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Rapid Communication

Functional significance of a hepta nucleotide motif present at the junction of *Cucumber mosaic virus* satellite RNA multimers in helper-virus dependent replication

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ABSTRACT

Satellite RNAs (satRNA) associated with *Cucumber mosaic virus* (CMV) have been shown to generate multimers during replication. We have discovered that multimers of a CMV satRNA generated in the absence of its helper virus (HV) are characterized by the addition of a hepta nucleotide motif (HNM) at the monomer junctions. Here, we evaluated the functional significance of HNM in HV-dependent replication by ectopically expressing wild type and mutant forms of satRNA multimers *in planta* either in (+) or (−)-strand polarity. Comparative replication profiles revealed that (−)-strand multimers with complementary HNM (cHNM) are the preferred initial templates for HV-dependent replication than (−)-strand monomers and multimers lacking the cHNM. Further mutational analyses of the HNM accentuate that preservation of the sequence and native length of HNM is obligatory for efficient replication of satRNA. A model implicating the significance of HNM in HV-dependent production of monomeric and multimeric forms of satRNA is presented.

Introduction

Satellite RNAs (satRNAs) are the smallest known infectious agents that depend on their helper viruses (HVs) to provide some of the proteins necessary for replication. Although many details of satRNA replication still remain unrevealed, accumulating evidence suggests that satRNA replication does not always involve the same mechanisms as replication of its HV (Buckley and Bruening, 1990; Huang et al., 2012; Masuta et al., 1988; Roossinck et al., 1992; Simon et al., 2004).

Cucumber mosaic virus (CMV), the type member of the genus *Cucumovirus*, is a small spherical plant virus capable of infecting a variety of hosts. CMV possesses a tripartite, single-stranded (ss) RNA genome. RNA 1 and 2 encode replicase proteins (1a and 2a, respectively) while RNA3 encodes movement and coat proteins (Palukaitis and Garcia-Arenal, 2003). Some CMV isolates have been found to contain satRNAs. CMV satRNAs utilize CMV-encoded proteins to complete the infection cycle. Like other viral satRNAs, CMV satRNAs, which consist of 5'-capped, noncoding, single-strand (ss) RNA genomes of 330–405 nucleotides, have little or no sequence homology with their HV genomic RNAs and exhibit a high degree of secondary structure with intramolecular base pairing (Palukaitis and Garcia-Arenal, 2003; Roossinck et al., 1992).

Several satRNA variants of CMV have been shown to generate multimeric forms during HV-dependent replication *in planta*, a feature that is commonly shared with satRNAs associated with taxonomically distinct plant viruses such as *Turnip crinkle virus* (TCV), *Tobacco ringspot virus* (TRSV), *Cymbidium ringspot virus* (CyRSV) and *Beet black scorch virus* (BBSV) (Hu et al., 2009). An early study involving the characterization of satRNA multimeric forms accumulated during CMV-dependent replication revealed that the junction regions in head-to-tail repeats of unit length satRNA were a mixture containing either precise fusions of monomer units or some contained deletions at either 5' end or 3' end and 5' end (Kuroda et al., 1997). However, our recent study has convincingly demonstrated that, in the absence of HV, a satRNA of Q-CMV (Q-satRNA) has a propensity to localize in the nucleus and generate multimeric forms of genomic and anti-genomic strands. Further sequence characterization revealed that junction regions of head-to-tail repeats of satRNA multimers formed in the absence and presence of HV are distinct (Choi et al., 2012); i.e. Q-satRNA multimers accumulated in the presence of HV were of the *Class* 1 type and characterized by lacking a 3' terminal C-residue of the first monomeric unit of head-to-tail repeats. By contrast, multimers of the *Class* 2 type generated in the absence of HV are characterized by a template independent polymerization of a hepta nucleotide motif (HNM; GGGAAAA) at the junction of head-to-tail repeats (Fig. 1A).

