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Inosine 5′-triphosphate can dramatically increase the yield of NASBA products targeting GC-rich and intramolecular base-paired viroid RNA

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ABSTRACT

Nucleic acid sequence-based amplification (NASBA) according to the standard protocol failed to amplify cRNA of viroids, probably because of their GC-rich and intramolecular base-paired structure. However, NASBA in the presence of inosine 5′-triphosphate successfully amplified the cRNAs to viroids in total nucleic acid extracts from citrus plants. As sequence specificity of the cRNA to viroids was confirmed by northern analysis, the amplification and fidelity of cRNAs are sufficient for the sensitive and specific detection of viroids.

Nucleic acid sequence-based amplification (NASBA) (1), which is also known as the self-sustained sequence replication technique (3SR) (2), is a method of *in vitro* amplifying target RNA. NASBA has been used for highly sensitive detection of several animal and plant pathogens. Viroids are the small, single-stranded, circular, plant pathogenic RNAs and five distinct citrus viroids (CVds), i.e. CVd-IV, -III, -II, -I and citrus exocortis viroid (CEVd), infecting citrus plants have been reported which exist in low concentrations in citrus tissues (3). We tried to detect viroids by NASBA, however, NASBA under standard conditions (4) failed to amplify cRNA of CEVd and hop stunt viroid-citrus (HSVd-cit: CVd-II) sufficiently (Fig. 1B, lane 2). The reason is probably that viroids and their initially synthesized cRNAs are GC-rich (60% in the CEVd sequence) and have an intramolecular base-paired structure (66% of the CEVd sequence are base-paired) resulting in a reduction in the processibility of AMV reverse transcriptase (RTase). In amplification of viroids by RT-PCR, the secondary structure may also inhibit the processibility of RTase. Heat-denaturation before RT and/or RT at elevated temperatures using thermostable RTase are thought to be efficient to overcome the problem in RT-PCR. However, these treatments cannot be used in the case of NASBA, since the NASBA process uses an amplified cRNA as the next template which has the potential to become almost the same structure as that of the target RNA, and by the combination of three enzymes (RTase, DNA dependent RNA polymerase and RNase H) of which none have been reported as thermostable except for RTase.

To overcome these problems, we investigated the effect of inosine 5′-triphosphate (ITP) on the stability or conformation of the secondary structure of amplified cRNA in order to increase the yield of NASBA products. Inosine can base pair with cytosine, thymine and adenine without de-stabilizing the helical structure, and this would allow the incorporation of ITP into amplified RNA in the NASBA reaction. In this study, pairs of oligonucleotide primers were designed to amplify cRNAs to both CEVd (5) and CVd-IV (6), or to HSVd-cit (7) (Fig. 1A). NASBA was basically carried out as described (4), except for an addition of ITP without change in other nucleotide concentrations. The effects of ITP concentration are shown in Figure 1B and C. An increase in the yield of NASBA products was observed at 1.5–4 mM (CEVd) and 0.5–3 mM (HSVd-cit) (Fig. 1B), peaking at 1.5–2 mM (Fig. 1C). The optimal ITP concentration was determined to be 2 mM, and in all the following experiments, NASBA was carried out in the presence of 2 mM ITP. The cRNAs of CEVd were next amplified from each serial dilution (100 pg–100 ag) of purified CEVd by modified NASBA. The sensitivity of the modified NASBA for detection of CEVd was analyzed by denatured polyacrylamide gel electrophoresis (dPAGE) (Fig. 2A) and northern hybridization probed with a full-length homologous RNA (hRNA) (Fig. 2B). A specific band was detected from a lane with as little as 1 fg of CEVd by both dPAGE and northern hybridization. The CEVd-hRNA probe did not hybridize with amplified cRNA of HSVd-cit (55% homology) under these hybridization conditions (data not shown). Nucleic acid hybridization analysis indicated that amplified cRNA could hybridize with the hRNA probe to detect respective viroids efficiently and specifically, although the stability between the amplified CEVd cRNA and the hRNA probe is thought to be reduced due to the incorporation of ITP into cRNA.

