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Genetic mapping of the compatibility between a lily isolate of *Cucumber mosaic virus* and a satellite RNA

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Five isolates of *Cucumber mosaic virus* (CMV) from *Lilium* sp. (lily), which were isolated from specimens in Japan, Korea and Taiwan, were unable to support satellite RNA (satRNA) accumulation. In order to map the CMV sequences that are involved in satRNA support, HL-CMV (Japanese lily isolate), Y-CMV (ordinary strain) and Y-satellite RNA (Y-sat) were used as the source material. The pseudorecombinants between Y-CMV and HL-CMV revealed that RNA1 was essential for satRNA replication in lily. The results of chimeric constructs and various mutations showed that two amino acid residues (at positions 876 and 891) in the 1a protein were the determinants for the inability of HL-CMV to support a satRNA. Specifically, Thr at position 876 had a more pronounced effect than Met at position 891. Specific changes in RNA sequence were also detected in the 3′ terminus of Y-sat and these particular alterations allowed it to be supported by HL-CMV. It is believed that, through evolution, the adaptation of CMV to lily resulted in the introduction of amino acid changes in the 1a protein, changes that coincidentally affected the ability of lily CMV to support satRNAs.

INTRODUCTION

Cucumber mosaic virus (CMV) is in the family *Bromoviridae* and is the type member of the genus *Cucumovirus*. The genome of CMV consists of three capped, plus-sense, single-stranded RNAs (RNAs 1–3). RNAs 1 and 2 encode the 1a and 2a proteins, respectively, which are components of the CMV replicase complex (Hayes & Buck, 1990). RNA3 encodes the 3a protein, which is known as the viral movement protein (MP). MP is involved in cell-to-cell movement and the viral coat protein (CP) is required for encapsidation and long-distance movement (Boccard & Baulcombe, 1993; Li *et al.*, 2001; Suzuki *et al.*, 1991). The CP is translated from the subgenomic RNA4, which is transcribed from the 3′ half of RNA3 (Schwinghamer & Symons, 1975, 1977). The 2b protein is translated from the subgenomic RNA4A molecule, which is generated from RNA2 (Ding *et al.*, 1994), and has been shown to be a suppressor of post-transcriptional

gene silencing (Brigneti *et al.*, 1998; Li *et al.*, 1999; Senda *et al.*, 2004).

CMV has a broad host range and a large number of CMV isolates have been reported (Palukaitis & García-Arenal, 2003). However, CMV isolates from lily are capable of infecting only a small number of host plant species, due to their specific adaptation to lily plants (Masuta *et al.*, 2002; Ryu *et al.*, 2002) and typical CMV strains are incapable of systemic spread in lily. CMV strains are divided into three groups (subgroups IA, IB and II) and these categories are based upon serological relationships and nucleic acid similarities (Roossinck, 2002; Roossinck *et al.*, 1999). Although CMV was isolated independently from different lily species in separate countries (Japan and Korea), RNA3 from these isolates showed a very high sequence similarity (Masuta *et al.*, 2002; Ryu *et al.*, 2002). Chen *et al.* (2001) also reported that nucleotide sequences of CMV isolated from lily in the Netherlands, France and Taiwan had high conservation of the CP gene sequence. These reports indicate that sequences of lily strains of CMV are similar, regardless of their geographical origins. Furthermore, these data suggest that lily strains of CMV became adapted to lily in their evolutionary history.

Several studies have mapped host-range determinants to RNA3 (Carrère et al., 1999; Ryu et al., 1998). However, some reports suggest that RNAs1 and 2 are responsible for further restriction in host range. For example, a local, hypersensitive response to CMV infection was mapped to the 2a gene in RNA2 (Kim & Palukaitis, 1997). Studies on a distantly related virus (Brome mosaic virus), the type member of the family Bromoviridae, also showed that host-specificity determinants existed on RNAs1 and 2, as well as on RNA3 (Allison et al., 1988; Mise et al., 1993). The determinants of the systemic infection in edible lily were mapped to the 5' untranslated region (UTR) and the region between the FbaI and SpeI sites in HL-CMV RNA1 (Yamaguchi et al., 2005).

Satellite RNAs (satRNAs) depend on helper viruses for replication and encapsidation and usually attenuate the diseases induced by the helper virus (Roossinck et al., 1992). Some satRNAs of CMV exacerbate the pathogenicity of CMV in specific hosts. For example, Y-satellite RNA (Y-sat) was found to induce bright yellow symptoms on tobacco plants (Takanami, 1981). In tomato, lethal necrosis is induced by CMV containing particular satRNAs (Kaper & Waterworth, 1977). Specific satRNA sequences involved in inducing such symptoms have been identified (Devic et al., 1989; Hidaka & Hanada, 1994; Jaegle et al., 1990; Kuwata et al., 1991; Masuta & Takanami, 1989; Sleat & Palukaitis, 1990).

There are some reports on the CMV strains that cannot support satRNAs (Gal-On et al., 1995; McGarvey et al., 1995; Roossinck & Palukaitis, 1991). For example, Sny-CMV can support replication of WL1-satRNA in tobacco, but not in zucchini (Gal-On et al., 1995; Roossinck & Palukaitis, 1991). Roossinck et al. (1997) found that the amino acid residue at position 978 of the 1a protein in Sny-CMV was important for WL1-satRNA replication in zucchini. It was apparent that lily CMV isolates were unable to support the replication of satRNAs in any of the host plants tested. In this study, we identified CMV sequences responsible for the inability of HL-CMV to support Y-sat.

METHODS

Virus isolates. HL-CMV was originally isolated from an edible lily (Lilium leichtlinii cv. Hakugin) in Hokkaido, Japan (Hagita, 1989; Hagita et al., 1989). Ly2-CMV and Li-CMV were isolated from Lilium longiflorum and Lilium tsingtauense, respectively, in Korea (Jung et al., 2000; Ryu et al., 2002). TW11-CMV and TW12-CMV were isolated from L. longiflorum in Taiwan. Y-CMV (Suzuki et al., 1991) has been maintained by successive inoculation onto tobacco at Hokkaido University, Japan. Two satRNAs, designated SA-sat and T73-sat, were isolated from soybean and tomato, respectively (Hanada et al., 1989; Masuta et al., 1990). These satRNAs and Y-sat (Takanami, 1981) were propagated in Nicotiana benthamiana.

Virus inoculation. The virus-free lily cultivar Hakugin, which had been grown from tissue-cultured bulbs, was supplied by the Hokkaido Central Agricultural Experiment Station (Naganuma-Cho, Hokkaido, Japan). The plants were dusted with carborundum, rub-inoculated and maintained in a greenhouse under natural light conditions (10–16 h) at 20–28 °C. Viral infection and satRNA

replication were confirmed by several methods, including ELISA, RT-PCR, immunocapture RT-PCR (IC-RT-PCR) and Northern blot hybridization.