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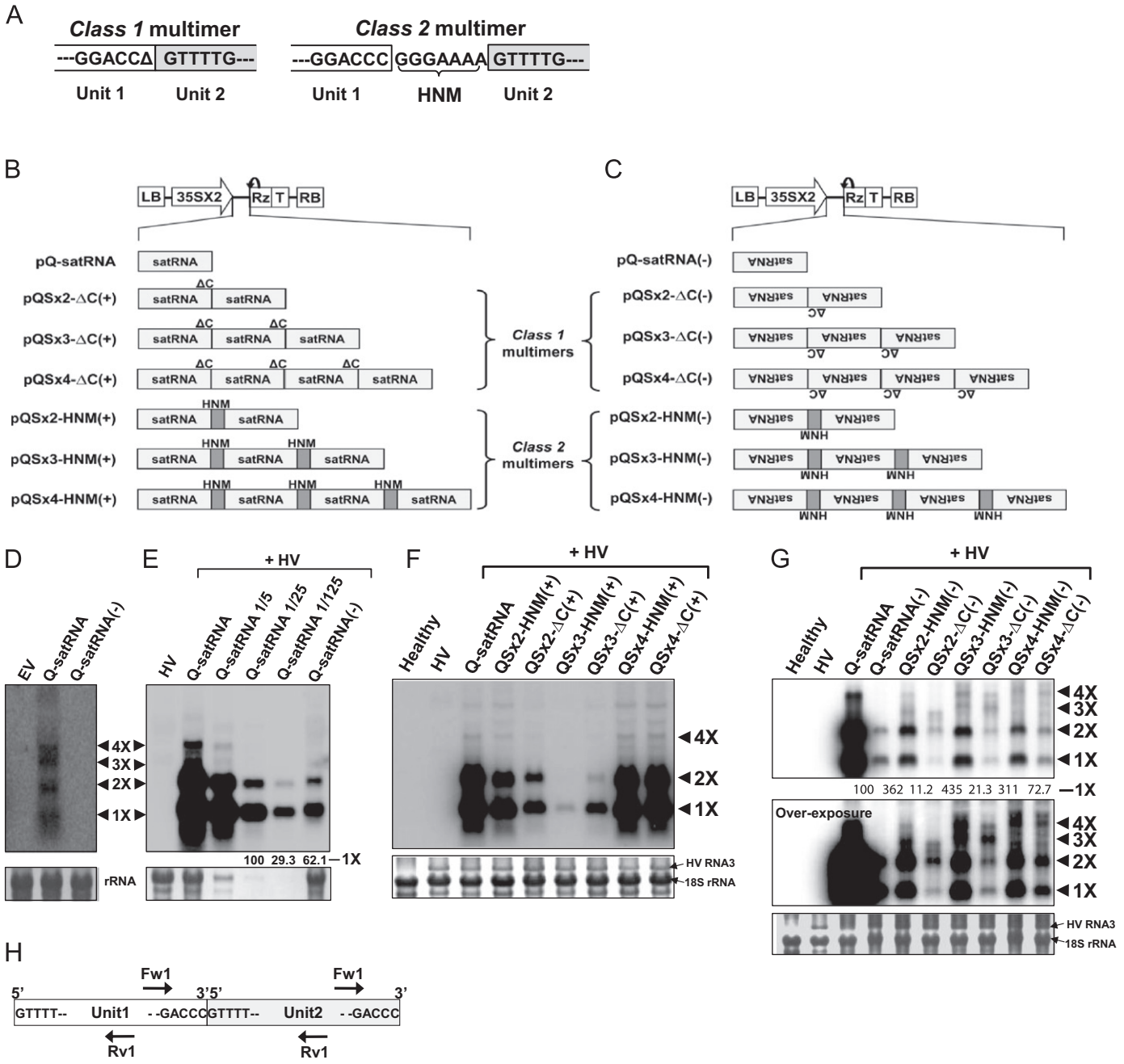

Fig. 1. Replication competence of the *Class* 1 and *Class* 2 Q-satRNA multimers of (+) and (−)-strand polarity. (A) Schematic representation showing the junction sequences of the *Class* 1 and *Class* 2 multimers of Q-satRNA. The *Class* 1 and *Class* 2 Q-satRNA multimers are characterized by having ΔC or the HNM at the junction. (B, C) Schematic representation of agroconstructs designed to transiently express (B) (+)-strand or (C) (−)-strand polarity transcripts of the *Class* 1 or *Class* 2 Q-satRNA multimers following agroinfiltration. x2, x3 and x4 in the construct names denote dimeric, trimeric and tetrameric forms of multimers, respectively. (+) and (−) in the construct names denote the expected polarity of the transcripts synthesized by the 35 S promoter *de novo*. The basal binary vector contains, in sequential order, a left border of T-DNA (LB), a double 35 S promoter (35SX2), indicated multimeric form, a ribozyme sequence (Rz), a 35 S terminator (Ter) and a right border of T-DNA (RB). The bent arrows represent ribozyme cleavage sites. (D) Northern blot analysis of total RNA recovered at 3 dpi from *N. benthamiana* leaves infiltrated with agrobacteria carrying empty vector (EV), pQ-satRNA or pQ-satRNA(−) using riboprobes detecting (+)-strand Q-satRNAs. (E) Northern blot analysis of replication competence of pQ-satRNA(−). (+)- or (−)-strand monomeric Q-satRNA transcript was co-expressed with HV in *N. benthamiana* cells by agroinfiltration. Total RNA was extracted from the infiltrated leaves at 3 dpi and subjected to Northern blot analysis using riboprobes detecting (+)-strand Q-satRNAs. To compare the replication competence of pQ-satRNA(−) with that of pQ-satRNA, total RNA extracted from the leaves expressing Q-satRNA with HV was five-fold serially diluted as indicated on the top of the image. Accumulation levels of monomeric Q-satRNA shown below the Northern blot were normalized against 1/25-dimulted Q-satRNA as 100%. (F, G) Northern blot analysis of replication competence of the *Class* 1 and *Class* 2 multimers of (F) (+)-polarity and (G) (−)-polarity. Each monomeric or multimeric Q-satRNA transcript indicated on the top of the images was co-expressed with HV in *N. benthamiana* cells by agroinfiltration. Total RNA was extracted from the infiltrated leaves at 3 dpi and subjected to Northern blot analysis using riboprobes detecting (+)-strand Q-satRNAs. In Fig. 1G, accumulation levels of monomeric Q-satRNA shown below the upper panel were normalized against Q-satRNA(−) as 100%; the middle panel shows an over-exposed autoradiogram of the upper panel. The positions of the Q-satRNA monomeric (1X), dimeric (2X), trimeric (3X), and tetrameric (4X) forms are indicated by arrowheads.The bottom panels show loading controls and accumulation of HV RNA3. (H) Schematic representation of the dimeric form of Q-satRNA and the location of primers for a divergent RT-PCR are shown.

