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Detection and identification of sweet potato viruses by the polymerase chain reaction

ABSTRACT

Among the numerous agents infecting tropical crops, such as sweet potato, several viruses belonging to the potyvirus group have been described. Two degenerate oligonucleotide primers, derived from conserved regions of the genome of potyviruses, have been designed in order to amplify a fragment including the non-conserved 5' terminal part of the coat protein cistron (which may greatly differ in length in distinct potyviruses) and the more conserved 3' terminal part of the RNA polymerase cistron. A combined assay of reverse transcription followed by the Polymerase Chain Reaction using these primers on total RNA extracted from different sweet potato clones from China, yielded one or several of three fragments, thus suggesting the presence of at least one to three distinct potyvirus, according to the sweetpotato clone. Sequence analysis of the three fragments confirmed the presence of mixed infections by distinct potyviruses in the sweet potato clones investigated. The cloned PCR products were used to develop a non-radioactive hybrization test, using the digoxigenin labelling system. The PCR procedure using degenerate primers is thus a rapid and valuable tool for the detection and characterization of potyviruses. The amplified fragments thus obtained can easily be cloned into a vector and sequenced for classification and taxonomical studies, or used for the development of nucleic acid hybridization tests to be used in certification procedure.

INTRODUCTION

The level of production of vegetatively propagated crops, such as sweet potato, is greatly reduced by infection with several viruses. In the short term, production of pathogen-free material and disease indexing are the first steps of a proper control strategy of viral diseases. Successful sanitation by meristem culture is exemplified by a case study dealing with freeing sweet potato clones originating from Guangdong province, China, from their viruses. Within the frame of a cooperative research program with the financial support of EEC, the production of virus free material through meristem tip culture allowed a significant increase of field yield of fresh storage root. The weight increment ranged from 15% to 100%, according to the cultivar, while the quality of the products was generally improved (unpublished results).

With vegetatively propagated clones, the economical value of virus-free clones relies on controlled schemes of field-propagated healthy seed material. Such supply of indexed material should take into account the epidemiological parameters and the nature of the viruses involved. Analysis of the rate of natural virus reinfection through successive field propagations is also necessary to determine the optimum number of successive field propagations sustaining acceptable yields. In this respect, the development of efficient certification procedures is necessary, based on a good knowledge of virus detection technology, of the etiology, and of the epidemiology of the viral agents involved.

The rapid progress achieved in molecular biology and medical diagnostics has generated a number of biochemical processes that have subsequently been successfully applied to crop protection and virus detection. The most significant progress in the area is the advent of the polymerase chain reaction (PCR) which has emerged as a powerful method for the identification of viruses. In the case of sweetpotato, viruses belonging to the Potyvirus genus have frequently been described. Therefore, the objective of our work was to develop a powerful broad spectrum PCR procedure to detect and characterize those potyviruses.

THE POLYMERASE CHAIN REACTION

The polymerase chain reaction relies on the ability of DNA polymerases to duplicate a DNA molecule and allows the amplification of a specific DNA region. PCR involves two oligonucleotides that flank the segment to be amplified. These primers hybridize to opposite strands of the target sequence and are oriented so that DNA synthesis by the polymerase proceeds across the region between the primers. Since the extension products are also complementary to the primers, the cycle can be repeated after a denaturation step. By repeated cycles of denaturation, annealing of the primers and elongation, there is a rapid exponential accumulation of the specific region bounded by the primers.

To amplify new or uncharacterized sequences related to a known family of genes, it is possible to use protein sequence data to design degenerate primers. These are a mixture of oligonucleotides varying in base sequence but having the same number of bases. The use of such degenerate primers designed from highly conserved regions of the viral genome has been shown to enable the detection and the identification of plant viruses belonging to the geminiviruses (12), the potyviruses (8,9) and the luteoviruses (12).

