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Heterologous replicase driven 3′ end repair of *Cucumber mosaic virus* satellite RNA

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ABSTRACT

To investigate the extent of the 3′ end repair in a satellite RNA of *Cucumber mosaic virus* (CMV) strain Q (Qsat) by a heterologous *Tomato aspermy virus* (TAV), a set of biologically active agrotransformants corresponding to the three genomic RNAs of TAV was developed. Analysis of *Nicotiana benthamiana* plants agroinfiltrated with TAV and either wild type or each of the six 3′ deletion mutants of Qsat revealed that (i) heterologous replicase failed to generate Qsat multimers, a hallmark feature of homologous replicase dependent replication of Qsat; (ii) manifestation of severe symptom phenotypes and progeny analysis suggested that heterologous replicase was competent to repair Qsat deletion mutants lacking up to 3′13 nucleotides (nt) but not beyond and (iii) comparative *in silico* analysis indicated that the 3′ secondary structural features of the repaired Qsat progeny from heterologous vs homologous driven replicases are remarkably very similar. The significance of these observations is discussed.

Introduction

The genome organization of *Tomato aspermy* virus (TAV) is identical to *Cucumber mosaic virus* (CMV), the type member of the *Cucumovirus* genus (Hull, 2013). TAV has a tripartite messenger-sense RNA genome and is divided among three species, RNAs 1 (3410 nt), 2 (3070 nt) and 3 (2386 nt) (Hull, 2013). Genomic RNAs 1 and 2 encode non-structural replicase proteins p1a and p2a respectively (Palukaitis and Garcia-Arenal, 2003). The two gene products, movement protein (MP) and capsid protein (CP), encoded by dicistronic RNA 3 are dispensable for replication, but are required for cell-to-cell and long distance movement (Palukaitis and Garcia-Arenal, 2003). Although MP is translated directly from the genomic RNA3, CP is translated from subgenomic RNA4 transcribed from progeny minus-sense RNA3 (Boccard and Baulcombe, 1993; O'Reilly et al., 1991). In addition to the three genomic RNAs and subgenomic RNA4, three additional RNA species are found associated with TAV infections (Shi et al., 1997a). These are RNA4A (702 nt), RNA3B (486 nt) and RNA5 (323 nt). RNA4A, which is also found in CMV infection (Ding et al., 1994) is a subgenomic RNA generated from the 3′ end of genomic RNA2 and encodes a 2b protein (Shi et al., 1997a). Protein 2b is the designated suppressor of RNA silencing (Li et al., 1999) and also play a role in cell-to-cell and systemic movement of cucumoviruses as well as in symptom induction (Ding et al., 1995). RNA3B and RNA5 were characterized to be subgenomic RNAs derived from genomic RNA3 (Shi et al., 1997a). RNA3B is unique to TAV while RNA5 is produced and packaged by CMV strains of subgroup II, but not subgroup I (Palukaitis and Garcia-Arenal, 2003). Although the biological significance of RNA3B is unknown (Shi et al., 1997a), recent genetic studies performed with RNA5 of CMV revealed that it is not a subgenomic RNA since it is produced by replication-independent manner and plays an important role in recombination (de Wispelaere and Rao, 2009). It remains to be determined whether the mechanism of RNA5 generation in TAV is analogous to that of CMV.

In addition to genomic and subgenomic RNAs, under natural conditions, a low molecular weight RNA referred to as satellite (satRNA) has been found to be associated with several strains of CMV (Hu et al., 2009) but not with TAV (Moriones et al., 1992). However, under experimental conditions, TAV is capable of acting as a helper virus (HV) for the replication of satRNAs of CMV albeit at a reduced level (Mossop and Francki, 1979a). Despite functioning as a HV, unlike CMV, TAV failed to repair the mutated 3′ end of the satRNA, suggesting that the repair was replicase-specific (Burgyan and Garcia-Arenal, 1998). A direct comparison of the extent of 3′ end repair of a satRNA of CMV strain Q (Qsat) by mechanical inoculation vs agroinfiltration revealed that the latter approach is more efficient in extending the 3′ repair (up to 3′ 18 nt vs 7 nt) because of its inherent ability to express the required constellation of viral components to initiate replication and repair in the same cell (Kwon et al., 2014). Therefore, we hypothesize the lack of 3′ end repair in satRNA mutants by TAV in a previous study

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(Burgyan and Garcia-Arenal, 1998) is due to mechanical inoculation that limits co-expression of TAV and satRNA in the same cell. Consequently, in this study, we sought to examine whether TAV replicase would promote repair of deleted nucleotides (nt) from the 3′ proximal region of Qsat if TAV and Qsat 3′ proximal deletion mutants were co-expressed by agroinfiltration in the same cell. The results of this study demonstrate that while functioning as a HV, TAV replicase undeniably repairs the engineered 3′ proximal deletions of Qsat although the extent of the 3′ ends repair and nt added varied with that of CMV. However, interestingly, the secondary structural features of repairing Qsat progeny resulting from TAV replicase vs CMV replicase are indistinguishable.

Results

Characteristic features and biological activity of TAV agrotransformants

The strategy used to construct the T-DNA based agrotransformant of each TAV genomic RNA into pCASS4-RZ is described under methods and schematically shown in Fig. 1. As a result of these cloning manipulations, following agroinfiltration into plant cells, the cauliflower mosaic virus 35S promoter would precisely initiate transcription at the authentic 5′ end of pTAV1 (pT1), pTAV 2 (pT2) and pTAV 3 (pT3) (Fig. 1). The 3′ end of each *de novo* synthesized RNA transcripts will terminate with +22-nt extensions beyond the natural 3′ CCA_{OH} due to the presence of a self-cleaving ribozyme (Fig. 1).