IC-RT-PCR. Samples from infected plants were homogenized in 10 mM sodium phosphate (pH 7·5). Homogenates were incubated for 1 h at 37 °C in a 96-well plate that was previously coated with anti-CMV antibody. After two subsequent washing steps with PBS/Tween buffer (1× PBS, 0·5 g Tween 20 l^{-1}), RT-PCR was performed as described previously (Ryu et al., 2002) by using the Core RT-PCR system (Perkin Elmer). In order to detect CMV and Y-sat, primer pairs consisting of CMCP5 (GAGTCATGGACAAATC-TGAATC) and CMCP3 (GGAACACGGAATCAGACTGG), and Y-sat5 (GATGGAGAATTGCGTAGAGG) and Y-sat3 (GGAGAATG-TATATAGACATTCAC), respectively, were used for the IC-PCR.

Cloning of the full-length cDNAs of HL-CMV and production of pseudorecombinant viruses. Pseudorecombinant viruses were created between HL-CMV and Y-CMV. The three genomic RNAs of HL-CMV and Y-CMV were designated H1–H3 and Y1–Y3, respectively. Cloning of the full-length cDNA for H1, H2 and H3 was described previously (Masuta et al., 2002; Yamaguchi et al., 2005). The transcripts were synthesized from the cDNA clones by T7 RNA polymerase (TaKaRa) in the presence of the m7G(5')ppp(5')G cap analogue (Invitrogen). Transcripts for each genomic RNA were mixed in various combinations and inoculated onto N. benthamiana.

Frameshift construct. For the construction of Yfs1d, the biologically active clone of Y-CMV RNA1 was digested with XbaI at nucleotide position 383 and the termini were blunt-ended by using a Blunting High kit (Toyobo). The 3' ends following nt 3138–3361 of Y1d and Yfs1d were deleted to restrict Y1 replication.

Viral transfection of protoplasts. Protoplasts were prepared from leaves of N. benthamiana and were used as the source of material for viral transfection. Specifically, the abaxial epidermis of the leaves was removed by peeling with forceps and the peeled leaves were immersed in an enzyme solution containing 2 % cellulase Onozuka R-10 (Yakult Honsha), 0·5 % Macerozyme R-10 (Yakult Honsha) and 0·5 M mannitol (pH 5·8). After incubation at 25 °C for 3 h, the protoplasts were collected by centrifugation at 400 r.p.m. for 3 min and were washed twice with 0·5 M mannitol solution. Approximately 1×10^6 protoplasts were inoculated with in vitro RNA transcripts in the presence of polyethylene glycol. After the mixtures were maintained for 15 min at 0 °C, the inoculated protoplasts were collected and washed with 0·5 M mannitol. Samples were subsequently incubated for 12, 20 or 24 h in the dark at 25 °C in incubation buffer (0·2 mM KH_2PO_4, 1 mM KNO_3, 1 mM $MgSO_4$, 1 μM KI, 0·1 μM $CuSO_4$, 10 mM $CaCl_2$ and 10 % mannitol) and the accumulation of Y-sat was detected by Northern blot hybridization analysis.

Northern blot anlysis. Detection of viral RNAs in the protoplasts was accomplished by Northern blot hybridization essentially as described previously (Masuta et al., 1998). Total RNAs were extracted from the infected protoplasts by using TRIzol reagent (Invitrogen) according to the supplier's instructions. In the satRNA detection, digoxigenin (DIG)-labelled RNA probes (in sense and antisense orientations) were in vitro-transcribed from the PCR-amplified Y-sat sequence with T7 polymerase. In the detection of CMV RNAs in protoplasts, a DIG-labelled antisense RNA was in vitro-transcribed from the PCR-amplified RNA4 sequence with T7 polymerase. For detection of CMV genomic RNAs in the upper leaves, Northern blot hybridization was performed to detect the genomic RNAs by using a DIG-labelled DNA probe that corresponded to the 3'-end sequence of Y-CMV RNA3 (352 bp).

RNA1 chimeric constructs. The chimeric structures of the RNA1 recombinants were created by using four unique restriction enzymes

(*Xba*I, *Fba*I, *Spe*I and *Stu*I). The *in vitro* transcripts from the RNA1 chimeric constructs were mixed with Y2 and Y3 and were inoculated onto *N. benthamiana*.

Point-mutation constructs. Point mutations at positions 862, 876 and 891 were introduced by recombinant PCRs using the following primer pairs: 5′-terminal primer (CCCTTCCGAAAGGAACCC-ATTC) and 3′-terminal primer (GAATGGGTTCCTTTCGG-AAGGG), 5′-terminal primer (GTTTCTCAATCTAAAGTGAGAAG) and 3′-terminal primer (CTTCTCACTTTAGATTGAGAAAC), and 5′-terminal primer (CGCTAGTGTGACATTGGTTGACTTG) and 3′-terminal primer (CAAGTCAACCAATGTCACACTAGCG), respectively. Underlined bases in the aforementioned primers reflect the nucleotides that were mutated. PCR products were subcloned into the pGEM-T Easy vector (Promega) and were sequenced fully in order to confirm their sequence integrity. The 200 nt fragment between the *Spe*I and *Stu*I sites containing the mutation was substituted for the corresponding region in pCHL1.

PVX vector construct. The PVX vector (Baulcombe *et al.*, 1995) was obtained from D. C. Baulcombe, The Sainsbury Laboratory, Norwich, UK, and Y-sat was amplified by RT-PCR. Plus- and minus-sense sequences of Y-sat were cloned into the PVX vector in order to create PVX-Y-sat(+) and PVX-Y-sat(−), respectively. The *in vitro* transcripts were then inoculated onto *N. benthamiana* leaves.

RT-PCR. Total nucleic acids were extracted from the infected leaves by the conventional phenol/chloroform method and reverse transcription was accomplished by using an RNA LA PCR kit (TaKaRa) according to the supplier's instructions. In order to amplify CMV RNA1, we used the primer pair 5′-terminal primer (GACTT-GTAAGTCTCTCTACTGGCG) and 3′-terminal primer (TGGTCT-CCTTGTGGAGCC), and we used the primer pair 5′-terminal primer (T7 promoter-GTTTTGTTTGATGGA) and 3′-terminal primer (GGGTCCTGTGTAGGAATGATAGACATTC) for amplification of Y-sat.

