Identification of a Peach Latent Mosaic Viroid Hairpin Able To Act as a Dicer-Like Substrate

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The ability of several viroids to induce posttranscriptional gene silencing has been demonstrated; however, the structure recognized by the Dicer enzyme(s) responsible for the initiation of this mechanism remains a mystery. Here, we show that the hairpin known to be implicated in the replication of peach latent mosaic viroid has the ability to trigger the Dicer enzyme(s). This domain, which is composed of a succession of several small stems separated by symmetrical bulges, is reminiscent of the precursor micro-RNAs.

Posttranscriptional gene silencing (PTGS) is a process known to play an important role in antiviral defense in plants (12, 21). PTGS is triggered by the presence of double-stranded RNAs (dsRNA) that are then cleaved by an RNase called Dicer (7, 19). There are at least four distinct Dicer-like (DCL) genes encoding predicted DCL enzymes in the *Arabidopsis* sp. and rice genomes (17). This cleavage leads to the formation of single-stranded RNAs, 21 to 25 nucleotides (nt) in length, called small interfering RNAs (siRNA). The siRNA are incorporated into a multisubunit protein complex, the RNA-induced silencing complex, and act as a guide to direct this RNA degradation machinery to its target RNAs. Moreover, the siRNA can serve as primers for an RNA-dependent RNA polymerase (RdRP), thereby causing an amplification phenomenon.

Viroids are small (~300-nt), single-stranded, circular RNA pathogens that infect higher plants (9). Based on the detection of siRNA of 21 to 25 nt, representing different regions of the viroid genome, species of the viroid families *Pospiviroidae* and *Avsunviroidae* have been shown to induce PTGS (6, 10, 12, 14, 15, 20). In order to determine how peach latent mosaic viroid (PLMVd), which belongs to the *Avsunviroidae* family (3), initiates PTGS, we adopted a model assay based on a wheat germ extract that contains DCL enzymes which convert dsRNA into siRNA (18). Initially, ^{32}P-radiolabeled PLMVd transcripts were synthesized in vitro in the presence of 50 μCi of [α-^{32}P]GTP (3,000 Ci/mmol) from the recombinant plasmid pPD1 that possesses two tandemly repeated PLMVd sequences (2). During transcription, RNAs of both polarities possessing hammerhead sequences were produced and self-cleaved efficiently, yielding 338-nt monomeric transcripts. Purified transcripts (0.5 pmol) of both plus and minus polarities were then heat denatured at 85°C and slowly cooled to room temperature in order to favor the formation of dsRNA (Fig. 1A). The resulting mixture was incubated with the wheat germ extract according to the manufacturer's recommendations (Promega) for 3 h at 25°C. The reaction was stopped by proteinase K treatment (15 min at 65°C) followed by phenol extraction. The nucleic acids

were then ethanol precipitated and fractionated on 10% denaturing polyacrylamide gels containing 7 M urea. The dsRNA-induced Dicer activity resulted in the formation of 21- to 25-nt siRNA. This confirms the hypothesis that dsRNA complexes formed by intermolecular base pairing of viroid strands of both polarities can serve as substrates for DCL enzyme(s) (10, 12, 14, 15). However, the incubation of PLMVd transcripts of either plus or minus polarity, both of which are highly structured RNA species (Fig. 1B) (4, 16), alone also resulted in the detection of siRNA (Fig. 1E, lanes 3 to 4). The latter structures are much more likely to be found in the cellular environment than are intermolecular dsRNA and can also be substrates for DCL. The cleavage of both the plus and minus strands produced different siRNA profiles (i.e., the former gave 21- and 24-nt siRNA, while the latter yielded 21-, 23- and 24-nt siRNA), probably due to small structural differences between the RNA species that either generated different products or recruited a different DCL. As controls, a characteristic hairpin formed by a 27-bp stem was efficiently cleaved, while a delta ribozyme motif that does not include a stem longer than 7 bp was not (Fig. 1C to E).

