Localization of cis-acting sequences essential for cymbidium ringspot tombusvirus defective interfering RNA replication

Zoltán Havelda, Tamás Dalmay and József Burgýan*

Agricultural Biotechnology Center, Plant Science Institute, PO Box 411, 2101 Gödöllő, Hungary

The smallest defective interfering RNA (DI-2) of cymbidium ringspot tombusvirus (CyRSV) was used to identify the cis-acting sequences necessary for its replication by making a series of deletions throughout the 404 nt long molecule and testing the biological activity of mutants. Deletion or substitution of the conserved sequence blocks (A, B and C) always yielded inactive molecules. The deletion of only a few nucleotides could be tolerated beyond the natural deletion sites in blocks A and B. However, either half of block C1 (34 nt) and the first 25 nt of C2 (102 nt) could be deleted without loss of infectivity. It was also demonstrated that either one of the two halves of block C1 was specifically required for replication. We suggest that the last 77 nt of the viral genome and either half of block C1 represent the complementary strand promoter sequence recognized by the viral replicase.

Cymbidium ringspot tombusvirus (CyRSV) is a member of the genus Tombusvirus, with a positive-sense, single-stranded RNA genome (4733 nt) that encodes at least four nonstructural proteins (33, 92, 22 and 19 kDa) and the coat protein (Russo et al., 1994). The CyRSV genome contains, as do other viral RNAs, cis-acting replication elements recognizable by viral replicase and probably by host cell factors necessary for replication. Cis-acting RNA replication signals have been identified for brome mosaic virus (French & Ahlquist, 1987) and beet necrotic yellow vein virus (Jupin et al., 1990) by deletion analysis of the smallest segmented genomic RNA, which is not required for viral RNA replication. However, the cis-acting replication signals of several other plant viruses, including tombusviruses have not been identified. A useful approach to identify cis-acting elements in viral RNA genomes is based on defective interfering (DI) RNAs (Schlesinger, 1988; Kim et al., 1993; Zhang & Simon, 1994). We have adopted such a strategy by using DI RNA of CyRSV for the analysis of cis-acting elements in the tombusvirus RNA genome.

DI RNAs are shortened forms of the viral genome that are generated by replicase errors during virus replication. DI RNAs are incapable of autonomous replication, but contain sequences necessary for replication. DI RNAs are ubiquitous among animal viruses (Holland, 1990) and are also found in association with several plant viruses (Roux et al., 1991).

A family of DI RNA molecules is generated during CyRSV genomic RNA replication; the largest of this group, composed of 679 nt (DI-13), is made up of three blocks (A, B and C) of sequences entirely derived from the viral genome (Burgýan et al., 1989, 1991). Block A contains the first 164 nt of genomic RNA including the 5' leader sequence and the start codon of the 33 kDa protein; block B (112 nt) represents the central part of the polymerase gene; block C is 403 nt long and corresponds to 49 nt of the carboxyl-terminus of the 22 kDa protein gene and the entire 3' noncoding region of 354 nt. Smaller DI RNAs have essentially the same sequence blocks but with a progressive reduction in size produced by the introduction of further deletions inside blocks A and C, whereas block B is almost unaffected showing only minor variations at the termini. The smallest DI RNA (DI-2) is 404 nt long and contains two short deletions (15 and 8 nt) in block A and one long deletion (235 nt) in the middle of block C dividing them into blocks A1, A2, A3 and C1, C2, respectively (Fig. 1a). Since the replication of CyRSV DI RNAs only needs trans-acting products of ORFs 1 and 2 of the viral genome (Dalmay et al., 1993; Kollár & Burgýan, 1994), it was suggested that it must contain all the cis-acting signals required for viral RNA replication (Russo et al., 1994).

In the present paper we describe the results of experiments whereby an infectious DI-2 RNA clone (Dalmay et al., 1995) was mutated, in order to map the cis-acting sequences essential for replication and accumulation.

