis in tobacco (Jaegle *et al.* 1990; Kuwata *et al.* 1991), and the B5-sat RNA, which induces chlorosis in tomato (Kurath and Palukaitis 1989a; Sleat and Palukaitis 1992). In both cases, sequences regulating chlorosis were localized to a similar and highly homologous region; however, it is not clear if chlorosis is induced by each satellite RNA via a similar mechanism, since it has also been shown that a nucleotide change in B5-sat RNA (position 153) to one present at the comparable position in Y-sat RNA destroyed the chlorosis-induction phenotype in B5-sat RNA (Sleat and Palukaitis 1992). Furthermore, a single nucleotide position (149) of B5-sat RNA regulated the host-specificity of chlorosis for either tobacco (C149) or tomato (U149) (Sleat and Palukaitis 1992).

Within the sequences containing the chlorosis domain (defined by restriction endonuclease sites at positions 97 and 191) (Fig. 1), two other positions (127 and 148) contain invariant sequences specific to chlorosis-inducing satellites (Sleat and Palukaitis 1992). In addition, various satellite RNAs show other alterations of the yellow chlorosis phenotype described for B5-sat RNA. B1-sat RNA induces yellow chlorosis in tomato with both LS-CMV and WL-CMV as helper viruses, while B5- and B3-sat RNA induce yellow chlorosis with LS-CMV but not with WL-CMV. WL2-sat RNA induces a yellow chlorosis in tomato with LS-CMV but a white chlorosis with WL-CMV (Palukaitis 1988; Kurath and Palukaitis 1989a). We assume that these perturbations in the chlorotic response by tomato plants are a consequence of minor sequence alterations within the chlorosis-induction domain. Therefore, we altered specific sequences within the chlorosis-induction domain to determine their effects on chlorosis induction per se, as well as on the type of chlorosis induced. The results enabled us to delimit further the chlorosis-induction domain and to define the role of particular satellite RNA sequences in various pathogenic responses.

RESULTS

Identification of satellite RNA sequences determining white versus yellow chlorosis.

We wanted to determine whether the white chlorosis in tomato induced by WL-CMV and the WL2-sat RNA is a more severe expression of the yellow chlorosis phenotype induced by B5-sat RNA or whether it is caused by sequence alterations outside the chlorosis-induction domain. Thus, we obtained a cDNA clone of the WL2-sat RNA (see Materials and Methods) and made two chimeric satellite constructs using the common *Nhe*I restriction endonuclease site at position 191. The chimeric constructs contained either nucleotides 1–191 of B5-sat RNA and nucleotides 192–3′ end from WL2-sat RNA (BW191-sat RNA) or the reciprocal arrangement (WB191-sat RNA). RNA transcripts of cDNA clones of both BW191-sat RNA and WB191-sat RNA induced yellow chlorosis in tomato when supported by LS-CMV, as expected. However, only WB191-sat RNA induced white chlo-

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rosis on tomato with WL-CMV. BW191-sat RNA, like B5-sat RNA (Table 1), did not show any chlorosis in tomato with WL-CMV. All satellite RNAs replicated to similar levels (data not shown). Thus, the sequences controlling white chlorosis are contained within the 5′ 191 nt, and the inability of B5-sat RNA to induce chlorosis with WL-CMV is not associated with the differences between B5-sat RNA and B1-sat RNA (from which B5-sat RNA was cloned) within the 3′ half of their respective sequences (Kurath and Palukaitis 1989b).

Sequences containing the chlorosis-induction domain for various satellite RNAs are shown in Figure 1. It is apparent that WL2-sat RNA only differs from B5-sat RNA at two positions (136 and 170); however, WL2-sat RNA does not differ from B3-sat RNA (which has the same phenotype as B5-sat RNA) at position 136, making position 170 the most likely site specifying the phenotypic differences between WL2-sat RNA and various other chlorosis-inducing satellite RNAs. Therefore, the cDNA clone of B5-sat RNA was modified by

