Nucleic Acids Research, 1993, Vol. 21, No. 5 1325–1326

Cloning biologically active geminivirus DNA using PCR and overlapping primers

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Received December 10, 1992; Accepted February 4, 1993

We report a new strategy for cloning DNA of the plant-infecting geminiviruses that is based on PCR amplification with primers overlapping the sequence of a restriction site in the viral genome. This method reduces the time and work necessary to obtain biologically active geminivirus DNAs and could be used for other DNA viruses having circular genomes, such as the animal-infecting circoviruses. Geminiviruses possess circular single-stranded DNA genomes that are monopartite [one DNA of ca. 2.6 kilobases (kb)] or bipartite (two DNAs designated DNA A and DNA B, each ca. 2.6 kb) and are encapsidated within twinned icosahedral particles (1). Replication of the viral genome occurs within plant cell nuclei via circular double-stranded replicative form (RF) DNA. RF DNA has been used to obtain infectious clones of several geminiviruses (2, 3, 4). Typically, RF DNA is isolated from infected plant tissue, a unique restriction site is identified within the DNA, and the digested full-length viral genome or genome component is cloned into a standard bacterial cloning vector. However, in our efforts to obtain full-length clones of the DNA A component of a bipartite tomato-infecting geminivirus from Costa Rica (given the provisional name TGV-CR), we were unable to identify a suitable unique restriction site in the DNA A RF for cloning. Therefore, an alternative PCR-based strategy was developed.

This cloning strategy first involved PCR amplification of a 1.1 kb fragment of TGV-CR DNA A from infected *Nicotiana benthamiana* plants with general (degenerate) geminivirus primers as described by Rojas (5). The 1.1 kb fragment was cloned using the TA Cloning System (Invitrogen), and the nucleotide sequences of the 5′ and 3′ ends of the fragment were determined by the dideoxy chain termination method with Sequenase (USB). DNA sequence analysis confirmed that the 1.1 kb fragment was a geminivirus DNA A fragment, and that TGV-CR was different from previously characterized geminiviruses (5). Further analysis of the sequence revealed an *Xba*I site that corresponded to nucleotide 255 in the DNA A of bean dwarf mosaic geminivirus (BDMV, 6). The *Xba*I and surrounding sequences were used to design overlapping primers for PCR amplification of the complete TGV-CR DNA A component. The primers and the strategy used for amplifying the DNA A component are shown in Figure 1. DNA was extracted from infected *N.benthamiana* plants as previously described (7). PCR was carried out according to Saiki *et al.* (8) with *Taq* polymerase (Promega) used according to manufacturers' recommendations in a Perkin-Elmer Cetus DNA Thermal Cycler. The final reaction volume was 100 μl with final dNTP and primer concentrations of 200 μM and 0.4 μM, respectively. DNA was amplified by 30 cycles of PCR with melting, annealing and polymerizing conditions of 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C, respectively.

An approximately 2.6 kb DNA was amplified from the infected plant extract. The 2.6 kb DNA was cloned using the TA cloning system, and the nucleotide sequences of the 3′ and 5′ ends of one clone (pCRA1) were determined. DNA sequence comparisons revealed that the 2.6 kb DNA was TGV-CR DNA A and that the primers annealed at the predicted locations, suggesting that this was a full-length clone. However, restriction enzyme analysis of pCRA1 revealed an additional *Xba*I site within the TGV-CR DNA A. To get a full-length insert to test for biological activity, partial *Xba*I digests of cesium-purified pCRA1 were made (9), and DNA was recovered by ethanol precipitation and checked for the linear 2.6 kb insert. Because the 5′ portion of the primers flanking the *Xba*I sequence contained randomly selected nucleotides and the 3′ portion contained viral sequences, partial digestion of the amplified fragment with *Xba*I would be predicted to release the full-length DNA A fragment from pCRA1 (Figure 1). To prove the biological activity of the amplified TGV-CR DNA A in the absence of the DNA B component, which is necessary for systemic infection of plants by bipartite geminiviruses (10), *N.tabacum* protoplasts were used. The partially digested pCRA1 DNA was electroporated into *N.tabacum* protoplasts prepared from a *N.tabacum* suspension culture essentially as described by Fromm *et al.* (11). Protoplasts were then incubated at room temperature in the dark, and aliquots collected at 0, 3, and 5 day intervals. Total genomic DNA was recovered from protoplasts and analyzed by Southern hybridization analysis with radiolabelled DNA A components of BDMV and bean golden mosaic geminivirus as probes. Clear evidence of the replication (biological activity) of the TGV-CR DNA A was seen at the 3- and 5-day time points, in which increased levels and new forms of TGV-CR DNA A were observed (Figure 2).