Determination of Y-sat(HL) sequence. Total nucleic acids were extracted from the plants in which Y-sat was detected. Single- and double-stranded RNAs of Y-sat(HL) were isolated from the agarose gels (1·5 % LowMelt agarose gel; BioExpress). The RNAs were boiled for 5 min and were then added to the reaction solution [40 mM Tris/HCl (pH 8·0), 8 mM MgCl$_2$, 5 mM dithiothreitol, 2 mM spermidine, 100 mM ATP and 10 U poly(A) polymerase (TaKaRa)]. After incubation at 37 °C for 30 min, the poly(A)-tailed RNAs were primed with an oligo(dT)$_{20}$-M4 adaptor primer (TaKaRa) at 40 °C for 1 h. A series of PCR fragments was then amplified by using pairs of several internal specific primers and the M4 primer (GTTTTCCCAGTCACGAC). The PCR products were cloned into the pGEM-T Easy vector (Promega) and sequenced for verification.

Dot-blot hybridization. DIG-labelled DNA probes were prepared from the Y-sat clones by PCR using the primer pair described above. Total nucleic acid extracts were mixed with an equal volume of the sample buffer containing 1× MOPS, 50 % formamide and 17·5 % formaldehyde. After denaturing at 65 °C, the samples were spotted onto Biodyne nylon membrane (Pall) and the blots were cross-linked with UV illumination for 5 min. The pre-hybridization buffer contained 5× SSC, 50 % formamide, 7 % SDS, 2 % blocking reagent (Roche Diagnostics) and 0·1 mg herring sperm ml^{-1} (Invitrogen). The blots were then hybridized with the probes overnight at 65 °C and positive signals were detected by using CDP-Star reagent according to the manufacturer's instructions (Tropix).

Computer analysis. Analysis of the 1a protein was performed by the PredictProtein program (http://www.predictprotein.org/), which is operated by the Columbia University Bioinformatics Center. Program SEG, which was developed by Wootton & Federhen (1996), was also utilized for data analysis.

RESULTS

Inoculation with CMV lily strains plus satRNAs

Due to the fact that the CMV lily strains that we collected did not contain any satRNAs, we investigated whether CMV lily isolates were capable of supporting satRNAs. Five CMV lily isolates (HL, Ly2, Li, TW11 and TW12) from different geographical origins were tested for their ability to support satRNAs (Table 1). Y-sat was inoculated onto *N. benthamiana* plants in the presence of the various CMV isolates. On non-inoculated upper leaves, Y-CMV and HL-CMV induced mosaic symptoms and mild mosaic symptoms, respectively [see Supplementary Fig. S1(a) in JGV Online]. Y-CMV plus Y-sat induced bright yellow symptoms, a phenomenon that was indicative of Y-sat replication [see Supplementary Fig. S1(a)]. We then analysed accumulation of Y-sat in the *N. benthamiana* plants inoculated with HL-CMV plus Y-sat. In the inoculated leaves, we hardly detected Y-sat by dot-blot hybridization. As a means to detect systemic accumulation of satRNAs, total RNAs were extracted from the non-inoculated upper leaves and satRNA was assayed by both dot-blot hybridization and RT-PCR 45 days after inoculation [see Supplementary Fig. S1(b) in JGV Online]. IC-RT-PCR was used to detect Y-sat in the plants inoculated with Li-CMV and Ly2-CMV plus Y-sat [see Supplementary Fig. S1(c) in JGV Online]. Y-sat was not detected in any of the plants that were inoculated with the five CMV lily strains (Table 1). To determine whether other satRNAs were supported by CMV lily strains, two satRNAs (SA-sat and T73-sat) were inoculated with HL-CMV or Ly2-CMV, but neither satRNA was detected in upper leaves (Table 1). These results suggest that

Table 1. Detection of satRNA in the *N. benthamiana* plants inoculated with satRNAs plus CMV lily strains

CMV strain	Country of origin	SatRNA detection		
		Y-sat	SA-sat*	T73-sat†
HL	Japan	0/20‡	0/6	0/6
Ly2	Korea	0/18	0/4	0/4
Li	Korea	0/18	−	−
TW11	Taiwan	0/12	−	−
TW12	Taiwan	0/12	−	−

*A 386 nt satRNA isolated from soybean. SA-sat contains the tomato necrogenic consensus sequence (NCS).

†A 330 nt satRNA isolated from tomato. T73-sat does not contain NCS.

‡No. satRNA-positive plants/no. inoculated plants. SatRNAs were inoculated with each CMV and detected by both dot-blot hybridization and RT-PCR 45 days after inoculation.
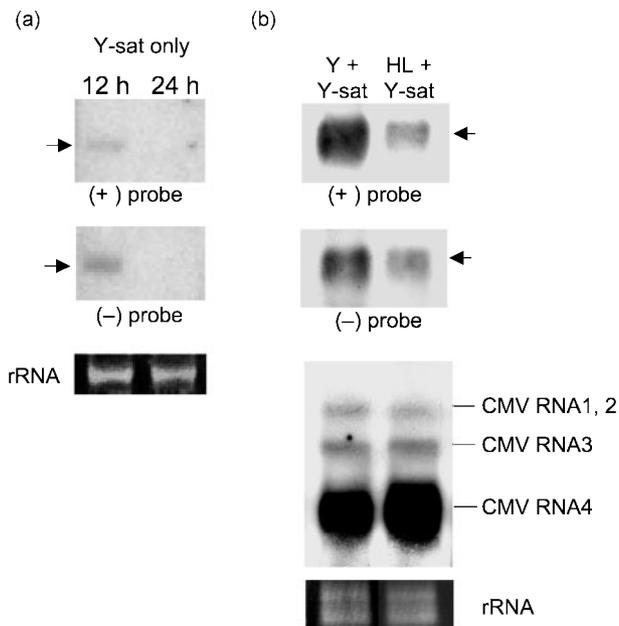
Fig. 1. Analysis of Y-sat accumulation in *N. benthamiana* proto-plasts transfected with either Y-CMV or HL-CMV together with Y-sat. (a) Y-sat stability in protoplasts. Y-sat (without a helper virus) was inoculated into protoplasts and total RNAs were extracted at 12 and 24 h post-inoculation. Y-sat was detected by Northern blot hybridization using the DIG-labelled sense (+) or antisense (−) RNA probe. Arrows indicate the satRNA positions. Exposure time was 10 min. rRNAs are shown as a loading control (bottom panel). (b) Y-sat accumulation in proto-plasts inoculated together with a helper virus. Total RNAs were extracted at 20 h post-transfection. Y-sat was detected as above. A prolonged exposure time (20 min) was taken to visua-lize CMV RNAs 1 and 2. CMV genomic RNAs of Y and HL were detected by Northern blot hybridization using the DIG-labelled antisense (−) RNA probe, which recognizes the RNA4 of Y and HL, respectively (third panel). rRNA is shown as a loading control (bottom panel).

lily strains of CMV cannot support the accumulation of (at least three) satRNAs, regardless of their geographical origin.