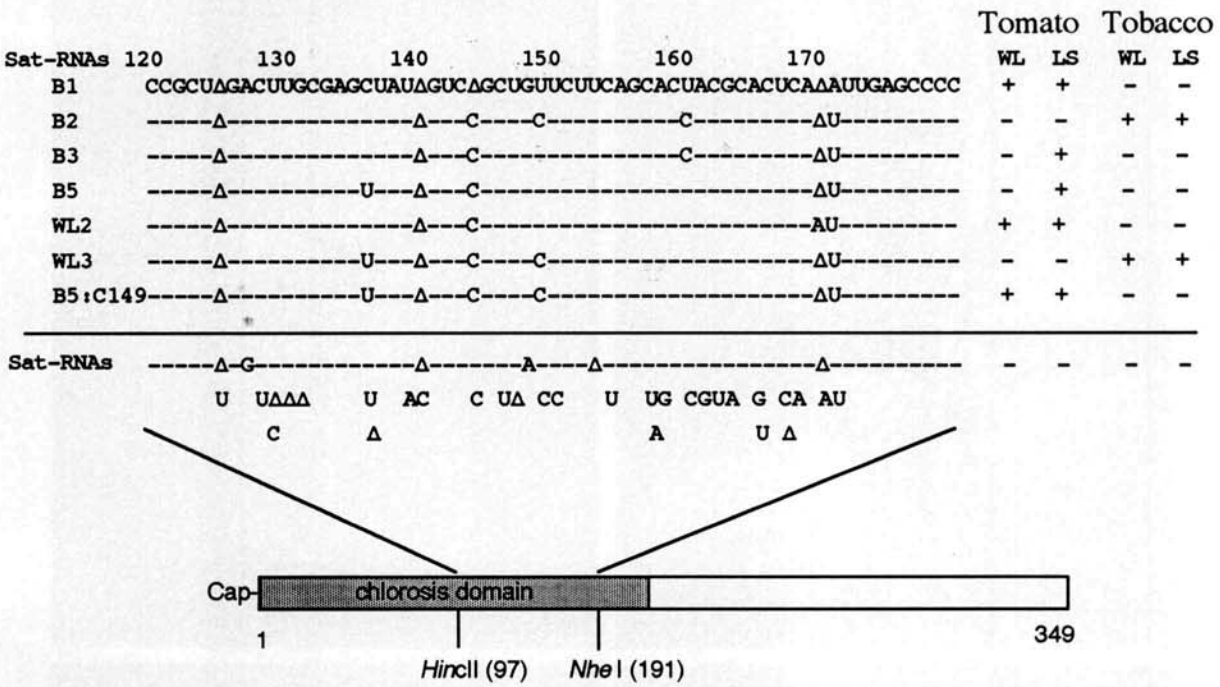

Fig. 1. Sequence comparison and pathogenic responses of CMV satellite RNAs. The chlorosis-induction domain as delimited by restriction sites for *Hin*cII and *Nhe*I is indicated. Sequence identities are indicated by dashed lines, and deletions by triangles. All chlorosis-inducing satellite RNA sequences are given above the solid line and sequence differences as well as alternative variations in all other satellite RNAs are given below the line. Chlorosis-induction on either tobacco or tomato, when supported by either LS-CMV or WL-CMV, is indicated on the right.

Table 1. Replication and pathogenicity of satellite RNAs of cucumber mosaic virus in tomato plants

Satellite RNA	Helper virus	Symptoms[a]	Satellite RNA replication and pathology[b]	
			Chlorosis/sat RNA	Total
B5	LS-CMV	yellow chlorosis	16/16	16
B5	WL-CMV	attenuation	0/8	8
WL2	WL-CMV	white chlorosis	7/7	8
BW191[c]	LS-CMV	yellow chlorosis	2/2	8
BW191[c]	WL-CMV	attenuation	0/8	8
WB191[c]	LS-CMV	yellow chlorosis	6/6	8
WB191[c]	WL-CMV	white chlorosis	8/8	8
B5:A170[d]	LS-CMV	yellow-chlorosis	6/6	8
B5:A170[d]	WL-CMV	white chlorosis	8/8	8
B5:G127[d]	LS-CMV	yellow chlorosis	8/8	8
B5:A148[d]	LS-CMV	CMV fernleaf	0/0	8
B5:A148/C149[d]	LS-CMV	attenuation	0/11	16
B5-U158/A170[d]	LS-CMV	attenuation	0/6	8
B5:U158/A171[d]	LS-CMV	attenuation	0/8	8

[a] Symptoms observed were either chlorosis (yellow or white) or attenuation of CMV fernleaf symptoms. Since B5:A148 did not replicate, the typical LS-CMV fernleaf symptoms were observed.