The major focus of this study is to evaluate the relative significance of *Class* 1 and *Class* 2 multimers in the replication of Q-satRNA, especially the HNM. Our proven ability to initiate (+)-strand synthesis by a viral replicase on ectopically expressed (−)-strands (Kwon and Rao, 2012) was extended to further test the contribution of (−)-strand Q-satRNA multimers in the generation of monomeric (+)-strand satRNA progenies.

Results and discussion

Biological activity of ectopically expressed (−)-strand monomeric satRNA

It was previously reported that mechanical inoculation of (−)-strand CMV satRNA monomeric transcripts synthesized *in vitro* could serve as templates for replication, *albeit* with reduced efficiency when compared to transcripts of (+)-strand satRNA monomer (Tousch et al., 1994). We have recently shown that, when expressed ectopically, (−)-strand transcripts of *Brome mosaic virus* (BMV) RNA3 were recognized as templates by BMV RNA-dependent RNA polymerase (RdRp) and initiated (+)-strand synthesis (Kwon and Rao, 2012). Thus, to verify whether ectopically expressed (−)-strand transcripts of satRNA would serve as templates for its HV RdRp, an agroconstruct, referred to as pQ-satRNA(−), was constructed (Fig. 1). Autonomous expression of pQ-satRNA(−) resulted in the synthesis of expected size of (−)-strand transcripts of satRNA (data not shown). Unlike (+)-strand satRNA, no multimer formation was detected for Q-satRNA(−) (Fig. 1D), since it is unable to be transported to the nucleus (Choi et al., 2012) for host-mediated transcription resulting in multimer formation. However, when complemented with HV, accumulation of monomeric and multimeric forms was evident (Fig. 1E), suggesting ectopically expressed Q-satRNA(−) did serve as a template for (+)-strand synthesis by HV RdRp. Consistent with the previous study (Tousch et al., 1994), quantitative analysis further revealed that the accumulation level of monomeric (+)-strand progenies resulted from providing Q-satRNA(−) as an initiate template was approximately 40 times lower than that resulting from providing Q-satRNA (+)-strand as an initial template (Fig. 1E).

Replication competence of the Class 1 and Class 2 Q-satRNA multimers in the presence of HV

To evaluate the relative contribution of the *Class* 1 vs. *Class* 2 Q-satRNA multimers (Fig. 1A) to HV-dependent replication, we assembled a series of agroconstructs corresponding to dimeric (2x), trimeric (3x) and tetrameric (4x) forms of the *Class* 1 and *Class* 2 multimers, amenable for ectopic expression of either (+) or (−)-strand polarity templates when agroinfiltrated into plants (Fig. 1B and C). Following co-expression of each set of Q-satRNA multimers with HV in *Nicotiana benthamiana* leaves by agroinfiltration, total RNAs were extracted from the infiltrated leaves at 3 days post-infiltration (dpi) and subjected to Northern blot analysis. HV-dependent replication profiles of Q-satRNA progenies when the (+)-strand multimers were provided as initial templates are shown in Fig. 1F. Among the three forms of the *Class*1 and *Class* 2 multimers, in relation to replication initiated on monomeric templates, expression of (+)-strand tetrameric forms of both classes [QSx4-ΔC(+) and QSx4-HNM(+)] resulted in the highest level of satRNA progeny accumulation followed by dimeric forms [QSx2-ΔC(+) and QSx2-HNM(+)]; whereas trimeric forms of both classes of multimers [QSx3-ΔC(+) and QSx3-HNM(+)] appeared to be the least preferred templates for HV-dependent replication (Fig. 1F). A likely explanation for the observed incompetence of trimeric forms to serve as templates for HV RdRp is that the secondary structure induced by the (+)-strand trimeric forms might not be in an optimal conformation.