Subsequently, we investigated which region(s) of PLMVd was (were) a substrate for DCL(s). Transcripts containing two different regions of PLMVd for both polarities were synthesized from the pPD1 plasmid, and their ability to be cleaved by Dicer-like enzymes was assessed. Regardless of the polarity of the transcripts, only those regions including the long P11 hairpin, which is composed of a succession of small stems separated by symmetrical bulges, resulted in the production of siRNA (Fig. 2E). These observations were confirmed by Northern blot hybridization using probes directed against either the P11 hairpin or the right domain of PLMVd (Fig. 2F and G).

In order to identify the important structural features of the P11 stem that trigger the DCL enzyme(s), cleavage experiments were performed using substrates derived from the P11 hairpin. The left portion of the P11 hairpin substrate (left-P11) of 83 nt in size and the left-P11-(-10bp) (as identified in Fig. 3) of 63 nt had almost the same levels of cleavage. These substrates contain, respectively, 34 and 24 bp, if we consider that the bulged nucleotides adopt a stacked, non-Watson-Crick base-pairing structure. Conversely, removal of a further 5 bp resulted in an uncleavable substrate [left-P11-(-15bp)] of 53 nt.

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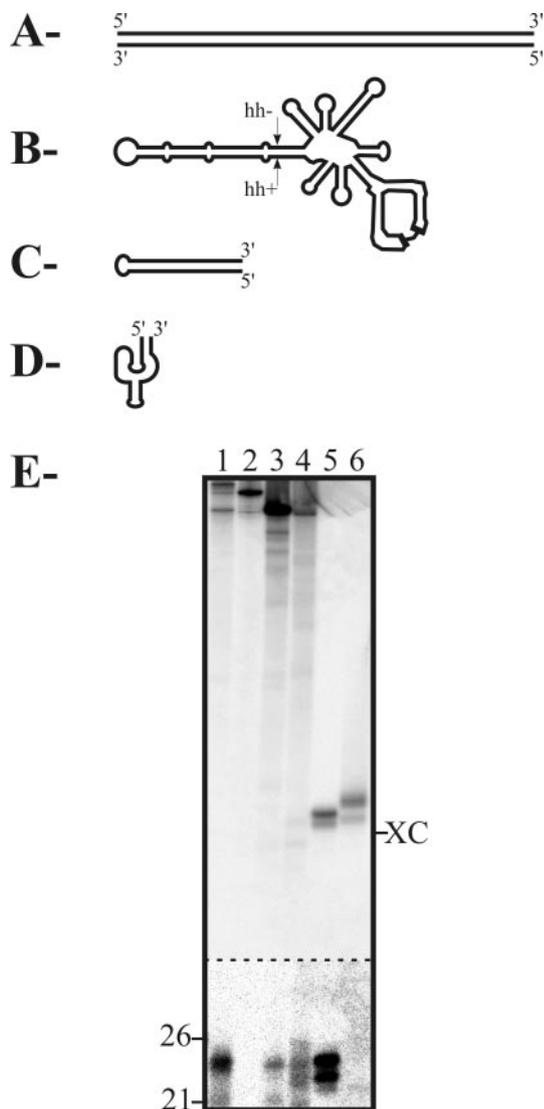
FIG. 1. Dicer activity assays performed using a wheat germ extract and various RNA substrates. (A to D) Schematic representations of the secondary structures of the tested RNAs. (A) Duplex of PLMVd strands of plus and minus polarity. (B) Single PLMVd strand of either plus or minus polarity. The hammerhead cleavage sites are identified (hh), and the + and − symbols designate their polarities. (C) Model hairpin including a 27-bp stem. (D) *Trans*-acting delta ribozyme (the nucleotide sequences of the latter two RNA substrates can be retrieved elsewhere) (11, 13). (E) Autoradiogram of a polyacrylamide gel electrophoresis (PAGE) gel of Dicer activity assays performed using a wheat germ extract. Lanes 1 and 2 are duplexes of the PLMVd strands shown in panel A. The reaction conditions were identical except that the wheat germ extract was inactivated by heat treatment prior to the incubation for lane 2. Lanes 3 and 4 are PLMVd strands of either plus or minus polarity, respectively. Lanes 5 and 6 are the assays with the model hairpin (R31-27) and delta ribozyme (Rz-12), respectively. To the left of the gel, the positions of the RNA markers of 21 and 26 nt are indicated. XC indicates xylene cyanol. The bottom part (below the dashed line) has been overexposed in order to reveal the bands corresponding to the small products.