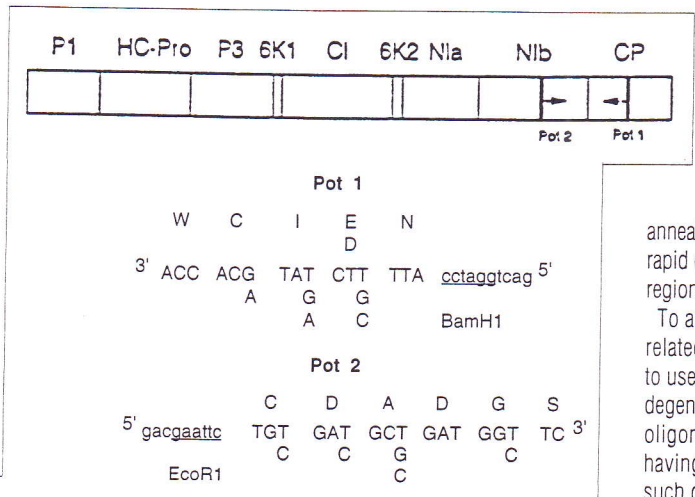

Figure 1 - Genetic map of the potyvirus genome showing the cleavage products of the polyprotein and the relative positions of the two degenerate primers based on selected conserved regions (Pot I and Pot 2)

PI: first protein (protease); HC-Pro: helper component/protease; P3: third protein; 6KI: 6k peptide; CI: cytoplasmic inclusion protein; 6K2: second 6k peptide; Nia: nuclear inclusion protein a; Nib: nuclear inclusion protein b (RNA polymerase); CP: coat protein.

THE POTYVIRUS GENUS

The Potyviridae family is the largest, economically most important and most widespread plant virus family in the world. 198 viruses are indexed so far in the Potyviridae, infecting a wide range of crops, especially in tropical and subtropical areas (15). The Potyviridae comprise a few genera from which the Potyvirus genus is by far the largest with a current membership of 180 viruses (15).

Viruses belonging to the genus Potyvirus share the same characteristics with respect to particle morphology and the organisation and expression strategy of their RNA genes. The flexuous and rod-shaped virus particles of 680 to 900 nm long and 11 to 15 nm wide consist of approximately 2000 units of one type of structural protein that encapsidate a non-segmented, single stranded RNA molecule of positive polarity and with a length of approximately 10000 nucleotides (11,15). The potyviral genome contains one large open reading frame (ORF) which is translated into a large polyprotein. This polyprotein precursor is cleaved by three virus-encoded proteases into at least eight polypeptides (Figure 1) (11,15).

Of the cleavage products of the polyprotein, coat protein is the most characterized. Complete amino acid sequences have been obtained for the coat protein of 89 strains of 28 distinct potyviruses (15). Sequence comparisons and biochemical analyses revealed that the N-termini of the coat protein of distinct potyviruses vary considerably in length and sequence, whereas the C-terminal two-thirds are highly similar (13,15). In contrast, the related strains of the same virus generally have coat proteins of the same length and their N-terminal sequences are highly homologous (13,15).

IDENTIFICATION OF DISTINCT POTYVIRUSES IN MIXED INFECTIONS

The PCR strategy using degenerate primers designed to amplify the variable 5'-terminal region of the coat protein cistron of potyviruses have been used to detect distinct viruses infecting sweetpotato. The amino acid sequences of the polyprotein of five potyviruses as well as the amino acid sequences of the coat protein of five other members of this genus have been compared. Two regions presenting a high percentage of homology and encoded by codons with low degeneracy were selected (Figure 1). Degenerate primers were synthesized representing all possible nucleotides found in these regions for the potyviruses under investigation. The region bounded by these primers, Pot 1 and Pot 2, spans the 5' terminal part of the coat protein cistron as well as the 3' terminal part of the RNA polymerase cistron (3).