[b] Number of plants showing chlorosis/number of plants containing satellite RNA (determined by dot blot hybridization); total number of plants inoculated.

[c] Chimeras formed between cDNA clones of B5-sat RNA and WL2-sat RNA, using the common *Nhe*I restriction endonuclease site at position 191 (see Fig. 1).

[d] Mutants of a cDNA clone of B5-sat RNA generated by site-directed mutagenesis.

inserting an A residue at position 170. WL2-sat RNA contains an A at this position, which is represented as a deletion in the other satellite RNAs (Fig. 1). RNA transcripts (B5:A170-sat RNA) of this modified cDNA clone replicated in tobacco and tomato with both LS-CMV and WL-CMV (Table 1, and results not shown). With LS-CMV, B5:A170-sat RNA induced the yellow chlorosis on tomato typical of that induced by B5-sat RNA (Fig. 2A and B) and less severe than that induced by WL2-sat RNA. However, with WL-CMV, B5:A170-sat RNA induced a white chlorosis (Table 1,

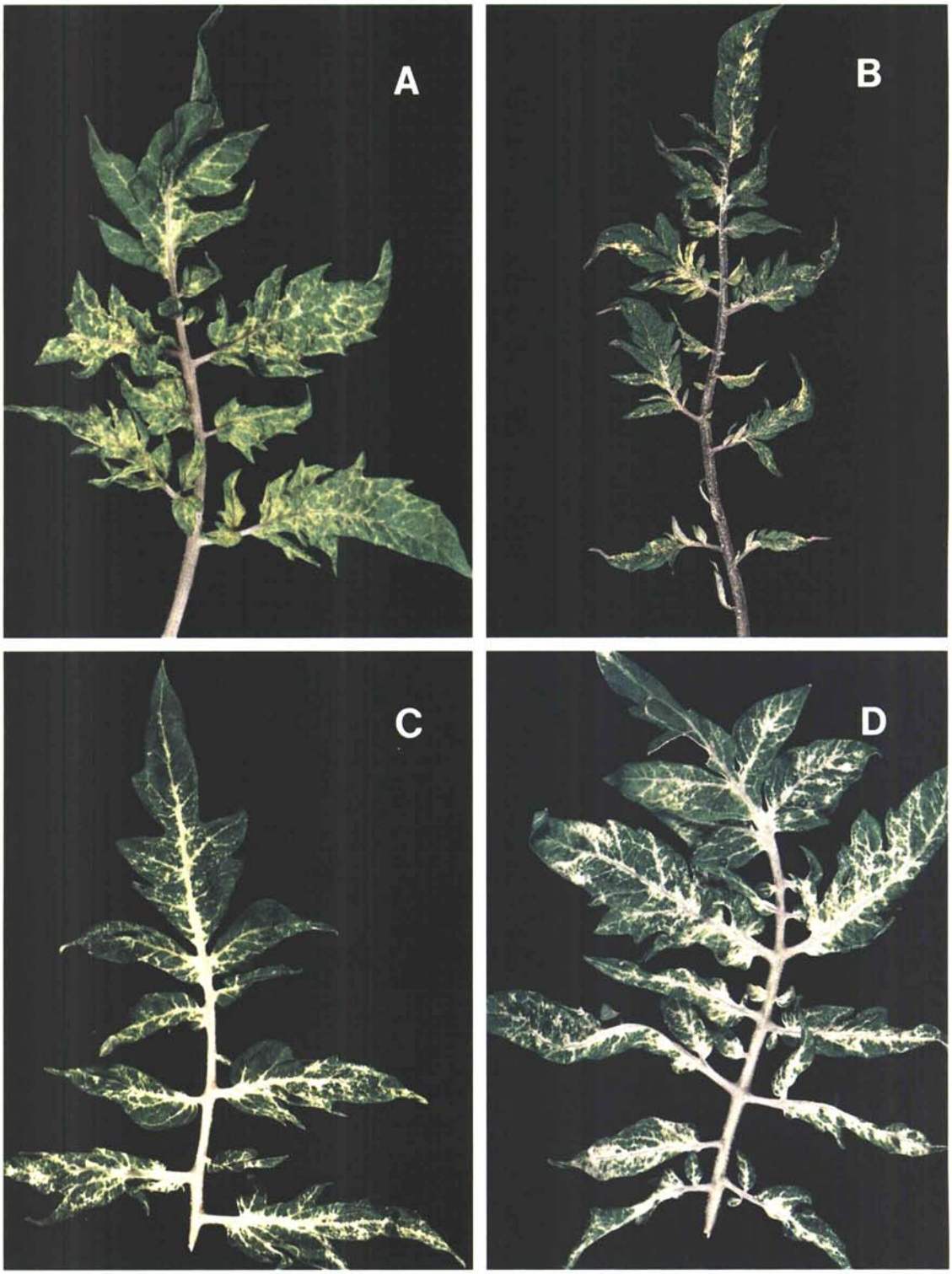

Fig. 2. Symptoms induced on tomato by CMV strains and satellite RNAs. Plants were inoculated with either LS-CMV (A and B) or WL-CMV (C and D) and various satellite RNAs: **A,** B5-sat RNA; **B and C,** B5:A170-sat RNA; and **D,** WL2-sat RNA. Leaves of plants scored positive for satellite RNA replication (Table 1), were photographed 3–4 wk postinoculation.

Fig. 2C) with the same intensity and over the same time course as that induced by WL2-sat RNA (Fig. 2D). Thus, position 170 controls both the type of chlorosis (yellow versus white leaves) and the helper virus strain specificity (LS-CMV alone versus LS-CMV and WL-CMV).

To determine whether the conversion of U171 in B5-sat RNA (Fig. 1) to the A171 present in B1-sat RNA would also broaden the strain specificity for chlorosis induction, this alteration was made in the cDNA clone of B5-sat RNA. However, while B5:A171-sat RNA still induced yellow chlorosis with LS-CMV in tomato, it did not induce chlorosis with WL-CMV (data not shown). There are other nucleotide differences between B5-sat RNA and B1-sat RNA that may have effects on chlorosis induction by B1-sat RNA and WL-CMV. These were not investigated.

