Use of group-specific primers and the polymerase chain reaction for the detection and identification of luteoviruses

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A general diagnostic assay for a number of distinct luteoviruses was developed using the polymerase chain reaction (PCR) and restriction enzyme analysis. Two minimally degenerate, group-specific primers were derived from previously published RNA sequences of three luteoviruses. This primer pair generated specific PCR fragments of about 530 bp from extracts of plants infected with potato leafroll virus, beet western yellows virus, or New York barley yellow dwarf virus (BYDV) serotypes MAV, PAV, RMV, RPV and SGV, which span much of the respective viral coat protein gene. Each virus was easily distinguished from the others by restriction enzyme analysis of the amplified DNA products. Samples from BYDV-infected oat and wheat collected in Nebraska were identified as containing PAV-like serotypes; micro-heterogeneity was detected in several samples. This method provides a rapid, sensitive and relatively inexpensive means of luteovirus detection and identification. It is the first test capable of simultaneously detecting all five BYDV serotypes.

Despite the fact that the aphid-transmitted luteoviruses have long been known to cause destructive diseases in numerous crops world-wide, these viruses still flourish in their respective plant hosts. For example, the most widely distributed virus disease of the Gramineae is caused by five serotypes of barley yellow dwarf virus (BYDV), designated MAV, PAV, RMV, RPV and SGV (Casper, 1988). Numerous strains of beet western yellows virus (BWYV) infect a wide range of economically important crop plants and potato leafroll virus (PLRV) occurs wherever potatoes are cultivated. Members of the luteovirus group are serologically inter-related to varying degrees and are subdivided into clusters on this basis (Casper, 1988; Waterhouse et al., 1988). The biology of luteoviruses complicates diagnosis and identification because they are confined to phloem tissue, occur in low concentrations and are not mechanically transmissible.

Luteovirus identification requires aphid transmission studies (Casper, 1988; Waterhouse et al., 1988), serological assays with several polyclonal and/or monoclonal antisera to the coat protein (Rochow & Carmichael, 1979; Diaco et al., 1986; D’Arcy et al., 1989; Forde, 1989; Martin & D’Arcy, 1990), or hybridization studies with cDNA probes (Waterhouse et al., 1986; Habili et al., 1987; Arundel et al., 1988; Falk et al., 1989; Fattouh et al., 1990; Martin & D’Arcy, 1990). However, such assays are limited in that several tests are required for virus identification and they are either time-consuming or use reagents (antisera or cDNA clones) which are expensive to produce and are often not widely available. These assays often yield inconclusive results and may not detect luteovirus infection. Animal virologists have recently used the polymerase chain reaction (PCR) (Saiki et al., 1986, 1988) to detect viruses; the PCR amplifies a specific genomic DNA fragment (Ou et al., 1988) which is then analysed using restriction enzymes (Torgersen et al., 1989). Here, we present a similar assay for the detection and identification of a range of distinct luteoviruses using appropriate group-specific primers. The viruses included in this investigation fall into the following clusters (Casper, 1988): (i) BWYV, BYDV-RMV and BYDV-RPV, (ii) BYDV-MAV, BYDV-PAV and BYDV-SGV and (iii) PLRV.

A number of group-specific primers were designed by comparing the deduced amino acid sequences of the conserved regions of the overlapping coat and 17K proteins of PLRV, BWYV and BYDV-PAV. These primers all amplified specific BYDV-PAV sequences (R. French & N. L. Robertson, unpublished results), but a primer pair amplifying the longest fragment was chosen for this study. The upstream primer Lu 1 (5’ CCAGTGGTTRTGGTC 3’), which is degenerate at one position, corresponds to bases 2938 to 2952 of BWYV (Veidt et al., 1988) and bases 3564 to 3578 of BYDV-PAV (Miller et al., 1988), bases 3687 to 3701 of PLRV (Van der Wilk et al., 1989). The downstream non-
degenerate primer Lu 4 (5' GTCTACCTATTTGG 3') can pair with bases 3455 to 3468, 4084 to 4097 and 4207 to 4220 of BYDV-PAV, BWYV and PLRV, respectively. The PCR products are predicted to be 531 bp long for BYDV-PAV and 534 bp long for both BWYV and PLRV.