The latter substrate included only a 19-bp stem, which is smaller than the 21 bp required for Dicer activity (8). Similar results were obtained when using the right portion of the P11 stem, to which a loop allowing the two strands to connect

was added [right-P11-(-10bp) and right-P11-(-15bp)]. The sequence composing the loop was also shown to be unimportant for cleavage to occur [i.e., left-P11-(loop)]. A substrate in which the bulged nucleotides from the upper strands were
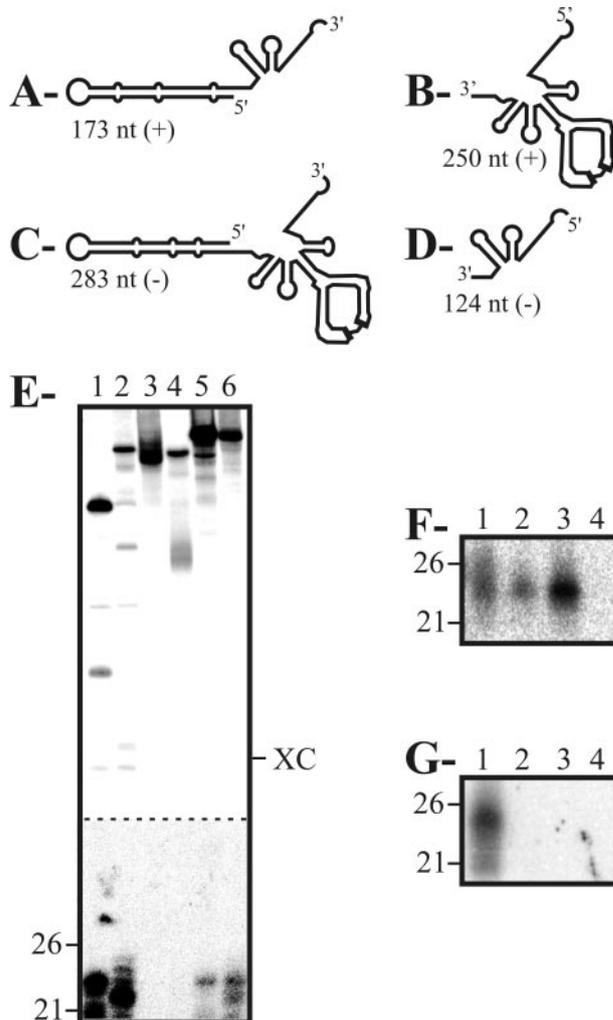