Identification of sequences affecting chlorosis induction.

Previous work in this laboratory (Sleat and Palukaitis 1992) showed that all of the chlorosis-inducting satellite RNAs listed in Figure 1 contain conserved sequences at three positions that differ from satellite RNAs that do not induce chlorosis: positions 127, 148, and 153. Deletion of U153 destroys the ability of satellite RNAs to induce chlorosis in either tobacco or tomato (Sleat and Palukaitis 1992). When position 127 of B5-sat RNA was modified from the A residue present in all chlorosis-inducing satellite RNAs to the G present in other satellite RNAs, there was no change in the ability of the resulting B5:G127-sat RNA to induce chlorosis in tomato with LS-CMV (Table 1). This result may indicate that the chlorosis domain does not include sequences 5′ of and including position 127.

When position 148 in B5-sat RNA was altered from a G (present in all satellite RNAs that induce chlorosis), the resulting satellite RNA (B5:A148-sat RNA) did not accumulate in either tobacco or tomato with LS-CMV (Table 1, and data not shown). Other helper virus strains were not tested. However, an examination of the sequences of satellite RNAs that do not induce chlorosis showed that all but one contained the sequence A148/C149, whereas the chlorosis-inducing satellite RNAs contained either G148/C149 or G148/U149 (Fig. 1); i.e., the sequence in B5:A148-sat RNA, A148/U149, did not exist in any natural satellite RNAs except WL1-sat RNA, which also has a unique deletion at position 147. Thus, we modified position 148 of the satellite mutant B5:C149-sat RNA (formerly B5*-sat RNA [Sleat and Palukaitis 1992]) to create B5:A148/C149-sat RNA. This satellite RNA was able to accumulate in both tobacco and tomato but was not able to induce chlorosis in either host (Table 1, and data not shown). Thus, position 148 is a determinant of chlorosis, and positions 148 and 149 are involved in satellite RNA accumulation.