Replication of Y-sat in protoplasts

In order to investigate the mechanism for the inability to support a satRNA, we first analysed the accumulation of Y-sat by using protoplasts. Specifically, we tested whether the viral replicase is capable of synthesizing the (−) strand from the (+) strand or, conversely, the (+) strand from the (−) strand. Y-sat was then transfected into protoplasts of *N. benthamiana* together with either Y-CMV or HL-CMV. This allowed us to compare the replication of satRNA in the presence of a helper CMV (Y and HL). As shown in Fig. 1(a and b), neither the (−) strand nor the (+) strand was detected well enough beyond the levels of residual inoculum (Y-sat), suggesting that HL-CMV was not able to recognize the 3′ end of Y-sat.

Table 2. Detection of satRNA in the *N. benthamiana* plants inoculated with satRNAs plus CMV lily strains

Pseudorecombinant*	Systemic infection in lily†	Y-sat detection (A_{405})‡
Y1H2H3	−	20/20 (1·39)
H1Y2Y3	+	0/15 (1·59)
H1Y2H3	+	0/10 (1·23)
Y1H2Y3	−	13/13 (1·36)
H1H2Y3	+	0/10 (1·37)
Y1Y2H3	−	10/10 (1·22)
H1H2H3	+	0/20 (1·24)
Y1Y2Y3	−	32/32 (1·47)

*The three genomic RNAs of Y-CMV and HL-CMV were designated Y1–Y3 and H1–H3, respectively.
†CMV was detected (+) or not (−) in the systemic leaves of lily plants (Yamaguchi *et al.*, 2005).
‡No. Y-sat-positive plants/no. inoculated plants. Y-sat was detected by both dot-blot hybridization and RT-PCR 10 days after inocula-tion. Mean ELISA values (A_{405}) of the infected plants are shown in parentheses.

Mapping viral genomic RNA for the inability to support Y-sat

To determine which viral genomic RNA(s) was essential for the inability to support the accumulation of Y-sat, eight different combinations of the synthesized RNA transcripts from the infectious clones were tested (Table 2). All pseudo-recombinant viruses were co-inoculated onto *N. benthami-ana* together with Y-sat. Total RNAs were extracted from the non-inoculated upper leaves 10 days after inoculation and Y-sat accumulation was detected by both dot-blot hybridization and RT-PCR analyses. H1Y2Y3, H1Y2H3, H1H2Y3 and H1H2H3 could not support the accumula-tion of Y-sat. On the contrary, Y1H2H3, Y1H2Y3, Y1Y2H3 and Y1Y2Y3 could support the accumulation of Y-sat and they induced bright yellow symptoms. Consequently, all of the pseudorecombinant viruses that contained RNA1 of Y-CMV supported the accumulation of Y-sat, suggesting that the failure to support the replication of Y-sat in *N. benthamiana* mapped to RNA1 of HL-CMV.

Y-sat replication in the protoplasts transfected with a frameshift mutant

To confirm whether it is the translated 1a protein from Y1 that is important for satRNA replication, rather than the Y1 RNA, we used a frameshift mutant (Yfs1d) in which the 1a protein was truncated and the 3′ end was deleted so that it could not be replicated *in trans*. As a control for this experiment, we created Y1d, which has the same 3′-end deletion as Yfs1d, but was capable of being translated and produced a functional 1a protein (Fig. 2a). *In vitro* RNA transcripts from these constructs were inoculated onto

N. benthamiana protoplasts together with H1H2H3 plus Y-sat. Total RNAs were extracted at 12 or 24 h post-transfection (p.t.) and Y-sat was detected by Northern blot hybridization analysis (Fig. 2b); CMV accumulation was confirmed by ELISA (Fig. 2c). At 12 h p.t., Y-sat was detected clearly in the Y1d-inoculated protoplasts. On the other hand, at 12 h p.t., Y-sat was barely detected in the Yfs1d-inoculated protoplasts and had disappeared by 24 h p.t. The experiments show that expression of the 1a protein from Y-CMV is sufficient and necessary for complementation of satRNA accumulation by HL-CMV.

Sequence of the domain responsible for the inability to support Y-sat

We constructed various chimeric RNA1 recombinants to test which affects the Y-sat support (Fig. 3). The synthesized transcripts generated from the recombinant clones were inoculated onto *N. benthamiana* plants together with the transcripts from the cDNA clones of Y-CMV RNA2, Y-CMV RNA3 and Y-sat, and total RNAs were extracted from the non-inoculated upper leaves 10 days after inoculation. Y-sat was detected by both dot-blot hybridization and RT-PCR. HL, YpH, HxYfH, HxYpH and HtY could not support the replication of Y-sat, whereas Y, HxY, YfHpY, YtH, HpYtH, HpY and HxYfHpY did support the replication of Y-sat and induced bright yellow symptoms (Fig. 3). Based upon these results, the inability to support the replication of Y-sat in *N. benthamiana* was mapped to the region between the *Spe*I and *Stu*I sites within the 3′ region of HL-CMV RNA1. Sequencing of the progeny of RNA1 recombinants revealed that there were no nucleotide changes in the region. In comparison to CMV-Y, nucleotide sequence analysis between the *Spe*I and *Stu*I sites revealed that there were seven nucleotide changes, with three amino acid differences in the 1a protein.