FIG. 2. Dicer activity assays performed with various PLMVd-derived fragments. (A to D) Schematic representations of the plus-polarity (A, B) and minus-polarity (C, D) PLMVd fragments tested. The length, in nucleotides, is indicated for each fragment. (E) Autoradiogram of a PAGE gel of Dicer activity assays performed as described above. Lanes 1 to 6 are the 173-nt (lane 1), 283-nt (lane 2), 250-nt (lane 3), 124-nt (lane 4), and 338-nt fragments of plus and minus polarity (lanes 5 and 6, respectively). Adjacent to the gel, the positions of the RNA markers of 21 and 26 nt are indicated. XC indicates xylene cyanol. The bottom part (below the dashed line) has been overexposed in order to reveal the bands corresponding to the small products. (F, G) Autoradiograms of Northern blot hybridizations of cleavage assays performed in the presence of nonradioactive PLMVd RNA fragments of plus polarity. The conditions for hybridization have been described previously (12). The 5′-^{32}P-oligonucleotides used as probes were complementary to a part of the P11 hairpin (5′-$_1$GCCCACTGATGAGC CGCTGAAATGCGGCGAAACTTTTGAT$_{40}$-3′) (F) and the right domain (5′-$_{142}$CCGCTTGGTTCCCGAAGGAAAAGTCCCACCTT ACCTCATTG$_{182}$-3′) (G) of PLMVd. Lanes 1 to 4 are the duplexes of PLMVd strands of plus and minus polarity (lane 1), the 338-nt fragments (lane 2), and the 173-nt (lanes 3) and 250-nt (lane 4) fragments of plus polarity. Adjacent to the gel, the positions of the RNA markers of 21 and 26 nt are indicated.
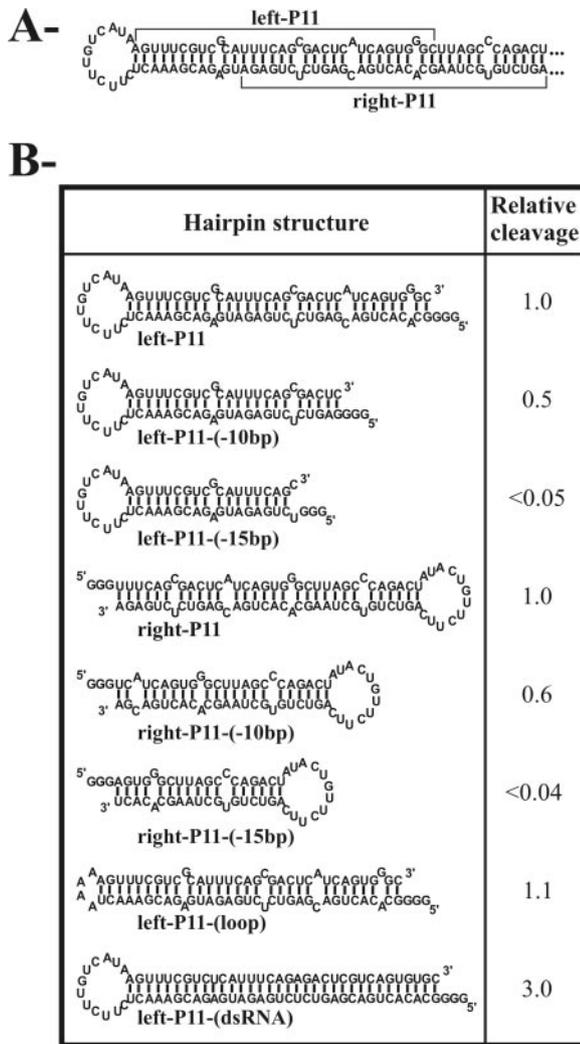
FIG. 3. Dicer activity assays performed with various transcripts derived from the P11 hairpin. (A) The sequence and secondary structure of the hairpin is illustrated. This is a variant sequence of the pPD1 insert sequence (see reference 2). Three guanosines were added at the 5′ end of each transcript to ensure efficient transcription from the oligonucleotides. (B) The Dicer assays were performed as described in the text. The cleavage levels were determined and are reported as relative values in comparison to a value of 1.0 that was arbitrarily assigned to the left-P11 substrate.