The functional significance of the HNM became apparent when replication profiles of (−)-strand multimer templates of *Class* 1 and *Class* 2 were compared (Fig. 1G). The presence of complementary HNM (cHNM) in (−)-strand multimers provided as initial templates significantly increased the replication competence of Q-satRNA over its absence. Interestingly, the (−)-strand transcripts of the *Class* 2 multimers [QSx2-HNM(−), QSx3-HNM(−) and QSx4-HNM(−)] served as more efficient templates than the (−)-strand monomeric Q-satRNA transcript [i.e. Q-satRNA(−)], resulting in higher accumulation of progenies (3.6, 4.3 and 3.1 times, respectively; Fig. 1G). However, co-expression of the (−)-strand transcripts of the *Class* 1 multimers [QSx2-ΔC(−), QSx3-ΔC(−) and QSx4-ΔC(−)] with HV resulted in lower accumulation of progenies than when Q-satRNA(−) was co-expressed with HV (Fig. 1G). Furthermore, it is interesting to note that the monomeric progenies accumulated lower than dimeric or trimeric progenies when the (−)-strand dimeric or trimeric *Class* 1 multimer [QSx2-ΔC(−) or QSx3-ΔC(−), respectively] was provided as an initial template (Fig. 1G). Thus, it appears that, in the absence of cHNM at the junction of the (−)-strand multimers, HV replicases might fail to efficiently terminate RNA synthesis at the junction to produce monomeric progenies, resulting in continued synthesis of the multimeric progenies. Collectively, our data suggest that HNM has a distinct role during the synthesis of (+)-strand satRNA progeny from (−)-strand multimeric templates by HV RdRp.

The efficient accumulation of dimeric progenies when the (−)-strand transcripts of the *Class* 2 multimers [QSx2-HNM(−), QSx3-HNM(−) and QSx4-HNM(−)] were provided as initial templates (Fig. 1G), prompted us to analyze the junction sequences of the accumulated multimeric progenies. To this end, a divergent RT-PCR using a primer pair (Fw1 and Rv1; Fig. 1H) was performed as described in our previous study (Choi et al., 2012). Interestingly, despite the *Class* 2 type multimers of (−)-strand were being provided as initial templates, the junction sequences in the dimeric progeny were of the *Class* 1 type (Table 1). This indicates that HV replicase prefers to synthesize *Class* 1 (+)-strand progeny from the (−)-strand *Class* 2 multimeric templates.

Table 1
Summary of the sequence analyses of the junction region of the Q-satRNA multimeric progenies.

Inoculum	Type of the junction sequence	Number of cDNA sequences*	
		Exp. 1	Exp. 2
HV+QSx2-ΔC(−)	*Class* 1	12/12	10/10
	Class 2	0/12	0/10
HV+QSx2-HNM(−)	*Class* 1	12/12	10/10
	Class 2	0/12	0/10
HV+QSx3-HNM(−)	*Class* 1	15/15	10/10
	Class 2	0/15	0/10
HV+QSx4-HNM(−)	*Class* 1	15/15	10/10
	Class 2	0/15	0/10
HV+QSx2-A7(−),	*Class* 1	12/12	12/12
	Class 2	0/12	0/12
HV+QSx2-G3T4(−)	*Class* 1	12/12	12/12
	Class 2	0/12	0/12
HV+QSx2-G1(−)	*Class* 1	10/10	12/12
	Class 2	0/10	0/12
HV+QSx2-G3(−)	*Class* 1	10/10	12/12
	Class 2	0/10	0/12
HV+QSx2-G3A2(−)	*Class* 1	10/10	12/12
	Class 2	0/10	0/12

* The values are the number of cDNA clones with a specific sequence/total number of cDNAs sequenced. Exp.—experiment.

Sequence specificity of HNM is required for dimeric progeny accumulation.