Since only one useful restriction site was available in
CyRSV genome

Fig. 1. Schematic representation and biological activity of genomic RNA, wild-type DI-2 RNA and mutants DI-RNAs of CyRSV. 
(a) Organization of CyRSV genomic RNA. The ORFs and the approximate molecular masses of the encoded proteins are indicated. Genomic RNA sequences conserved in DI-2 RNA are represented below as shaded boxes and deleted regions are depicted as lines. Dashed lines show the origin of sequences conserved in DI RNA. Numbers above the shaded areas are the sizes (in nt) of the conserved genomic RNA sequence blocks; numbers of deleted bases are shown below the lines. Arrowheads indicate the position of introduced restriction sites in DI-2 RNA. (b) Schematic representation of DI-21 RNA and its deletion mutants. The DI RNA genomes are shown as open boxes and lines indicate the deleted regions between two restriction sites. Restriction sites: X, XhoI; A, AccI; B, BstBI; E, EcoRI; N, NcoI; Bg, BglII. (c) Northern blot analysis of RNA extracted from leaves inoculated with CyRSV genomic RNA (G11) plus each of the DI-21 RNA deletion mutants as indicated above the pairs of lanes. G, sg1, sg2 and DI show the position of genomic, subgenomic and DI RNAs, respectively. Northern hybridization was done using a 32P-labelled nick-translated probe of DI-2 RNA.
the original DI-2 sequence, five additional unique sites (XhoI, BstBI, EcoRI, NcoI and BgII) were introduced at the positions of the natural deletion points by site-directed mutagenesis (Kunkel et al., 1987). The following oligonucleotides were used (new restriction sites are underlined and mutated nucleotides are italicized): 5' TCCAGGACACCTCTGGATTCCGGTG 3', 5' TTCTTTCTTCTCCAGGGTTC 3', 5' AAGCGAAAAATGACATGTTCGTTCAAGGGGT 3', 5' TGGAG- AGCTCTCCCTGTTCGAATCAGGGTTT 3', 5' AAGCGAGCAGATCTGTGATGGGAAACCA 3', which are homologous to positions 6-34, 65-90, 139-166, 262-276, respectively.

A series of deletion mutants of DI-21 was prepared by deleting regions between XhoI and BstBI, XhoI and AccI, BstBI and AccI, BstBI and EcoRI, AccI and EcoRI, EcoRI and NcoI or NcoI and BgII (Fig. 1b). In vitro RNA transcripts of DI-21 RNA mutants mixed with synthetic helper genome were used to inoculate Nicotiana clevelandii plants and protoplasts as previously described (Kollár et al., 1993; Dalmay et al., 1995). Total RNA was extracted from infected plants and the accumulation of DI RNAs was analysed by Northern hybridization (Sambrook et al., 1989) using a 32P-labelled nick-translated probe of a clone containing only the central translated probe of a clone of DI-2 RNA (Burgyán et al., 1991).

The results shown in Fig. 1(c) indicate that the replication of mutant DI-21 RNA is not affected significantly by the introduction of new restriction sites as compared with wild-type DI-2 RNA. Conversely, none of the deletion mutants obtained by deletion of conserved sequence blocks yielded biologically active transcripts. The results were confirmed by transfecting N. clevelandii protoplasts with deletion mutants in the presence of genomic RNA transcripts (not shown). These observations demonstrate that each of the conserved sequence blocks in the DI-2 RNA contain cis-acting elements necessary for viral RNA replication. Furthermore, no significant difference in the stability of the mutant and wild-type DI RNAs was observed in the control protoplasts transfected with DI RNAs without helper transcripts (not shown).

In order to identify more precisely the cis-acting replication signals of CyRSV DI RNAs, a number of mutants was prepared by extending the natural deletions into conserved blocks. The DI-21 plasmid was digested with the appropriate restriction enzymes, and then treated with Bal 31 at 30 °C for 1, 2, 3 and 5 min. The ends of the shortened plasmids were made flush with Klenow enzyme, religated, and used to transform Escherichia coli DH5α (BRL) competent cells. Several recombinant clones were sequenced with modified T7 DNA polymerase (Sequenase, USB) and mutant clones carrying a set of nested deletions (Fig. 2) were selected and used to inoculate N. clevelandii plants in the presence of in vitro transcripted genomic RNA. The accumulation of mutant DI RNAs was tested by Northern blot analysis in four replicates using a 32P-labelled nick-translated probe of a clone containing only the central block B of DI-2 RNA (Burgyán et al., 1991).

As shown in Fig. 2(a) deletions as long as 5 nt in block A1 are tolerated without affecting infectivity (XL5; Fig. 3), whereas mutants lacking 10 (XL10) or all of the first 5'-terminal 19 nt (XL19) were not infectious. This indicates that the first 5'-terminal 14 nt of DI RNA are essential for accumulation. Analysis of the second conserved block, A2, between the XhoI and BstBI sites, showed that the first 5 nt (XR5) or 10 nt (XR10, Fig. 3) can be deleted at the XhoI site without loss of infectivity but deletions longer than 10 nt are not tolerated. Similarly, deletions of 6 nt (BSL6) or 20 nt (BSL20) at the BstBI site in the same block (A2) were not permitted (Fig. 2b). Only 6 nt from the 5' end (BR6) and 4 nt (EL4) from 3' end of the third conserved region, A3 (BstBI-EcoRI), could be deleted without abolishing DI RNA replication (Fig. 2b, c).

In conclusion, these results indicate that a few nucleotides in block A are not essential for DI RNA replication, since natural deletions in block A could be further extended by a further few nucleotides without detectably altering DI RNA accumulation. In particular, mutant EL4 lacks the AUG start codon of the 33 kDa protein which is retained in nearly all reported tombusvirus DI RNA sequences (Burgyán et al., 1991; Knorr et al., 1991; Finnen & Rochon, 1993). It was suggested that the presence of this AUG could confer stability to DI RNA molecules by allowing binding to ribosomes (Rochon et al., 1994). Our results do not exclude this, but clearly show that the presence of the start codon for the 33 kDa protein is not essential for DI RNA survival, at least in the case of in vitro synthesized DI RNA. It cannot be ruled out, however, that this may be advantageous during de novo generation of DI RNA.