hairpin. In plants, the siRNA can subsequently be used as a primer by an RdRP, thereby producing more dsRNA susceptible to the activity of DCL enzyme(s) (7, 19). Incubation of a full-length PLMVd RNA with a $5′$-^{32}P-radiolabeled RNA primer complementary to the left-terminal P11 loop produced RNA fragments of the predicted sizes, confirming the presence of the RdRP activity in the wheat germ extract (Fig. 4, lane 1). Similar results were obtained using other RNA primers complementary to various regions of the viroid and to different PLMVd fragments (e.g., Fig. 4, lane 2). Moreover, the production of dsRNA by RdRP from a nonradioactive PLMVd fragment and an RNA primer was monitored by the detection of siRNA by Northern blot hybridization after incubation in the wheat germ extract. Using a probe against the right domain of PLMVd, a faint tiny signal was observed in the presence of the RNA primer, while no signal was detected if PLMVd was incubated alone (data not shown). This shows that the amplification took place in the wheat germ extract, although inefficiently compared to that in the plant cell. The amplification is presumably at a higher level in plants than in wheat germ extracts because the enzymatic extract is exhausted after several minutes, while the phenomenon of accumulation in the living cell occurs for an extended period of time.

In summary, this study identifies the P11 hairpin of PLMVd as the domain recognized by the DCL enzyme(s), thereby initiating PTGS. It seems unlikely that the P11 hairpin evolved specifically to be a Dicer substrate, because the viroid evades PTGS-mediated destruction by replicating in the chloroplast (3, 12). The selective pressure on the P11 sequence and structure came primarily from its implication in the mechanism of
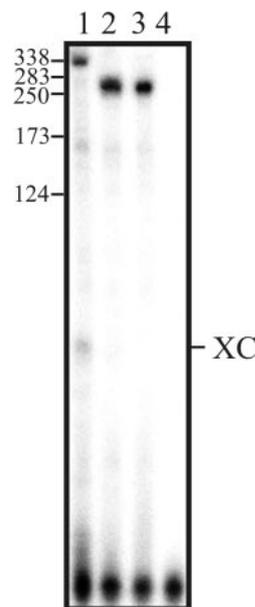
substituted by residues permitting the formation of Watson-Crick base pairs was cleaved significantly more efficiently than the initial imperfect stem [i.e., 3.0 for the left-P11-(dsRNA)], demonstrating DCL's preference for perfect dsRNA. Similar results were obtained when the experiments were repeated using transcripts derived from the P11 hairpin of minus polarity as well as changing the positions and identities of the bulged nucleotides (data not shown). Taken together, these data demonstrated that DCL enzymes prefer dsRNA of at least 21 bp in length, regardless of their sequence, but can tolerate the presence of bulged nucleotides.

Previous studies indicated that accumulated siRNA in infected plants form a population of sequences representing the entire PLMVd genome (12, 14), whereas in wheat germ extract they appeared to be restricted to the sequences of the P11

FIG. 4. Autoradiogram of a 10% PAGE gel of primer extension by the RdRP in wheat germ extract. Lane 1 is the extension of primer P11 (5′-GGG$_6$UUUGAUGAUAUGAGUUUCGUC$_{324}$-3′) on the 338-nt PLMVd template. Lane 2 is the extension of primer P10 (5′-GGG$_{290}$ACUCAUCUUCCAGAAUCACU$_{270}$-3′) on the 250-nt PLMVd fragments. Lanes 3 and 4 are controls with DNase digestion and without template, respectively. To the left of the gel, the positions of the RNA markers are indicated. XC indicates xylene cyanol.

replication. It seems most likely that PTGS induction occurs during the cytoplasmic phase of the viroid's life cycle (e.g., as it moves from cell to cell). On the other hand, the structure of P11 recalls that of the pre-micro-RNAs (pre-miRNA), which are bulged hairpins of variable sizes, sometimes with branched structures, that are cleaved by DCL1 to give miRNA (1, 5, 17). These miRNA subsequently interact with specific mRNAs in order to regulate their translation. It seems reasonable to suggest that this mechanism might be involved in the pathogenesis of this viroid (20); however, physical evidence remains to be reported. In contrast to the DCL2 and DCL3 that have been associated with cytoplasmic siRNA biogenesis, DCL1 was demonstrated to be a nuclear enzyme (17, 21, 22). However, this localization is incompatible with the hypothesis that PLMVd might take in the miRNA biogenesis pathway. Clearly, additional experimentation should reveal many other intimate details related to the viroid's PTGS mechanism.

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