It is interesting to note that, in the absence of HV, a template independent, pre-determined HNM sequence (i.e. GGGAAAA) was added by a host polymerase yet to be identified (Choi et al., 2012). The data shown above clearly demonstrates the importance of HNM in HV-dependent replication of satRNA. The question that needs to be addressed is: How significant is the sequence specificity of HNM in relation to Q-satRNA replication? To find an answer to this question, we engineered a series of substitution mutations into the HNM located at the junction of the (+)- and (−)-strand *Class* 2 type dimeric background (Fig. 2A). The (+)- or (−)-strand of each mutant dimeric form was co-expressed with HV in *N. benthamiana* leaves by agroinfiltration. At 3 dpi, total RNAs were extracted from the infiltrated leaves and subjected to Northern blot analysis. As shown in Fig. 2C, the progeny accumulation levels of each mutant were indistinguishable when the (+)-strand dimeric substitution mutants [QSx2-A7(+), QSx2-G3T4(+)] were provided as initial templates. This is expected because the (+)-strand *Class* 1 dimer [QSx2-ΔC(+)] also served as an efficient template for the replication (Fig. 1F and C). On the other hand, when the (−)-strand dimeric substitution mutants [QSx2-A7(−), QSx2-G3T4(−),] were provided as initial templates for HV-dependent replication, we observed alterations in the ratio of monomer/dimer accumulation (1X/2X), compared to that detected in co-expression of HV with Q-satRNA or QSx2-HNM(−) (Fig. 2D). The ratio of monomer/dimer accumulation was greatly increased when mutations were introduced into the HNM (Fig. 2D). Basically, this alteration was caused by significant reduction of accumulation of the dimeric progenies compared to that detected in co-expression of HV with Q-satRNA or QSx2-HNM(−) (Fig. 2D). We also analyzed the junction sequences of the multimeric progenies accumulated when the (−)-strand dimeric substitution mutants were provided as initial templates (Table 1). The divergent RT-PCR and sequencing results revealed that the accumulated multimeric progenies were of the *Class* 1 type. However, this accumulation of *Class* 1 multimers might occur by the recruitment of monomeric progeny into the replication cycle but not due to reversion of the introduced mutations at the junction because HV-dependent Q-satRNA replication initiated with (−)-strand multimeric forms of Q-satRNA results in accumulation of monomeric Q-satRNAs (Fig. 1G and D). It is also possible that the *Class* 1 multimeric progeny might be synthesized directly (but inefficiently) from the (−)-strand dimeric mutants of Q-satRNA. Therefore the specific sequence of the HNM (GGGAAAA) is likely to be required for efficient synthesis of the *Class* 1 dimeric Q-satRNA from (−)-strand templates since disturbance of this sequence specificity resulted in significant decrease of accumulation of dimeric progenies.

The length of the junction sequence between monomeric units of the Q-satRNA multimer affects monomeric progeny accumulation

The data shown above exemplify the importance of the HNM sequence. Next, we sought to address the significance of the length of the junction sequence between each monomeric unit of the Q-satRNA multimers on (+)-strand progeny synthesis from (−)-strand multimeric templates. To this end, we assembled a set of three mutants involving deletion of two (pQSx2-G3A2), four (pQSx2-G3), or six nucleotides (pQSx2-G1) encompassing the HNM sequence located at the junction of the (−)-strand *Class* 2 dimer (Fig. 2B). Each of the three deletion mutants was co-expressed as a (−)-strand dimeric form with HV in *N. benthamiana* leaves by agroinfiltration. At 3 dpi, (+)-strand