In the course of mutating positions 170 and 171, some of the mutants generated by the polymerase chain reaction also contained a second mutation converting position 158 from an A to a U. These RNAs containing the double mutants (B5:U158/A170-sat and B5:U158/A171-sat) were able to replicate to similar levels as B5-sat RNA in both tobacco and tomato (data not presented), but neither double mutant was able to induce chlorosis in either tomato or tobacco with LS-CMV as the helper (Table 1). Thus, like those at positions 148 and 153, the nucleotide at position 158 is an important determinant in the induction of chlorosis.

DISCUSSION

The chlorosis domain of the B5-sat RNA of CMV has been localized between nucleotide positions 97 and 191 by the construction and analysis of satellite RNA chimeras. Sequence analysis and site-directed mutagenesis studies have shown that the nucleotides between positions 148 and 171 control various aspects of the chlorosis-induction phenotype. Sequences flanking this region may also be critical to the interactions leading to chlorosis. However, sequences directly 3′ of the core chlorosis domain are identical in satellite RNAs that induce or that do not induce chlorosis, and the sequences directly 5′ of the core chlorosis-induction domain are either identical or nearly identical between these two groups of satellite RNAs. For example, the sequence at one position (127) in this latter region was found not to be critical to chlorosis induction, even though the sequence was always an A in chlorosis-inducing satellite RNAs and a G in all other satellite RNAs. Thus, the borders for all sequences essential to chlorosis induction cannot be ascertained.

Within the chlorosis domain (Fig. 3B), positions 148 and 149 affected aspects of both accumulation and chlorosis induction. In addition, positions 148, 153, and 158 were all shown to be important for chlorosis induction per se, while position 149 determined the host specificity of chlorosis induction (tobacco versus tomato), and position 170 controlled both the strain specificity of chlorosis and the extent of chlorosis (white versus yellow leaves and stems). Thus, many of the nucleotides within the chlorosis domain have pleiotropic effects on various aspects of chlorosis induction as well as satellite RNA accumulation. The effect on accumulation could be due to an impairment of replication, encapsidation, or movement. These were not investigated further.

Y-sat RNA induces chlorosis in tobacco (Takanami 1981). The chlorosis domain of Y-sat RNA has also been investigated intensively, and the core of this domain was shown to be in a region similar in location to the one delimited for B5-sat RNA (Jaegle *et al.* 1990; Kuwata *et al.* 1991). However, Y-sat RNA and several other satellite RNAs have 30 or more nucleotides inserted immediately 5′ of the chlorosis induction domain in B5-sat RNA (Hidaka *et al.* 1984, 1988; Devic *et al.* 1989; Masuta and Takanami 1989; Masuta *et al.* 1990) (Fig. 3A). Furthermore, there is considerable sequence variation in the region containing the chlorosis induction domain between Y-sat RNA and the other chlorosis-inducing satellite RNAs shown in Figure 1. We previously suggested that the core domain for chlorosis induction may be shifted in Y-sat RNA relative to B5-sat RNA (Sleat and Palukaitis 1992). This would explain two observations: 1) alteration of nucleotides 191–193 in Y-sat RNA to sequences present in S19-sat (and B5-sat) RNA abolished chlorosis (see Fig. 3A) (Kuwata *et al.* 1991); and 2) the inability of the smaller I17N′-sat RNA to be converted to a chlorosis-inducing satellite RNA by alteration at two nucleotides (185 and 186 in Y-sat RNA, equivalent to 157 and 158 in B5-sat RNA; see Fig. 3A) (Jaegle *et al.* 1990) which are not in the core domain specified by nucleotides 148-156 of B5-sat RNA. Although nucleotides 157 and 158 are part of the expanded core domain (nucleotides 148–171; Fig. 3B), the A residue at position 148 and the deletion at position 153 in I17N′-sat RNA would also have to be altered for this satellite to induce chlorosis in tobacco. It is interesting

that position 157 reverted from a C back to a U in the double mutant, I17N′:C157/U158-sat RNA (Jaegle *et al.* 1990). In our studies, the A to U mutation at position 158 was stable, although we did not alter position 157.

