Size-dependent cell-to-cell movement of defective interfering RNAs of *Cymbidium ringspot virus*

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Defective interfering (DI) RNAs are deletion mutants of viral genomes that have generally lost all essential viral genes. Therefore, DI RNAs require the presence of a helper virus to provide *trans*-acting factors necessary for replication and movement. DI RNAs accumulate at the expense of the helper virus from which they are derived and this interference generally results in remarkable symptom attenuation. DI RNAs have been reported for a wide variety of plant and animal viruses, and are thought to occur universally in animal and plant virus infections (Roux et al., 1991; White, 1996). The 41 kDa coat protein (ORF 3) is translated from the subgenomic RNA 1 (sg1 RNA) and the two small nested ORFs (ORF 4 and 5) encoding 22 kDa and 19 kDa proteins, respectively, are translated from the subgenomic RNA 2 (sg2 RNA) (Russo et al., 1994). The 22 kDa protein is required for cell-to-cell movement (Rochon & Johnston, 1991; Dalmay et al., 1993) and has also a role in symptom development on certain hosts (Scholthof et al., 1995a). The 19 kDa protein has a crucial role in necrotic symptom development on certain hosts (Scholthof et al., 1995b). Moreover, the 19 kDa protein is able to suppress post-transcriptional gene silencing (PTGS) in *Nicotiana benthamiana* (Voïnet et al., 1999).

CymRSV DI RNAs are generated *de novo* during virus replication (Burgyán et al., 1991). Although, the length of DI RNAs is variable, sequence analysis revealed that all CymRSV DI RNAs have a common structural arrangement containing three (A, B, C) conserved blocks (Fig. 1a; Russo et al., 1994). The generation and evolution of DI RNAs involves a series of replicase-mediated deletions (Roux et al., 1991; Russo et al., 1994; White, 1996). The large DI-13 RNA (679 nt) represents the early stage, while the short DI-2 and DI-3 RNAs (Fig. 1a) represent the final stage of CymRSV DI RNA evolution (Havelda et al., 1997). Co-inoculation of *N. benthamiana* plants with *in vitro* transcripts of both genomic and short DI RNA monomers of different tombusviruses including CymRSV resulted in the accumulation of head-to-tail dimers of DI RNAs in the inoculated leaves (Dalmay et al., 1995; Finnen & Rochon, 1995). Inoculation of plants or protoplasts with artificial dimers of short DI RNAs led to the appearance of both dimer and monomer molecules, indicating that monomer DI RNA molecules may be generated from dimers. It has been speculated also that plus-sense DI RNA dimers accumulating in the infected leaves may represent important intermediates in the replication of monomer molecules (Dalmay et al., 1995; Finnen & Rochon, 1995). However, the accumulation of CymRSV DI RNA dimers is size dependent, since the large DI-13 RNA did not form dimers (Dalmay et al., 1995), while the...
artificial minimal DI RNA (BB38, Fig. 1a) (366 nt) accumulated exclusively in dimeric form in the infected plants (Havelka et al., 1995).

Plant viruses have evolved the ability to use plasmodesmata – the natural connection of cytoplasm between adjacent cells (Lucas & Wolf, 1999; Tzfira et al., 2000) – for movement in
viruses in infected plants. In the last decade, there has been a remarkable accumulation of knowledge about the intracellular transport of nucleic acid, including plant viral RNA. It is known that viral movement proteins (MP) facilitate the cell-to-cell movement of both viral and foreign RNAs (reviewed recently by Tziria et al., 2000). However, little is known about the requirements of the viral RNA itself for efficient cell-to-cell transport.