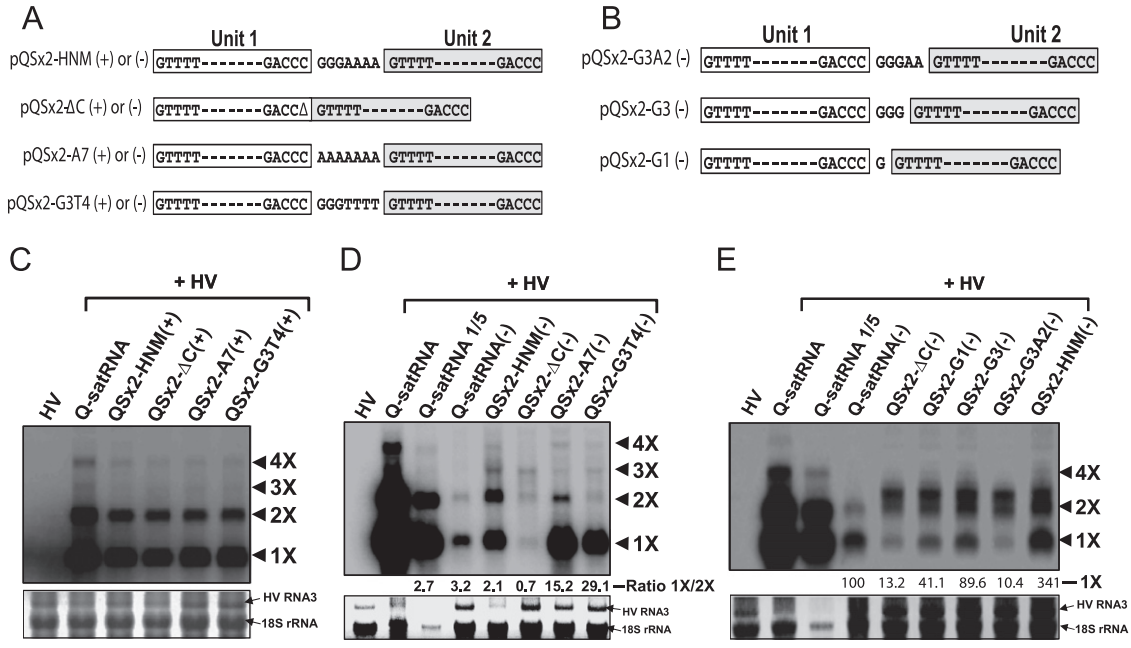

Fig. 2. Schematic representation and replication competence of the dimeric mutants of Q-satRNA containing substitutions or deletions at the junction. The dimeric mutants of Q-satRNA containing nucleotide substitutions (A) or deletions (B) at the junction were constructed using the binary vector as shown in Fig. 1B and C. (+) and (−) in the construct names denote the expected polarity of the transcripts synthesized by the 35 S promoter *de novo*. Northern blot analysis of replication competence of the dimeric mutants of Q-satRNA containing nucleotide substitutions (C and D) or deletions (E) at the junction. Each monomeric or dimeric Q-satRNA transcript indicated on the top of the images was co-expressed with HV in *N. benthamiana* cells by agroinfiltration. Total RNA was extracted from the infiltrated leaves at 3 dpi and subjected to Northern blot analysis using riboprobes detecting (+)-strand Q-satRNAs. In Fig. 2D, the ratios of the accumulation level of monomer progenies to that of dimeric progenies (1X/2X) are shown below the Northern blot. In Fig. 2E, accumulation levels of monomeric Q-satRNA shown below the Northern blot were normalized against Q-satRNA(−) as 100%. The positions of the Q-satRNA monomeric (1X), dimeric (2X), trimeric (3X), and tetrameric (4X) forms are indicated by arrowheads. The bottom panels show loading controls and accumulation of HV RNA3.

progeny accumulation in the infiltrated leaves was analyzed by Northern blot hybridization. Results are shown in Fig. 2E. Compared to the intact HNM sequence, deletion of as little as two nucleotides from the HNM, as exemplified from the replication profiles of QSx2-G3A2(−), had profound influence on the accumulation of both monomeric and dimeric progenies (Fig. 2E). These results accentuate the importance of an intact length of the junction sequence between monomeric units of the Q-satRNA multimer for efficient production of both monomeric and dimeric (+)-strand progenies from (−)-strand multimeric templates. Furthermore, only the *Class* 1 type junction sequences were detected when the junction sequences of progenies generated by providing the three deletion mutants as initial templates were analyzed (Table 1).

Formation and accumulation of multimeric forms of satRNAs associated with a selective group of taxonomically distinct plant viruses was documented (Hu et al., 2009). The mechanism regulating the generation of satRNA multimers followed by the accumulation of monomeric forms varied between virus groups. For example, in nepo-and sobemoviruses, production of multimers is mediated through a rolling circle mechanism followed by accumulation of monomeric progeny as a result of autocatalytic cleavage of the multimeric forms. This indicates that multimers in these satRNAs are intermediates (Forster and Symons, 1987; Gerlach et al., 1986; Kiefer et al., 1982). By contrast, in satRNA of TCV, generation of multimers was implicated to re-initiation of replication by HV replicases before release of the nascent strand (Carpenter et al., 1991). In satRNAs of CMV, it was hypothesized that dimeric forms are generated by self-ligation of double-stranded RNA monomers since no circular intermediates have been detected (Linthorst and Kaper, 1984; Roossinck et al., 1992). We recently found, even in the absence of HV, a CMV Q-satRNA can produce multimeric forms containing HNM at the junction due to host mediated transcription in the nucleus; whereas a majority of multimers accumulated in the presence of HV have a deletion of a 3' terminal C-residue at the junction (Choi et al., 2012). Template independent addition of HNM at the junction suggests that multimers formed in the absence of HV are not the products of self-ligation .