Nicotiana glutinosa is the preferred host for the maintenance and propagation of TAV (Habili and Francki, 1974). In this host, TAV induces severe mottling symptoms (Shi et al., 1997b). However, *N. glutinosa* is not amenable for agroinfiltration. *Nicotiana benthamiana*, an amenable host for agroinfiltration, has been reported to be a susceptible host for TAV (Asaoka et al., 2010; Masuta et al., 1998; Suzuki et al., 2003). In this host, unlike QCMV, as reported previously (Masuta et al., 1998), TAV induced severe mosaic symptoms (Fig. 2A). Therefore, to test the biological activity of TAV agrotransformants, leaves of *N. benthamiana* plants were infiltrated with either individual or a mixture of either two or three agrotransformants. At 5 dpi, total RNA and CP was isolated and respectively subjected to Northern and Western blot analysis. Results are shown in Fig. 2B–D. As expected, accumulation of detectable levels of (+) and (−)-strand progeny RNA profile characteristic of TAV (Shi et al., 1997a) was observed in leaves infiltrated with either pT1+pT2 or pT1+pT2+pT3 but not with pT1 or pT2 or pT3 (Fig. 2B and C). It is important to note that, as observed previously with QCMV (de Wispelaere and Rao, 2009), in the absence of RNA3, the replication levels of T1+T2 are higher than those in the presence of RNA3 (Fig. 2B, compare lanes 4 and 6). This is because viral replicase is available exclusively for amplifying T1 and T2 only. Western blot analysis detected the expected size of monomeric (1 ×) and dimeric (2 ×) forms of the CP in leaves infiltrated with a mixture containing pT1+pT2+pT3 but not with other inocula (Fig. 2D). The generation of faster-migrating bands could be due to the cleavage of the monomeric form (Fig. 2D). Furthermore, symptom phenotype induced by a mixture containing all three TAV agrotransformants was indistinguishable from plants mechanically inoculated with purified TAV virions (Fig. 2A). Electron microscopic analysis of TAV virions purified from agroinfiltrated leaves revealed the presence of icosahedral virions of 28 nm in diameter (Fig. 2E).

An interesting profile was observed when total and virion RNAs in *N. benthamiana* and *N. glutinosa* infected with TAV was comparatively analyzed by Northern blot hybridization (Fig. 2F). A profile characteristic of TAV was observed in total RNA preparations from *N. benthamiana* and *N. glutinosa* and contained three genomic (RNAs 1, 2 and 3) and four subgenomic RNAs (RNAs 4, 4A, 3B and 5) (Fig. 2F, lane 1 in each panel). Among these four subgenomic RNAs, as reported previously (Shi et al., 1997a), the accumulation level of RNA4A is significantly less than others. Interestingly, although virions purified from *N. glutinosa* contained all three genomic and four subgenomic RNAs, those purified from *N. benthamiana* packaged only three genomic and a subgenomic RNA4 while packaging of subgenomic RNAs 4A, 3B and 5 is severely debilitated (Fig. 2F, lane 2 in each panel). The reasons for this host-specific packaging phenotype are currently not known.

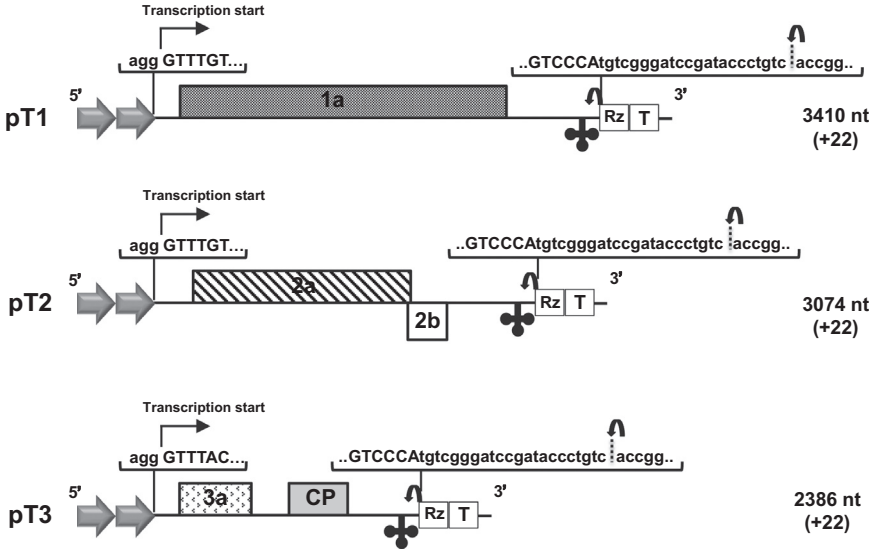

Fig. 1. Characteristic features of pCASS-based TAV agrotransformants. Schematic representation of agrotransformants of TAV RNA1 (pT1), RNA2 (pT2) and RNA3 (pT3). Solid lines and open boxes respectively show non-coding and coding regions. A clover leaf-like structure at the 3′ end represents a tRNA-like structure. The position of the double 35S promoters (arrows) at the 5′ end and the positions of the ribozyme (Rz) cassette derived from *satellite tobacco ringspot virus* and the 35S terminator (T) at the 3′ ends are shown. At the 5′ junctions, the nucleotide sequence of the 35S promoter (lowercase letters) and the 5′ sequence of the genomic RNA (uppercase letters) are shown. A bent arrow indicates the expected transcription start site. At the 3′ end, viral sequences (uppercase letters) left after self-cleavage by the Rz are shown. A bent arrow shows the predicted self-cleavage site. The sizes of wt TAV genomic RNAs and the number of non-viral nt left after self-cleavage by ribozyme (shown in brackets) are indicated.