A combined assay of reverse transcription, followed by the Polymerase Chain Reaction using primers Pot 1 and Pot 2 on preparations of total RNA extracted from *Ipomoea purpurea* infected with a sweet potato feathery mottle virus isolate from China (SPFMV-CH) or *Nicotiana benthamiana* infected with a sweet potato latent virus isolate from Taïwan (SPLV), amplified 1.35 kb and 1.30 kb fragments respectively (4). Amplification of the unconserved 5' terminal part of the coat protein cistron from transcripts of total RNA extracted from different sweet potato clones from China, yielded one or both of these fragments together with a 1.45 kb fragment, thus suggesting the presence of a third potyvirus in these sweet potato clones. The three PCR-amplified fragments were cloned into a plasmid and partially sequenced. Comparison of the amino acid sequences coded by the amplified fragments with those of the C-terminal part of the RNA polymerase and the N-terminal part of the coat protein published for several other potyviruses, revealed the presence of consensus motifs (1,6,10) which confirm the presence of mixed infections by distinct potyviruses in the sweet potato clones investigated (5). The name sweet potato virus G (SPV-G) has been tentatively assigned for the virus from which amplification yielded a 1.45 kb fragment (5).

Sequence information from the coat protein and the 3' non-coding region is generally used for the classification of potyviruses. Therefore a modification of the PCR technique, known as the RACE (Rapid Amplification of cDNA Ends, 7) procedure was used to clone a fragment corresponding to the 3'-terminal part of the coat protein cistron together with the 3' non-coding region of SPVG. Sequence comparisons revealed that the coat protein of SPV-G was only distantly related to that of known potyviruses, with the exception of the sweet potato feathery mottle virus (SPFMV). Indeed, sequence identity in the C-terminal three quarters of the coat protein (more than 80%) and in the 3' untranslated region (more than 70%) indicate that SPV-G should be considered as closely related to, though distinct from SPVG (5).

The cloned PCR products were used to develop non-radioactive probes for nucleic acid spot hybridization tests which allow to detect viruses from crude extracts of infected plant material.

CONCLUSIONS

Certification of vegetatively propagated crops infected with several known viruses relies on the availability of multiple detection methods, including inoculation of test plants, serological tests or nucleic acid hybridization. For practical use, any method of diagnosis should be evaluated in terms of sensitivity, specificity, reliability and cost, according to the problem encountered.The certification is hampered with a crop such as sweet potato because the etiology of many viruses remains unclear. In this case, availability of one rapid broad spectrum assay, allowing the detection of known or even unknown viruses in one test, would be very helpful. In this respect, a first strategy could be the production of monoclonal antibodies specific to epitopes that are well conserved within a virus group. PCR amplification based on primers designed from conserved fragments in the genome of a target group of viruses constitutes another possible strategy to develop broad spectrum virus detection tests.

Therefore, a PCR procedure using degenerate primers was developed and allowed the detection of different members of the Potyvirus genus infecting sweet potato. This procedure is based on the amplification of the region corresponding to the non-conserved N-terminal region of the potyvirus coat protein and proved to be a valuable tool for the detection of mixed infections by distinct potyviruses and the identification of viruses not described so far. Apart from sweetpotato, this PCR strategy has been applied successfully for the identification and the characterization of potyviruses infecting yam and banana. This polyvalent approach does not require any preliminary fastidious work of separation and purification of the components of the viral complexes, and is therefore particularly useful when the primary objectives of tests performed for quarantine or certification purposes, are to identify potyvirus-infected samples, whatever the identity of the involved viruses. Moreover, the amplified fragments obtained can be cloned and sequenced by routine procedures for subsequent classification and epidemiological studies, or to develop virus-specific nucleic acid hybridization tests.

To date, one drawback to the use of probes, as commonly applied, is linked to their radioactive markers. Radioactive probes predominated because they are more reliable than chromogenic-based probes. This creates a problem for the development of user-friendly kits; while radioactive probes are reliable and safe in well-equipped laboratories, they are not suitable for use elsewhere. The digoxigenin-labelled probes, combined with detection of hybridization by chemoluminescence, overcome that problem and allowed us to detect virus infection in crude plant extract.

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