In the present study, we examined the significance of the satRNA multimers formed HV-dependently or independently (i.e. the *Class* 1 vs. *Class* 2 type multimers, respectively). The significance of HNM of *Class* 2 multimers could not be discerned when multimers of (+)-strand were provided as initial templates (Fig. 1F and C). This is not surprising since previously it was shown that artificial (+)-strand CMV satRNAs containing a monomeric unit flanked by extensive non-satRNA sequences at the 5' and 3' ends were biologically active to restore wild type satRNA progenies including monomers and dimers (Baulcombe et al., 1986; Tousch et al., 1994). This finding suggests that CMV replicase is capable of initiating synthesis of satRNA (−)-strands from (+)-strand inoculum templates by internal initiation at the penultimate C residue of 3' CCC_{OH} (Sivakumaran et al., 2000). However, the significance of HNM in satRNA replication became obvious when the (−)-strand *Class* 2 multimers were provided as initial templates. Higher levels of Q-satRNA progeny accumulated when the (−)-strand *Class* 2 multimers were provided as initial templates compared to those accumulated from (−)-strands of either monomeric satRNA or *Class* 1 multimers (Fig. 1G). These findings suggest that the *Class* 2 multimers of (−)-strand polarity generated in the absence of HV are the preferred templates for HV-driven replication. In addition to strand polarity, the sequence specificity of HNM and preservation of its length are also required for efficient accumulation of monomeric and/or dimeric progeny (Fig. 2D and E). Taken together, these observations suggest that the HNM has a regulatory role in HV-driven Q-satRNA replication,

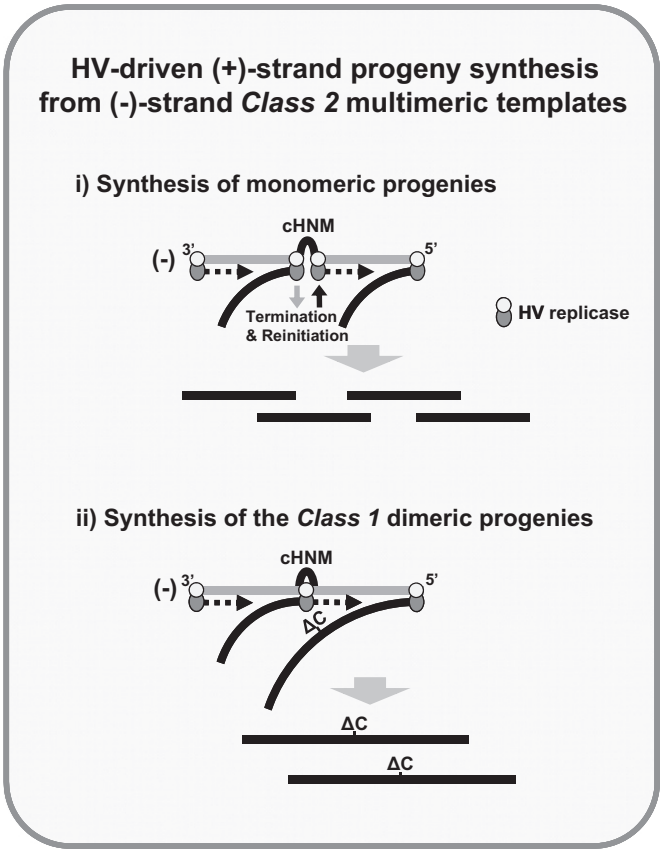

Fig. 3. Hypothetical model for HV-driven synthesis of (+)-strand progenies from (−)-strand *Class* 2 multimeric templates in Q-satRNA. Envisioned mechanism regulating the synthesis of (i) monomeric and (ii) dimeric *Class* 1 (+)-strand progeny from (−)-strand dimeric templates having distinct cHNM at the junction (See Discussion section for details).

and most importantly is required for (+)-strand progeny synthesis from (−)-strand multimeric templates.

Based on our results, we propose a mechanism involving HNM located at the junction of the *Class* 2 Q-satRNA multimers as integral for HV-driven synthesis of (+)-strand progenies from (−)-strand *Class* 2 multimeric templates. Our proposed mechanism, as schematically shown in Fig. 3, is different from that of either a rolling circle mechanism as proposed for circular satRNAs or replicase re-initiation as in TCV (Carpenter et al., 1991; Gerlach et al., 1986; Kiefer et al., 1982). According to our model, HV replicase initiates (+)-strand progeny synthesis at the 3' end of (−)-strand *Class* 2 multimers. The cHNM at the junction allows proper termination and re-initiation of RNA synthesis to generate monomeric progenies. However, occasionally, HV replicase might bypass the cHNM by an unknown mechanism and continue RNA synthesis. This could result in the accumulation of *Class* 1 dimeric progenies. In addition, our results also provide a new insight into the understanding of how satRNA multimers generated in the absence of HV contribute to HV-driven replication of a satRNA.