In this study we have analyzed the role of DI RNA dimers in the colonization of leaves. To examine the role of dimer formation in the DI RNA life-cycle, we monitored the accumulation of DI RNA dimers and monomers during the time-course of infection. *N. benthamiana* plants were co-infected with *in vitro* transcripts of the helper virus CymRSV and DI RNA monomers (DI-2, -3, -13; Dalmay et al., 1995) which were different in size (Fig. 1a). Northern hybridization of RNAs isolated from the infected plants confirmed our previous results (Dalmay et al., 1995) that accumulation of the dimeric form of DI-2, -3 RNAs was rapid: at 3 days post-inoculation (p.i.) DI RNA dimers were detectable in the inoculated leaves (Fig. 1b, c). Moreover, dimer-size RNAs accumulated preferentially over the corresponding monomers in samples taken at 3–5 days p.i. (Fig. 1b, c). However, from 5 days p.i. the relative level of monomeric DI-2 RNA started to increase and by 7 days p.i. the abundance of monomeric and dimeric DI RNAs was equal. (Fig. 1b, c). A similar pattern of DI RNA dimer and monomer accumulation was found in leaves co-inoculated with CymRSV and DI-3 RNA. DI-3 RNA dimers accumulated rapidly, and then the ratio of dimers/monomers tended to equalize. These data showed that inoculation of *N. benthamiana* with short (DI-2, -3) DI RNA monomers resulted in early DI RNA dimer accumulation in virus-infected tissue. In contrast, no dimeric DI RNA was observed in helper- and DI-13 RNA-infected plants (Fig. 1d). We also tested the stability of DI-2, -3 RNA monomers and dimers in transfected protoplasts. The results obtained (data not shown) indicated that there is no significant difference in the stability of monomer and dimer forms of the same DI RNA molecules.

The early accumulation of DI-2 RNA dimers, and the subsequent increase in the DI-2 RNA monomer level (Fig. 1b) might be explained by a model in which dimers are replication intermediates of DI RNA monomers. Alternatively, DI RNA dimers may have an advantage over monomers in cell-to-cell movement, allowing dimeric molecules to spread and accumulate more efficiently during the early phases of infection. To differentiate DI RNA replication from movement, *N. benthamiana* protoplasts were prepared (Kollár et al., 1993) and transfected with CymRSV and DI-2 RNA transcripts. Northern blot analysis of RNA isolated from infected protoplasts at 6 to 30 h p.i. showed that, the dominant molecule, independent of sample timing, was the DI-2 RNA monomer (Fig. 2a). This result suggested that the replication of DI RNA is not responsible for the preferential accumulation of dimeric molecules in the inoculated leaves, and indicated that the dimers were not required for DI-2 RNA monomer accumulation in single cell systems.

To gain a better understanding of DI RNA dimer accumulation in whole plants, transgenic plants expressing biologically active DI-2 RNA were also prepared (Kollár et al., 1993) and inoculated with CymRSV helper RNA transcripts. This experimental system offers an obvious advantage in that one can follow the accumulation of DI RNAs *in planta*. In DI RNA-expressing transgenic plants the importance of cell-to-cell movement of DI RNA molecules is significantly reduced, because all of the cells are expressing DI-2 RNA monomers. Therefore, replication of monomer DI RNA can start immediately after the helper virus (with or without DI RNA dimer molecules) enters a plant cell. The accumulation of DI RNA forms in CymRSV-infected DI-2 RNA-expressing transgenic plants was monitored by Northern hybridization. Fig. 2(b) shows that DI-2 RNA monomers were first detected at 4–5 days p.i. (lanes 5, 6) and the majority of DI RNAs were in monomeric form.

These data obtained in a DI RNA movement-independent system (protoplast) or in DI-2 RNA-expressing transgenic plants showed that in the absence of movement short DI RNAs accumulate as monomers in plant cells. These results
RNA dimers accumulated in the infected protoplasts (Fig. 3a, was co-transfected with the helper genome, only BB38 DI monomers. In contrast, if BB38 dimer (BB38Dim) transcript dimers could easily be generated from BB38 DI RNA BB38 DI RNA replicate efficiently in protoplasts, and that the This result showed that both the monomer and dimer forms of BB38 DI RNA monomer- and CymRSV-infected protoplasts revealed Northern hybridization of RNAs extracted from BB38 DI infected with BB38 DI RNA in the presence of helper virus. In a previous study we observed that inoculation of N. benthamiana plants with monomers of an artificial minimal DI RNA (BB38, Fig. 1a) resulted in the exclusive accumulation of DI RNA dimers (Havelda et al., 1995). To clarify whether the exclusive accumulation of BB38 DI RNA dimers was due to replication or more effective movement, protoplasts were transfected with RNA extracts isolated from either BB38 or BB38Dim + helper-infected plants contained only dimers of BB38 DI RNA.