Point mutations to support Y-sat

To determine which amino acid residue(s) between the *Spe*I and *Stu*I sites of HL-CMV RNA1 was responsible for the inability to support the replication of Y-sat, point mutations were introduced into the 1a protein of HL-CMV via site-directed mutagenesis (as shown in Fig. 4). There are three amino acids that are different between HL and Y in this region. The amino acid transitions were as follows: for HLpm1, Arg to Lys at position 862; for HLpm2, Thr to Lys at position 876; for HLpm3, Met to Leu at position 891; for HLpm123, all three transitions; for HpYtHpm1, Lys to Arg (862); for HpYtHpm2, Lys to Thr (876); for HpYtHpm3, Leu to Met (891). The synthesized transcripts that were generated from the constructs were inoculated onto *N. benthamiana* together with the transcripts from the cDNA clones of Y-CMV RNA2, Y-CMV RNA3 and Y-sat. The results of ELISA and Northern blot hybridization indicated that these mutant constructs accumulated to a similar level in *N. benthamiana* (Fig. 4; Supplementary Fig. S2 in JGV Online). Total RNAs were extracted from the non-inoculated upper leaves 10 and 45 days after inoculation. Y-sat was detected by both dot-blot hybridization and RT-PCR analyses (Fig. 4). HLpm1, HLpm3 and HpYtHpm2 could not support the replication of Y-sat throughout the experiments. HLpm2 and HpYtHpm3 could not support the replication of Y-sat 10 days after inoculation, but Y-sat was detected 45 days after inoculation. Sequencing of RNA1 of HLpm2 and Y-sat revealed that there were no
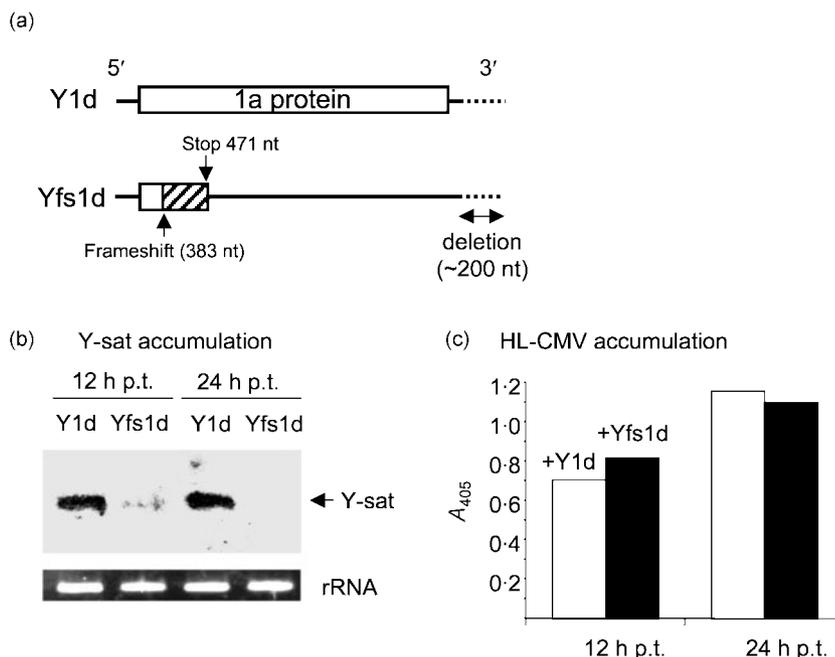
Fig. 2. Analysis of Y-sat accumulation in *N. benthamiana* protoplasts inoculated with either Y1d or Yfs1d together with H1H2H3 plus Y-sat. (a) Schematic representation of the two Y1 constructs. A frameshift mutation was introduced in the 1a protein of Yfs1 (at nt 383). To restrict Y1 replication, the 3′ ends of Y1 and Yfs1 were deleted by ~200 nt, creating Y1d and Yfs1d, respectively. After position 383, the authentic 1a protein cannot be translated. (b) Analysis of Y-sat accumulation in *N. benthamiana* protoplasts transfected with either Y1d or Yfs1d together with H1H2H3 plus Y-sat. Total RNAs were extracted at 12 or 24 h post-transfection (p.t.). Accumulation of Y-sat was detected by Northern blot analysis using the DIG-labelled antisense (−) RNA probes. Exposure time was 10 min. rRNA is shown as a loading control (bottom panel). (c) Accumulation of HL-CMV in protoplasts transfected with HL-CMV plus Y1d or Yfs1d. Virus was detected by ELISA (A_{405}) at 12 and 24 h p.t. and mean values of two replicates are shown.

RNA1 recombinants

N. benthamiana
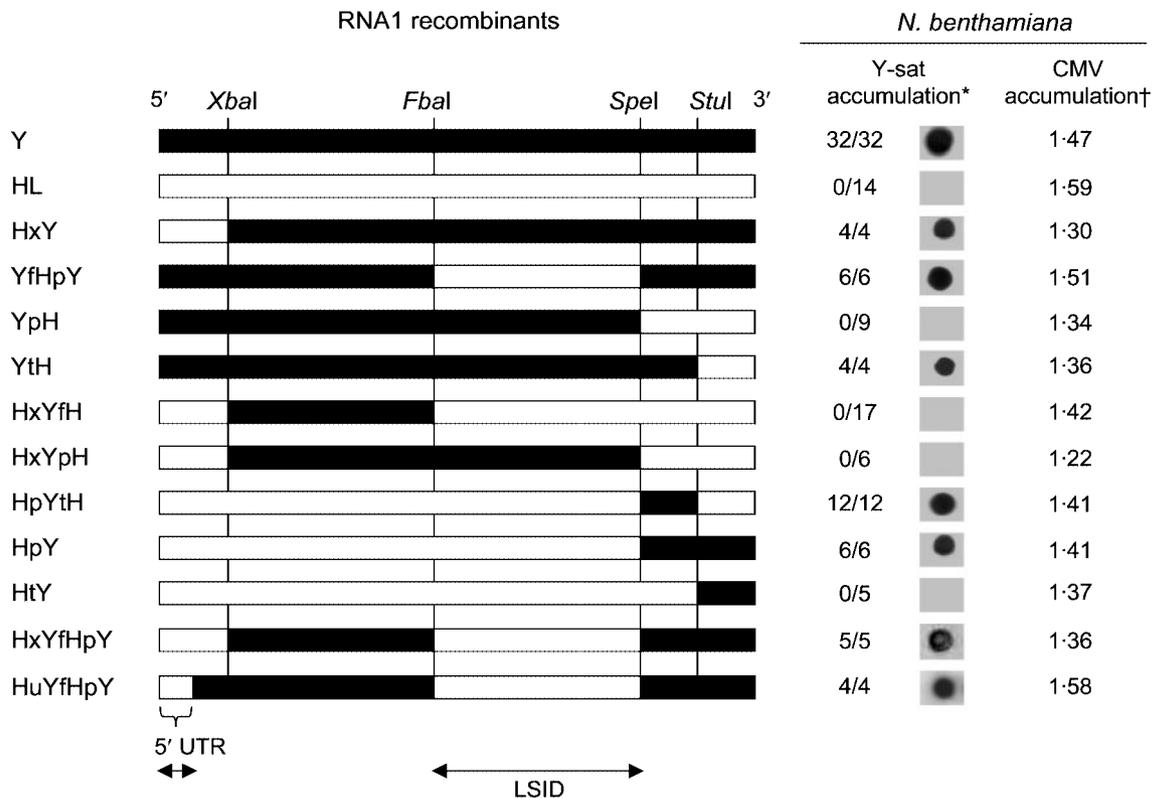
Fig. 3. Chimeric structures of the RNA1 recombinants and their ability to support a satRNA. The restriction enzymes used to create the constructs were *Xba*I (nt 381), *Fba*I (nt 1547), *Spe*I (nt 2599) and *Stu*I (nt 2821). CMV-Y sequences are represented as filled boxes and HL-CMV sequences as open boxes. Transcripts from the recombinant clones were inoculated onto the *N. benthamiana* plants together with Y-CMV RNA2, Y-CMV RNA3 and Y-sat. Total RNAs were extracted from the non-inoculated upper leaves 10 days after inoculation. The sap was inoculated onto the lily plants. CMV replication was confirmed by ELISA (A_{405}) 20 days after inoculation. The lily systemic-infection domain (LSID) is shown, which is essential for the systemic infection of HL-CMV in edible lily (Yamaguchi *et al.*, 2005). *, No. Y-sat-positive plants/no. inoculated plants; dot-blot hybridizations were performed to confirm the replication of Y-sat. †, Mean ELISA values (A_{405}) of the infected plants.