The unique natural restriction site (AccI) in the DI-21 RNA sequence prompted us to create mutants carrying deletions internally in the third (A3) conserved region; however, replication of these mutants was not detected (Fig. 2f).

As for block B, similarly to block A it was also sensitive to long deletions. In fact, deletions of 8 nt (ER8) and 13 nt (NL13) at the 5' and 3' ends, respectively, of block B were tolerated but mutants with longer deletions were not (Fig. 2c, d).

It was shown above that deletion of the 34 nt long conserved block C1 was lethal (mutant N-Bg, Fig. 1c). However, deletion of either half of the C1 sequence did
Fig. 2. For legend see opposite.
not abolish the accumulation of DI RNA (mutants NR17, and BB28 or BB38, respectively (Fig. 2d, e; Fig. 3). Incidentally, mutants BB28 and BB38 accumulated almost exclusively in the dimer form, which was in line with our recent results showing that short CyRSV DI RNAs accumulate preferentially as dimers (Dalmay et al., 1995). The observation that either half of the C1 block could be deleted suggested that the C1 block could merely be a spacer element rather than a specific recognition signal for viral replication. To test this hypothesis, the sequence of the C1 block was replaced with a foreign sequence. A cDNA clone from potato virus Y (PVY-H; Thole et al., 1993) was digested with AvaI and a 39nt long fragment between positions 8782–8819 was eluted and ligated into NcoI and BglII digested, blunted and dephosphorylated DI-21 plasmid. Two recombinants were selected, in which the entire C1 block of DI-21 was replaced by the 39 nt long PVY-H sequence in both positive DI-C1Y(+) and negative DI-C1Y(−) orientations. Contrary to our expectations, neither DI-YCI(+1) or (−) mutants were able to replicate in the presence of helper virus in inoculated protoplasts (not shown), indicating that either one of the two halves of the C1 block was specifically required for replication other than simply restoring a size suitable for replication. This is in contrast with a report on turnip crinkle virus (TCV) DI RNA where the biological activity of a lethal TCV DI RNA deletion mutant was restored by replacing the deleted sequence with foreign non-viral nucleotides (Zhang & Simon, 1994). It should be noted that the same PVY-H sequence inserted between the C1 and C2 blocks did not affect DI RNA replication: therefore it was not lethal per se (Dalmay et al., 1995).

Finally, it was shown that only the last 77 nt out of the 3′-terminal 102 nt are necessary for replication (mutant BR28) (Fig. 2e), whereas longer deletions in the C2 block completely abolished DI-21 RNA replication. It is tempting to suggest that the last 77 nt of the viral genome and either half of the C1 sequence block represent the complementary strand promoter sequences which are recognized by the viral replicase.

Those DI RNA deletion mutants which were able to replicate in inoculated plants in the presence of synthetic helper genome were isolated, RT–PCR amplified, cloned and sequenced as described in Dalmay et al. (1995). Each of them was identical with the respective input inoculum. Those mutants which failed to replicate in inoculated leaves were also used to transfect protoplasts, but none of them replicated, indicating that the lack of accumulation of these mutant DI RNAs is not a consequence

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**Fig. 2.** Nucleotide sequences of DI-21 RNA deletion mutants around the deletion sites. Deletion mutants are grouped (a–f) according to the restriction enzyme used for linearization before Bal 31 digestion as indicated on the top of each sequence group. Numbers in parentheses show the position of the sequence block in the DI-21 RNA genome. The designations of individual mutants are given on the left. + and − indicate whether the mutants DI RNAs are able to replicate (+) or not (−).
of insufficient movement but rather the lack of replication. In addition, no difference in the degradation of mutant and wild-type DI RNAs was detected in protoplast transfected with DI RNAs without helper genome (not shown).

In the present study, we have shown that the naturally formed smallest DI-2 RNA still contains sequences which are not required for replication and the natural deletions can be extended a few nucleotides beyond the natural ends of deletions. However, deletions created internally at the AccI site in block A3 abolished DI RNA infectivity.

Our observation that block C1 is essential for replication is inconsistent with a report on DI RNAs of tomato bushy stunt and cucumber necrosis tombusviruses, for which the entire block III (corresponding to the NcoI–BglII region in DI-21 RNA of CyRSV) can be deleted without loss of biological activity (Borja et al., 1994). Unfortunately, the results are not sufficiently detailed in that paper, so it is not possible to fully discuss these contradictory observations.

In this study, we mapped those sequences which are essential for CyRSV DI RNAs replication; however, it seems very likely that the same sequences are required for genomic RNA replication also. Experiments are in progress to determine whether the nucleotide sequence and/or the structures of these regions are involved in cis in the viral genome replication.

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References


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