Based on these data, we conclude that although generation of the dimeric form of BB38 DI RNA is very efficient from the BB38 DI RNA monomer template, replication doesn’t explain the exclusive accumulation of the dimeric form of BB38 DI RNA in the infected plants. Therefore, efficient movement of DI RNA dimers plays a decisive role in the exclusive accumulation of dimers from short DI RNA in the inoculated leaves.

Dimers of DI or satellite RNAs have been reported in association with many viruses including tombusviruses (Dalma et al., 1995; Finnen & Rochon, 1995; Russo et al., 1994), carmoviruses (Roux et al., 1991), cucumoviruses (Kuroda et al., 1997) and tospoviruses (Inoue-Nagata et al., 1998) but no biological role has been assigned to dimeric forms of subviral RNAs (Carpenter et al., 1991; Dalma et al., 1995; Finnen & Rochon, 1995). However, the evidence presented here suggests that dimers of small DI RNAs have an important role in cell-to-cell spread. We propose that dimers can move more efficiently than monomeric forms from cell to cell because in infected plants dimeric DI-2 RNA accumulates in early stages of infection, while in transfected protoplasts and in infected DI-2 RNA-expressing transgenic plants mainly monomers were found. Moreover, BB38 RNA monomers replicate as efficiently as dimers in protoplasts; however, only dimers were detectable in plants. The reason why monomer and dimer forms of the same RNA molecule differ in cell-to-cell spread is not clear yet.

In the inoculated leaves of CymRSV-infected plants viral RNAs do not move in the encapsidated form (Dalma et al., 1992, 1993) and DI RNAs including dimeric forms are poorly encapsidated. In addition, the accumulation of monomer and dimer forms of DI-2 RNA in the presence of a coat protein-deletion mutant (CP1) of CymRSV (Dalma et al., 1992) was the same as in wt virus-infected plants (J. Burgýán, unpublished also suggested that preferential DI RNA dimer accumulation in plants was due to the effective movement of dimers rather than the consequence of efficient replication of dimers versus monomer molecules.

In a previous study we observed that inoculation of N. benthamiana plants with monomers of an artificial minimal DI RNA (BB38, Fig. 1a) resulted in the exclusive accumulation of DI RNA dimers (Havelda et al., 1995). To clarify whether the exclusive accumulation of BB38 DI RNA dimers was due to replication or more effective movement, protoplasts were transfected with RNA extracts isolated from either BB38 or BB38Dim + helper-infected plants contained only dimers of BB38 DI RNA.
results). Moreover, Rochon et al. (1994) showed in the case of a related tombusvirus, *Cucumber necrosis virus* (CNV), that CNV coat protein is not required for efficient accumulation of CNV DI RNA in plants. Therefore, it is unlikely that the observed differences of monomer and dimer DI RNAs in cell-to-cell spread are the result of altered encapsidation. The primary structure of the viral RNA does not explain differential accumulation of monomers and dimers, since dimer DI RNA is identical to monomer, only in a duplicated form. Finally, it is also unlikely that the repetition itself would be responsible for higher dimer accumulation, because DI-13, the largest RNA is important for efficient cell-to-cell spread. Consistently, mutant DI-2 RNAs with heterologous insertions longer than 150 nt accumulated in monomeric forms (Dalmay et al., 1995).

Movement of viral RNAs through plasmodesmata requires the activity of viral MPs (Tzfira et al., 2000). MPs bind cooperatively to viral RNA forming a nucleoprotein complex, which can pass through the plasmodesmata upon interacting with the plasmodesmatal trafficking apparatus (Carrington et al., 1996). The mechanism of size-selection in viral RNA transport is not known. So we hypothesize that similarly to other viral MPs, CymRCSV MPs bind viral RNAs, such that the size of DI RNA would limit the number of interacting movement proteins. If efficient cell-to-cell spread requires binding of a minimal amount of movement protein, size-constraints would exist for MP-mediated RNA transport. The size-constraint of viral MPs for RNA mobilization could be an adaptive virus strategy to reduce the competitiveness of small, quickly replicating parasitic RNA molecules. However, this size exclusion of short parasitic RNAs can be overcome by DI RNA dimerization.

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References


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