Compared with other detection techniques, the modified NASBA was found to be more sensitive than dot blot hybridization with a cRNA probe, with the ability to detect as little as 10–100 pg of CEVd RNA (unpublished data). It was also more sensitive than RT-PCR, which was carried out basically as described (8) with the following modifications: 40 µl of PCR premix was directly added to total RT products synthesized in 10 µl of mixture and 40 cycles of PCR were performed, using a pair of primers, PCEV-1P and PCEV-1M (almost the same sequence as T7PCEV-1M without the T7 promoter sequence) and *Tth* DNA polymerase (Toyobo). When 20% of the total RT-PCR products was analyzed by agarose gel electrophoresis and ethidium bromide staining, as little as 100 fg of CEVd could be detected (unpublished data).

For practical diagnosis, the cRNAs of CEVd and CVd-IV were amplified from the total nucleic acid extracts of an infected citrus leaf, by modified NASBA. Figure 3 shows that a band considered as cRNAs of CEVd and CVd-IV, respectively, could be detected, although two other artifact bands were also detected in lane 3.

Consideration must be given to the potential detrimental effects of ITP on the fidelity of polymerization by RTase and T7 RNA

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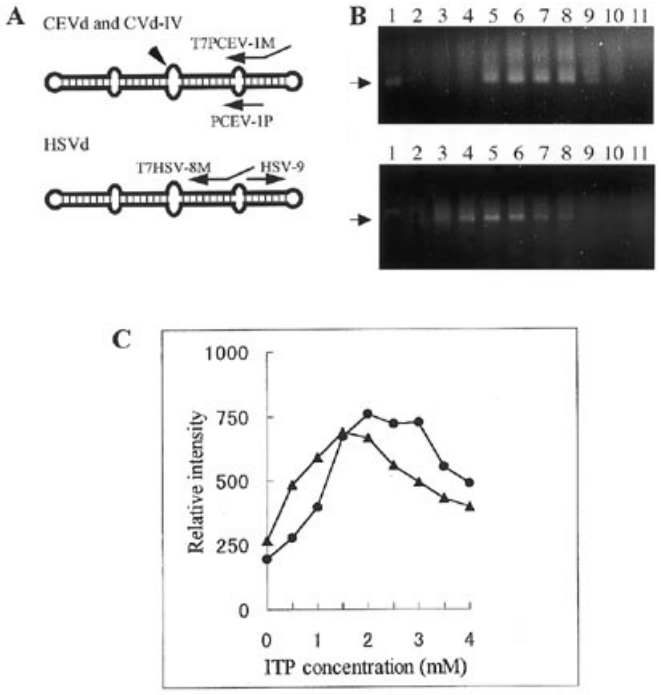

Figure 1. (**A**) Schematic representation of the secondary structures of viroids, including the localization of NASBA primers on CEVd and CVd-IV, or on HSVd. The expected sizes of amplified fragments complementary to viroid sequences are 334 nt for CEVd (consisting of 371 nt) (5) and 247 nt for CVd-IV (consisting of 284 nt) (6) with primers PCEV-1P (homologous: 5′-GCTCCACATCCGATCG-TC-3′) and T7PCEV-1M (complementary: 5′-*AATTCTAATACGACTCACTATAG-GG*GCTGGACGCCAGTGATCCGCGGC-3′), and 290 nt for HSVd-cit (consisting of 302 nt) (7) with primers HSV-9 (homologous: 5′-CGCGGTGCTC-TGGAGTAGA-3′) and T7HSV-8M (complementary: 5′-*AATTCTAATACGACT-CACTATAGGG*ACGCCTCTCGCTGGATTCTGAG-3′) (T7 promoter sequence is shown in italic letters). An arrowhead indicates a *Bam*HI site used for cloning of a full-length cDNA and generating a linear full-length hRNA *in vitro* to CEVd. (**B**) NASBA products amplified from 1 ng of purified CEVd (upper panel) and HSVd (lower panel). NASBA reaction mixture (25 µl) contained 40 mM Tris–HCl pH 8.5, 12 mM $MgCl_2$, 42 mM KCl, 5 mM DTT, 15% DMSO, 1 mM of each dNTP, 2 mM of each rNTP, increasing concentrations (0–4 mM) of ITP, 0.1 µg/µl BSA, 0.1 U RNase H (TaKaRa), 40 U T7 RNA polymerase (Gibco BRL), 8 U AMV RTase (Seikagaku), 0.2 µM each of complementary and homologous primers. NASBA was carried out at 41°C for 90 min. Lane 1 is RNA transcripts from CEVd RT-PCR products with PCEV-1P and T7PCEV-1M by T7 RNA polymerase; lanes 2–10 are 20% of the total NASBA products in the presence of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 mM of ITP, respectively; and lane 11 is in the absence of the purified viroid. These products were denatured and analyzed by electrophoresis on a 2% agarose gel containing 0.615 M formaldehyde and stained with ethidium bromide. (**C**) Effect of ITP concentration on relative intensity of the NASBA products, amplified in (B) to CEVd (●) and HSVd-cit (▲), determined by densitometric analysis (ATTO Densitograph 4.0).