One Y-sat RNA mutant failed to replicate after modification to resemble I17N′-sat (and fortuitously B5-sat) RNA between nucleotides 167 and 175 (corresponding to nucleotides 137–146 of B5-sat RNA; Fig. 3A), but another Y-sat RNA mutant was able to replicate and induce chlorosis in tobacco, after modification of nucleotides 176 to 180 (nucleotides 147–150 of B5-sat RNA) (Jaegle *et al.* 1990). Hence, it appears that sequences determining replication are quite different in this region for B5-sat RNA and Y-sat RNA. Thus, the effects of various sequence changes seem to be influenced by the surrounding sequence context, and Y-sat RNA may not have an extended core domain for chlorosis induction of similar sequence or organization to that of the other chlorosis-inducing satellite RNAs.

The yellow leaves and stems that result from infection of tomato plants by CMV and various satellite RNAs are due to the loss or breakdown of chlorophyll, which exposes the presence of the yellow pigment xanthophyll. White tomato plants must therefore result from the loss of both pigments. Since these two pigments are synthesized by different pathways, the single nucleotide change common to B5:A170-sat RNA and WL2-sat RNA might influence a common localization or cofunction rather than the biosynthesis of the pigments themselves. On the other hand, the absence of symptoms in tomato leaves that contain satellite RNA but were already formed at the time of inoculation (P. Palukaitis, unpublished) argues against a direct effect on developed structures but rather an effect on structures (e.g., organelles) formed during development (compare van Loon *et al.* 1990). However, the absence of chlorosis in tomatoes infected with WL-CMV plus B5-sat RNA indicates that more than just the satellite RNA is required for the induction of chlorosis; i.e., the helper virus plays more than just a passive role in replicating the satellite RNA to high levels. This is also the case for chlorosis induction in tobacco, where chlorosis appears to involve specific RNAs (or their gene products) of some strains of CMV (Sleat and Palukaitis 1990). Elucidation of the specific viral component in the interactions leading to chlorosis induction should provide testable models for how specific satellite sequence alterations can have such a dramatic effect on the expression of the chlorosis phenotype.

MATERIALS AND METHODS

Propagation, purification, and characterization of CMV and satellite RNAs.

The origin, propagation, and purification of WL-CMV and LS-CMV helpers were described previously (Palukaitis 1988). RNA transcription from cDNA clones of satellite RNA, satellite RNA purification, and the construction of the B5-sat RNA and of chimeric satellite DNA clones involving the common *Nhe*I site of B5-sat and WL2-sat RNAs were all described (Kurath and Palukaitis 1987, 1989a,b; Sleat and Palukaitis 1992). A cDNA clone of the WL2-sat RNA was obtained from D. Gonsalves (Geneva, NY), and its construction and characterization have been described elsewhere (Kearney 1989). Tomato (*Lycopersicon esculentum* Mill. 'Rutgers') and tobacco (*Nicotiana tabacum* L. 'Xanthi-nc')