Based on the successful amplification of biologically active TGV-CR DNA A, it appears that annealing of the primers at the complementary *Xba*I sequences did not prevent amplification, and that nonvital sequences flanking the TGV-CR DNA A were removed by the *Xba*I digestion of pCRA1. By using the TA cloning system, we were able to clone the entire amplified TGV-CR DNA A despite the presence of the additional *Xba*I site within TGV-CR DNA A, although partial digests were needed to

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recover the full-length fragment for infectivity studies. While it is possible that the PCR-generated TGV-CR DNA A clone contained errors introduced by the *Taq* polymerase, the biological activity of the clone suggests that no errors were introduced, or that any introduced errors were silent (in respect to replication functions). The use of DNA polymerases having increased fidelity of DNA synthesis (e.g. *Pfu* DNA polymerase, 12) could greatly reduce the probability of introducing such errors.

ACKNOWLEDGEMENTS

We thank Margaret Sanger for assistance in preparation of protoplasts, and Jeff Hall for assistance in preparation of the figures. This work has been supported in part by the College of Agricultural and Environmental Sciences, University of California-Davis and by grants from the Asgrow and Petroseed Companies.

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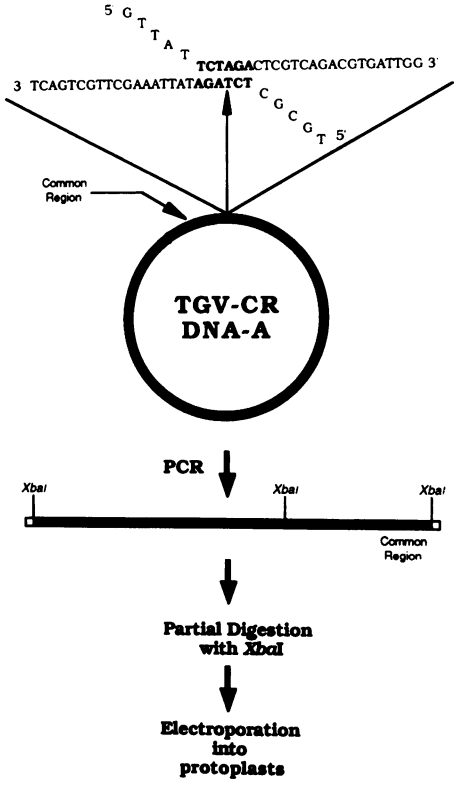

Figure 1. Strategy for PCR amplification, cloning and testing biological activity of the DNA A component of the bipartite geminivirus, TGV-CR.

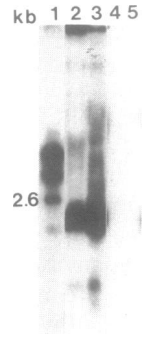

Figure 2. Replication of the PCR-amplified TGV-CR DNA A in *N.tabacum* protoplasts. Southern blot analysis of total DNA extracted from protoplasts and hybridized with radiolabelled DNA A components of bean dwarf mosaic geminivirus and a bean golden mosaic geminivirus. Lane 1, DNA extracted from protoplasts immediately after electroporation with pCRA1 partially digested with *XbaI* (2.6 kb linear monomer of TGV-CR is indicated); lanes 2 and 3, DNA from protoplasts electroporated with partially digested pCRA1 after 3 and 5 day incubation, respectively; lanes 4 and 5, DNA from mock-inoculated protoplasts after 3 and 5 day incubation, respectively.