nucleotide changes. The sap from this plant was reinoculated onto eight plants and Y-sat was detected only in two plants out of eight at 10 days post-inoculation. As a result, it was concluded that Y-sat was not supported in the other six plants. HpYtHpm3 supported the replication of Y-sat in two of the five inoculated plants at 45 days after inoculation. The sap from one of the two plants was reinoculated onto five plants and, after a 10-day period, Y-sat was detected only in four plants. There were no nucleotide changes in the Y-sat progeny. It is important to note that only HpYtHpm1 could support the replication of Y-sat at 10 days after inoculation. Therefore, the inability to support the accumulation of Y-sat in *N. benthamiana* was mapped to Thr at position 876 and Met at position 891 in the 1a protein of HL-CMV. However, we still need to study the involvement of the other four (silent) nucleotide changes that are found in the domain between the *Spe*I and *Stu*I sites.

To test whether these mutants can infect edible lily systemically, HLpm2 and HpYtHpm1 were inoculated onto lily plants. CMV was detected by ELISA and RT-PCR 20 days after inoculation and the results showed that both constructs could infect lily plants systemically.

Accelerated generation of Y-sat supported by HL-CMV

As shown by the schematic illustrations of various constructs in Fig. 5(a), a PVX vector was used to express Y-sat. Plus- and minus-sense sequences of Y-sat were cloned into the PVX vector in order to create PVX-Y-sat(+) and PVX-Y-sat(−), respectively. The synthesized transcripts generated from the PVX vector constructs were inoculated onto *N. benthamiana* and thereby enabled Y-sat sequences to be supplied continuously in the PVX-infected plants. Seven days after PVX inoculation, HL-CMV was super-inoculated onto the same plants that had been pre-infected with the PVX vector constructs. satRNA replication by CMV could be discriminated by the detection of the strand complementary to that expressed from the PVX vector,

Fig. 4. Point mutations in the 1a protein of HL-CMV.

Point-mutation constructs — *N. benthamiana*

Gain of function by a single mutation (HL to HLpm2)

Positions 862, 876, 891 (between SpeI and StuI sites, 5′ to 3′)

Construct	862	876	891	Y-sat accumulation 10 d.p.i.	45 d.p.i.	CMV accumulation
HL	R	T	M	0/14	0/14	1·59
HLpm1	K	T	M	0/5	0/5	1·53
HLpm2	R	K	M	0/8	1/8‡	1·31
HLpm3	R	T	L	0/4	0/4	1·34
Y	K	K	L	32/32	32/32	1·47

Loss of function by a single mutation (HpYtH to HpYtHpm2)

Construct	862	876	891	Y-sat accumulation 10 d.p.i.	45 d.p.i.	CMV accumulation
HpYtH	K	K	L	12/12	12/12	1·41
HpYtHpm1	R	K	L	4/5	5/5	1·36
HpYtHpm2	K	T	L	0/5	0/5	1·16
HpYtHpm3	K	K	M	0/5	2/5	1·15

Transcripts were inoculated onto *N. benthamiana* plants together with Y-CMV RNA2, Y-CMV RNA3 and satRNA. Total RNAs were extracted from the non-inoculated upper leaves 10 and 45 days post-inoculation (d.p.i.). CMV replication was confirmed by ELISA (A_{405}) 20 days after inoculation. *, No. Y-sat-positive plants/no. inoculated plants; dot-blot hybridizations were performed to confirm the replication of Y-sat. †, Mean ELISA value (A_{405}) of infected plants. The results of Northern blot hybridization are shown in Supplementary Fig. S2, available in JGV Online. ‡, The sap from the plant showing the Y-sat symptoms was inoculated successively onto eight new plants. Only two plants showed the Y-sat symptoms.

either by Northern hybridization or by dot-blot hybridization. The plants infected with Y-CMV and the PVX vector constructs induced bright yellow symptoms within 1 month of Y-CMV inoculation. On the other hand, the plants infected with HL-CMV and the PVX vector constructs did not show such bright yellow symptoms within 1 month, indicating no Y-sat accumulation. However, when observed for 2 months after HL-CMV inoculation, three out of the four plants infected with HL-CMV plus PVX-Y-sat(−) induced bright yellow symptoms, indicating that Y-sat accumulation occurred. Total RNAs were extracted from the plants as a method to confirm Y-sat accumulation. Sequencing of the amplified Y-sat revealed three nucleotide differences at the 3′ end, compared with the authentic Y-sat (Fig. 5b). We designated this mutant as Y-sat(HL). Y-sat(HL) was isolated from the plants infected with HL-CMV plus PVX-Y-sat(−), but it was not generated with HL-CMV plus PVX-Y-sat(+). We then confirmed that HL-CMV could support the replication of the transcript from a cDNA clone of Y-sat(HL). The 3′ end of Y-sat(HL) actually reverted in the progeny Y-sat when a transcript of Y-sat(HL) was inoculated with Y-CMV. We also tested whether Y-sat(HL) amplified by HL-CMV was maintained

in lily by inoculating it onto the surface of lily leaves. Although Y-sat(HL) was created in *N. benthamiana*, we detected Y-sat accumulation in the systemically infected lily leaves (data not shown).