Materials and methods

Construction of the Q-satRNA multimers for agroinfiltration

Positive-strand Q-satRNA multimers of the *Class* 1 and *Class* 2 types were amplified by double-joint PCR. Briefly, forward monomeric Q-satRNAs of the *Class* 1 and *Class* 2 types were amplified by PCR using two primer sets, U1Fw/U1Rv1 and U1Fw/U1Rv2,

respectively (U1Fw, 5'-GTTTTGTTTGTTAGAGAATTGCGTAGAG-3; U1Rv1, 5'-CTCTAACAAACAAAACGGTCCTGGTAGGGAATG-3'; U1Rv2, 5'-CAAAACTTTTCCCGGGTCCTGGTAGGGATTG-3'). Reverse monomeric Q-satRNAs of the *Class* 1 and *Class* 2 types were amplified by PCR using two primer sets, U2Fw1/U2Rv and U2Fw2/U2Rv, respectively (U2Fw1, 5'-ACCAGGACCGTTTTGTTTGTTAGAGAATT-GCGT-3'; U2Fw2, 5'–GACCCGGGAAAAGTTTTGTTTGTTAGAGAAT-TGCGT-3'; U2Rv, 5'-CATGCCATGGGGTCCTGGTAGGGAATGAT-3'; *Nco*I site is underlined). These two PCR products of forward and reverse monomeric Q-satRNAs were mixed and joined by PCR. This PCR reaction produced a ladder of multimeric fragments including dimer, trimer and tetramer in the same reaction tube. Each multimeric fragment, which had a size of dimer, trimer or tetramer, was purified by gel-extraction, digested with *Nco*I, and inserted into a binary vector (pCassHDV) digested with *Stu*I and *Nco*I. To produce an authentic 3' terminus of (+)-strand Q-satRNA multimers, the resulting plasmids were digested with *Nco*I, treated with mung bean exonuclease (New England Biolabs, USA), and self-ligated . The resulting clones, expressing (+)-strand dimeric, trimeric or tetrameric *Class* 1 Q-satRNA by agroinfiltration, were referred to as pQSx2-ΔC(+), pQSx3-ΔC(+) and pQSx4-ΔC(+), respectively. The clones which can express (+)-strand dimeric, trimeric or tetrameric *Class* 2 Q-satRNA by agroinfiltration, were referred to as pQSx2-HNM(+), pQSx3-HNM(+) and pQSx4-HNM(+), respectively. To amplify the (−)-strand
Q-satRNA multimers of the *Class* 1 and *Class* 2 types, the corresponding agro-constructs of the (+)-strand Q-satRNA multimers were used as templates for PCR with a primer pair (5'-**GTTCATTTCATTTGG*CCATG***GTT-3' and 5'-**TGGAGATGCCAGGCC-TACCC**G-3'; nucleotide sequences corresponding to vector sequences are shown in bold, mutated nucleotides to engineer restriction enzyme sites are in italics and restriction sites *Nco*I and *Stu*I are underlined). The resulting PCR products were digested with *Nco*I and *Stu*I, and inserted into pCassHDV digested with *Stu*I and *Nco*I. To terminate (−)-strand Q-satRNA multimers with authentic 3' termini, the resulting plasmids were digested with *Nco*I, treated with mung bean exonuclease, and self-ligated . The clones, which can express the (−)-strand dimeric, trimeric or tetrameric *Class* 1 Q-satRNA by agroinfiltration, were referred to as pQSx2-ΔC(−), pQSx3-ΔC(−) and pQSx4-ΔC(−), respectively. The clones, which can express the (−)-strand dimeric, trimeric or tetrameric *Class* 2 Q-satRNA by agroinfiltration, were referred to respectively as pQSx2-HNM(−), pQSx3-HNM(−) and pQSx4-HNM(−). We constructed the *Class* 2 dimeric mutants containing substitution or deletion mutations at the junction based on double-joint PCR as described above by introducing the corresponding mutations into the PCR primers (The composition of the primers used in the construction of the *Class* 2 dimeric mutants is available upon request). The construction and characteristic features of agrotransformants corresponding to monomeric Q-satRNA and three genomic RNAs of CMV strain Q are as described previously (Choi et al., 2012).

Agroinfiltration and progeny analysis

Following transformation into *Agrobacterium tumefaciens* strain GV3101, agrotransformants were infiltrated into *Nicotiana benthamiana* leaves as described previously (Choi et al., 2012). Total RNAs were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Plus-strand Q-satRNA progenies in the total RNA extracted from the infiltrated leaves were analyzed by Northern hybridization using a (+)-strand Q-satRNA specific ^{32}P-labeled riboprobes (Choi et al., 2012). Junction sequences of the Q-satRNA multimeric progenies were analyzed by reverse transcription (RT)-PCR using a primer pair (Fw1, GCGGAATTTCGAAAGAAAC-3; Rv1, GTTTTGCTAGCGAACT-GAGCG;GGGG) as described previously (Choi et al., 2012).

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