polymerase. However, conversions of the amplified cRNA sequence from the original one may only be G→I substitutions (9). Since amplified cRNA could indeed be detected efficiently not only by dPAGE but also by northern hybridization, the modified NASBA maintained fidelity sufficiently for the detection and identification of CEVd. Additionally, the modified NASBA was more sensitive than RT-PCR for the detection of CEVd. Another advantage of NASBA over RT-PCR is that a target RNA is amplified very quickly [to achieve the 10^5-fold amplification observed in the first 15 min (2)] in one reaction, in one tube, and at one temperature. The modified NASBA is also thought to be useful for amplifying cRNA and for sensitive detection of other GC-rich and intramolecular base paired RNAs such as satellite, ribosomal and transfer RNAs.

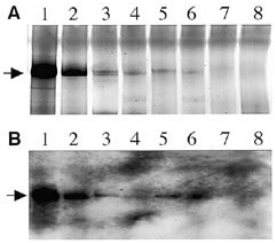

Figure 2. Sensitivity for the detection of CEVd by NASBA in the presence of 2 mM ITP. (**A**) NASBA products were denatured at 50°C for 1 h in glyoxal/DMSO solution (10 mM sodium phosphate, pH 7.0, 50% DMSO, 1 M glyoxal), then precipitated with ethanol. 20% of the total NASBA products was analyzed by electrophoresis on a 5% denatured polyacrylamide gel containing 8 M urea, and stained with silver as described (10). (**B**) 4% of the total NASBA products was electrophoresed as in panel A, and electrophoretically transferred to a nylon membrane Hybond-N (Amersham) and hybridized with a digoxigenin-labeled full-length CEVd-hRNA basically according to ref. 11, but hybridization was performed at 50°C (in the presence of 50% formamide) and washing was performed at 60°C in 0.1× SSC–0.1% SDS without the RNase A digestion step. Lanes 1–8 are NASBA products amplified from 100, 10, 1 pg, 100, 10, 1 fg, 100 ag and absence of purified CEVd, respectively.

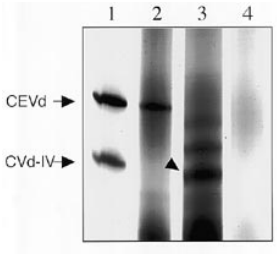

Figure 3. Detection of CEVd and CVd-IV in total nucleic acids extracted from infected citrus leaves by NASBA in the presence of 2 mM ITP. Denatured PAGE analysis of NASBA products amplified from 3 U of full-length linear RNA of CEVd and CVd-IV generated *in vitro* (lane 1), total nucleic acids equivalent to extracts from 10 mg of citrus leaf infected with CEVd and CVd-III (lane 2) or HSVd-cit, CVd-III and CVd-IV (lane 3), and absence of total nucleic acids (lane 4). Each of the NASBA products was analyzed as in Figure 2A. In lane 3, an arrowhead indicates a band considered to be cRNA to CVd-IV.

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