DISCUSSION

None of the five lily CMVs tested, which were originally isolated in Japan, Korea and Taiwan, could support the replication of Y-sat in *N. benthamiana* (Table 1). None of the three satRNAs was detected in the plants that were co-inoculated with HL-CMV. At the present time, there are only a few reports on CMV strains that are unable to support satRNAs. For example, Sny-CMV supports the replication of WL1-satRNA in tobacco, but not in zucchini (Gal-On *et al.*, 1995; Roossinck & Palukaitis, 1991). HL-CMV is different from Sny-CMV in that it cannot support the replication of satRNAs in any plants that have been tested, including lily, *N. benthamiana* and *Nicotiana tabacum*. McGarvey *et al.* (1995) reported that Ix-CMV could not support the replication of a necrogenic satRNA, which causes lethal necrosis in tomato, whilst it could support the replication of a non-necrogenic satRNA. HL-CMV is
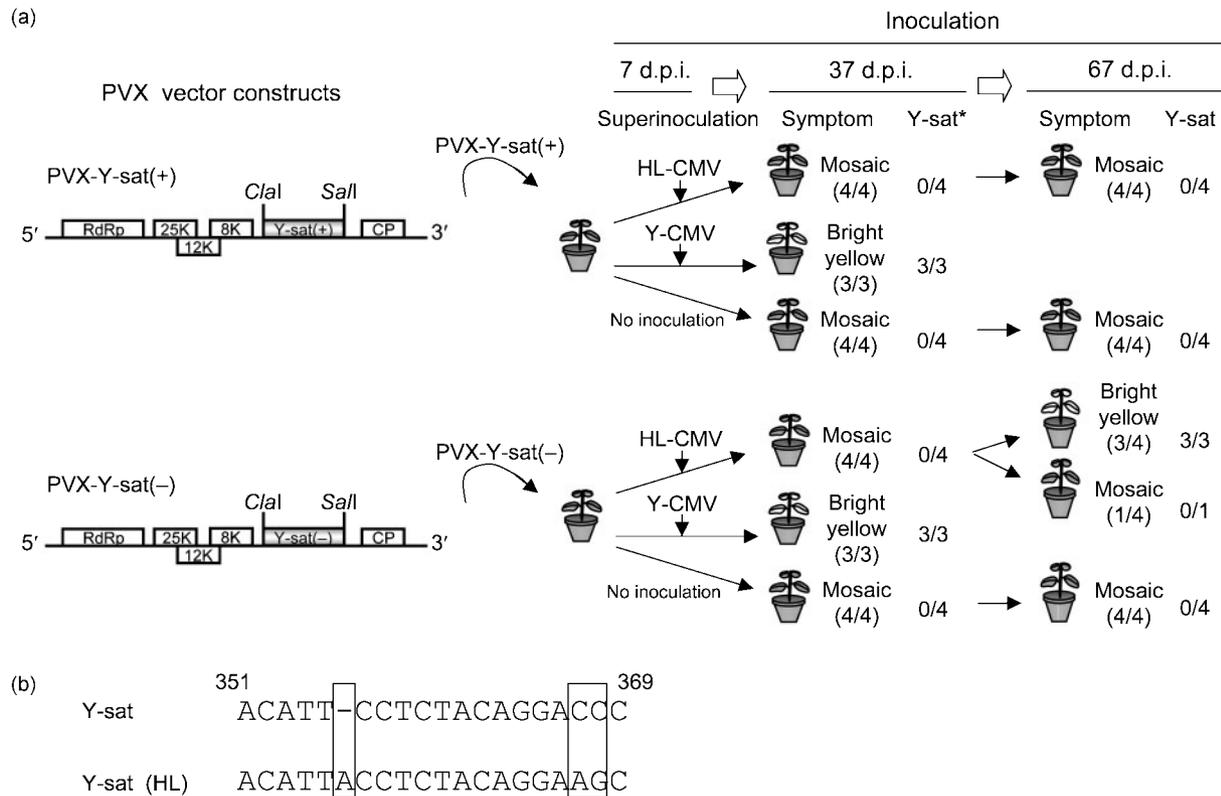
Fig. 5. Accelerated generation of Y-sat supported by HL-CMV in the plants inoculated with PVX-Y-sat(−) followed by HL-CMV inoculation. (a) The left column shows schematic illustrations of the PVX vector constructs to express Y-sat. *N. benthamiana* plants were inoculated first with the PVX vector constructs and then, 7 days later, with HL-CMV. Symptoms on the non-inoculated upper leaves were observed 30 and 60 days after CMV inoculation. *, No. Y-sat-positive plants/no. inoculated plants. Y-sat replication was confirmed by Northern blot. (b) Comparison of the nucleotide sequences at the 3′ end of Y-sat. Nucleotide changes observed after inoculation with HL-CMV plus PVX-Y-sat(−) are shown in open boxes. It has been confirmed that HL-CMV can support a transcript from a cDNA clone of Y-sat(HL), the Y-sat mutant eventually isolated from the *N. benthamiana* plants infected with HL-CMV plus PVX-Y-sat(−).

different from Ix-CMV in that it cannot support the replication of either necrogenic (Y-sat and SA-sat) or non-necrogenic satRNAs (T73-sat). Y- and SA-satRNAs contain the necrogenic consensus sequence (NCS) at their 3′ end, but T73-satRNA does not (Devic *et al.*, 1989; Hidaka & Hanada, 1994; Masuta & Takanami, 1989; Sleat & Palukaitis, 1990). For Ix-CMV, the NCS may be important for the viral replicase to recognize a satRNA.

In the next line of our investigation, we demonstrated that a frameshift mutation in the 1a protein of CMV-Y abolished the ability to support Y-sat (Fig. 2). This result revealed that the protein translated from Y1, rather than the Y1 RNA itself, was essential for Y-sat replication. Roossinck & Palukaitis (1991) showed previously that the inability of Sny-CMV to support WL1-satRNA in zucchini also mapped to RNA1. McGarvey *et al.* (1995) mapped the inability of Ix-CMV to support the replication of a necrogenic satRNA to RNA1. In addition, the inability of a strain of *Peanut stunt virus* (PSV-W) to support the replication

of PSV-satRNA in tobacco was also mapped to RNA1 (Hu *et al.*, 1998). As PSV is another cucumovirus, it is therefore concluded that the 1a proteins of cucumoviruses are important for satRNA replication. Beyond the data presented in the previously mentioned studies, we successfully identified two amino acid residues (at positions 876 and 891) in the 1a protein responsible for the inability of HL-CMV to support Y-sat in the present study. It is apparent that Thr at position 876 seems to be more important than Met at position 891. This conclusion was reached due to the observation that a single amino acid transition at position 876 allowed Y-sat to replicate with HL-CMV, although the ability was still poor (Fig. 4). Although Roossinck *et al.* (1997) suggested previously that the amino acid residue at position 978 in Sny-CMV was important for satRNA replication in squash, it is not important in the combination of HL-CMV and Y-sat in *N. benthamiana*.