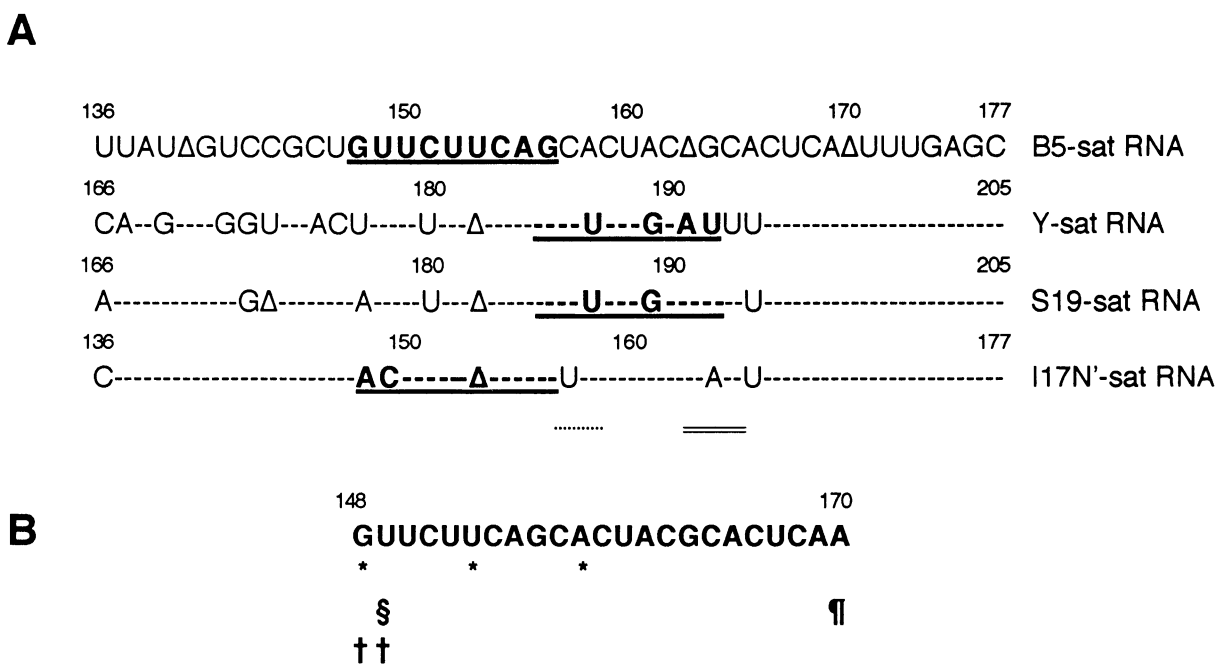

Fig. 3. Nucleotide sequence of the core and expanded chlorosis-induction domain. **A,** Nucleotide sequence comparison of chlorosis-inducing satellite (B5-sat and Y-sat) RNAs and satellite (S19-sat and I17N'-sat) RNAs that do not induce chlorosis used to delimit the core chlorosis domain (bold and underlined). The dotted line represents sequences modified in I17N'-sat RNA to resemble Y-sat RNA (Jaegle *et al.* 1990), and the double underline, sequences modified in Y-sat RNA to resemble S19-sat RNA (Kuwata *et al.* 1991). **B,** The core chlorosis domain was expanded for B5-sat RNA to take into account data presented in this study. Symbols indicate sequences essential for chlorosis-induction (*), accumulation (†), host specificity for chlorosis (§), and helper virus strain-specificity for chlorosis, as well as the extent of chlorosis (¶) (an A vs. a deletion at position 170).

plants were inoculated with either LS- or WL-CMV RNAs and transcripts of one of the satellite RNA chimeras or site-directed mutants. The presence of replicating satellite RNA was confirmed by dot blot hybridization. Virus was purified, and satellite RNAs were characterized by partial nucleic acid sequencing, all as previously described (Kurath and Palukaitis 1987, 1989a; Sleat and Palukaitis 1992). Each chimera or mutant was inoculated at least twice to groups of eight tomato plants with each helper virus.

Mutagenesis of B5-sat RNA.

A cDNA clone of B5-sat RNA (Kurath and Palukaitis 1989a,b) was used to construct a series of site-directed mutants via the two-step polymerase chain reaction–based procedure of Higuchi *et al.* (1988), using vector-complementary primers and the conditions described by Shintaku *et al.* (1992). The satellite-specific primers were: 5′-GGACCGCTGGCTTGCGAGTTATG-3′ (+) and 5′-CATAACTCGCAAGCCAGCGGTCC-3′ (–) for B5:G127-sat RNA; 5′-GTTATGTCCGCTATTCTTCCAGCAC-3′ (+) and 5′-GTGCTGAAGAATAGCGGACATAAC-3′ (–) for B5:A148-sat RNA; 5′-CTACGCACTCAATTTGAGCCCCCG-3′ (+) for B5:A170-sat RNA; and 5′-CTACGCACTCAATTGAGCCCCCGC-3′ (+) for B5:A171-sat RNA. For B5:A170-sat and B5:A171-sat RNAs, the (–) polarity primer was 5′-GGTAACGGCAAACC-3′. This same (–) polarity primer and a new (+) polarity primer (5′-GTTATGTCCGCTACTCTTCAGCAC-3′) were used with the cDNA clone of B5:C149-sat RNA (Sleat and Palukaitis 1992) to create B5:A148/C149-sat RNA. (All mutated nucleotides are underlined in the primers.) Mutant cDNAs were selected by nucleic acid sequencing of plasmid DNAs (Tabor and Richardson 1987).

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