To determine whether these mutations affect the structure of the 1a protein, we analysed the 1a protein of HL-CMV

by using an online program for secondary-structure prediction (PredictProtein). Among the predicted secondary structures, we found one unique difference in the locations of the low-complexity region (LCR) (Wootton & Federhen, 1996). Because LCR can signify interdomain regions of protein structures, LCR is usually found to reside between two domains. It is important to note that the 1a protein of HL-CMV and Ly2-CMV has an LCR between motifs IV and V in the helicase domain, separating the two motifs spatially (Fig. 6a). On the other hand, there is no LCR in the helicase domains of the other CMV strains (Fig. 6b). The structural changes in the helicase domain of lily CMVs caused by inserting an LCR may have coincidentally affected the ability to support satRNAs; motif IV was thought to be important for eIF-4A helicase activity (Buck, 1996). Although the amino acid residue at position 891 of Ly2-CMV was Leu instead of Met, Thr at position 876, which induces an LCR between motifs IV and V, was also found in Ly2-CMV (Fig. 6b).

Because the 1a protein of lily CMVs is important to viral systemic infection in lily (Yamaguchi et al., 2005), it is reasonable to consider that the 1a protein must possess some structural constraint to adapt to lily plants and this constraint would have resulted in selection for the amino acid changes at positions 876 and 891. The amino acids important for satRNA replication do not occur in the domain for systemic infection in lily, thereby suggesting

that they may not be essential for viral adaptation to lily (Yamaguchi et al., 2005). However, we believe that they are certainly preferred in the lily environment, because the 1a protein sequences of at least three lily CMV isolates available in GenBank [HL, Ly2 and LILY (accession no. AJ276582)] contain Thr at position 876, whereas the other CMVs contain a different amino acid (Lys).

In this study, we demonstrated that transient expression of Y-sat sequences through a PVX vector accelerated generation of Y-sat(HL) that was supported by HL-CMV (Fig. 5). Although all of the plants inoculated with HL-CMV plus PVX-Y-sat(+) induced simple mosaic symptoms without Y-sat, three out of the four plants inoculated with HL-CMV plus PVX-Y-sat(−) showed bright yellow symptoms with Y-sat (Fig. 5a). Continuous supply of the minus strand of Y-sat resulted in the generation of the replicative Y-sat [Y-sat(HL)] in the presence of HL-CMV. Sequencing of Y-sat(HL) revealed that there were three nucleotide changes at the 3′ end (Fig. 5b). Considering that the synthesis of the (−) strand of Y-sat was hardly detected in the protoplast experiment, it is likely that the HL replicase may not recognize the 3′ end of Y-sat efficiently. It is conceivable that the replicase could bind to the 3′ end of the (−) RNA and this binding would allow it to copy the satRNAs. The 3′ end of (+) satRNA will eventually be selected to be fit for replication by accumulation of random mutations. In fact, Burgyán & García-Arenal (1998) reported
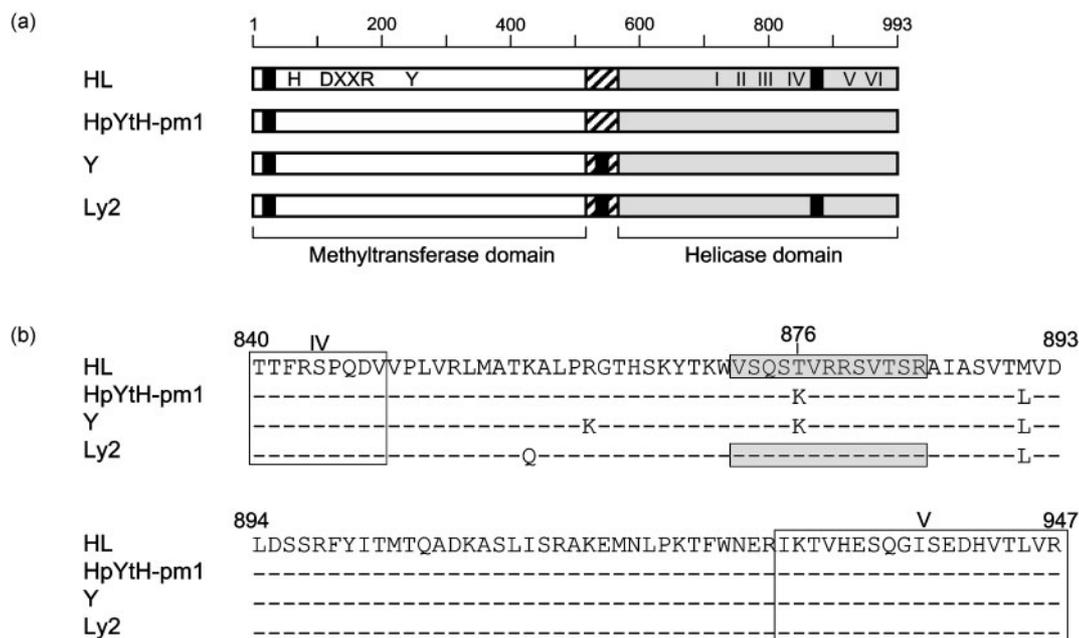
Fig. 6. SEG-predicted low-complexity region (LCR) in CMV 1a proteins. (a) Locations of LCR and highly conserved motifs in 1a proteins. LCR that can signify interdomain regions are shown as filled black boxes. The highly conserved motifs for methyltransferase and helicase are shown as H, DXXR and Y (located in the methyltransferase domain) and I–VI (located in the helicase domain). Amino acid positions in the 1a protein are shown. Point mutations were introduced in the 1a protein of HL-CMV to create HpYtH-pm1. (b) Comparison of the 1a amino acid sequence between motifs IV and V (shown in open boxes). Identical amino acid residues are shown by hyphens. LCR are shown in shaded boxes.

previously that the 3′ end of satRNA lacking the last three Cs was repaired *in planta* in the presence of CMV, indicating that CMV has an ability to restore the 3′ end of a satRNA. In addition, Moriones *et al.* (1991) reported that minor sequence changes might have a major effect on the fitness of sequence variants of satRNAs in different hosts.

However, unless a satRNA and its helper virus at least coexist, no satRNA variants will be generated. It is important to consider here the rather peculiar lily propagation through bulbs; lily CMV isolates are transmitted vertically to progeny plants, although they are occasionally carried by aphids. Once a CMV strain has adapted to lily plants by changing some amino acid residues in the 1a protein and, accordingly, lost the ability to support satRNAs, any challenge inoculation by foreign CMV may be defeated through cross-protection. This can be concluded because most ordinary strains of CMV cannot infect lily plants efficiently. There is thus little chance that a satRNA can be incorporated successfully into lily CMV from the outside.

We believe that there must be a specific host factor(s) in lily that should be somehow different from the corresponding factor in many other plants, which associates with the 1a protein and affects the ability of CMV to support satRNAs. After all, without a certain level of initial replication by using lily factor(s), a satRNA cannot accumulate random mutations on its RNA sequence to increase fitness to lily CMV isolates.

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