Crude nucleic acid extracts were obtained from 0.5 g leaf samples from oats (Avena byzantina cv. Coast Black) infected with each of the five New York serotypes of BYDV (MAV, PAV, RMV, RPV and SGV) by grinding tissue with liquid nitrogen, adding 4 ml of buffer (0.1 M-glycine, pH 9.5, 0.1 M-NaCl, 10 mM-EDTA) and emulsifying the extract with an equal volume of phenol. After centrifugation, nucleic acids were precipitated from the aqueous phase by addition of sodium acetate to 0.3 M and 2.5 vol. ethanol. The pellets were washed with 70% ethanol, vacuum-dried and resuspended in 100 μl distilled water or TE (10 mM-Tris-HCl pH 7.2, 1 mM-EDTA). Healthy oat and wheat tissue, as well as Nebraskan field samples of BYDV-infected wheat and oat plants, and Physalis floridana infected with either BWYV or PLRV, were all processed in the same manner except that the PLRV RNA extraction procedure contained an additional LiCl precipitation step after extraction with phenol. Lithium chloride (0.2 vol.; 10 M) was added to the aqueous phase and the solution was incubated overnight at 4 °C. After centrifugation, the resulting pellet was resuspended in 300 μl TE and ethanol-precipitated as described above.

cDNA was synthesized by heating 1 μl nucleic acid extract with 10 pmol primer Lu 4 in H2O (total volume 12.5 μl) for 5 min at 95 °C, incubating at 40 °C for 10 min and chilling on ice. An equal volume of 2 × reaction buffer, containing either 0.5 units (U) of avian myeloblastosis virus (Boehringer Mannheim) or 10 U/μl of Moloney murine leukemia virus (BRL) reverse transcriptase, 4 mM each of dATP, dCTP, dGTP and TTP, 100 mM-Tris–HCl pH 8.0, 10 mM-2-mercaptoethanol, 20 mM-MgCl2 and 140 mM-KCl, was then added before incubating the reactions at 40 °C for 60 min. The volume of the mixtures was increased to 50 μl with H2O after which they were boiled for 10 min.

DNA amplification was carried out in a 100 μl reaction using 5 μl of the cDNA preparation, 5 pmol each of Lu 1 and Lu 4, 2.5 U Taq DNA polymerase (Cetus) in reaction buffer provided with the enzyme (10 × PCR buffer contains 100 mM-Tris–HCl pH 8.3 at 25 °C, 500 mM-KCl, 15 mM-MgCl2 and 0.01% w/v gelatin) and 0.2 mM of each dNTP. Samples were overlaid with 100 μl mineral oil and placed in a Perkin Elmer Cetus thermal cycler programmed to give one cycle at 95 °C (1 min), 41 °C (2 min) and 72 °C (20 min), followed by 40 cycles at 94 °C (1 min), 41 °C (1 min), 41 °C to 72 °C (3 min) and 72 °C (2 min), with a final cycle of 72 °C (10 min). The PCR products were visualized by electrophoresis of 5 μl of the PCR reaction product on 10% polyacrylamide gels followed by staining with 0.15 μg/ml ethidium bromide for 20 min. A major PCR product of about 530 bp was present in samples from all the luteoviruses, but not from healthy controls (Fig. 1) or from plants infected with barley stripe mosaic virus or brome mosaic virus (not shown). These results show that primers Lu 1 and Lu 4 can specifically amplify sequences from each of the luteoviruses tested.

To characterize the amplified DNAs from each of the viruses, 5 μl aliquots of the crude PCR reaction products were digested with Sau3AI and analysed on polyacrylamide gels. All five New York BYDV serotypes, PLRV and BWYV produced distinctive patterns (Fig. 2). The BYDV serotype MAV and PAV, and PLRV profiles were identical to those predicted from published sequences (Rizzo & Gray, 1990; Miller et al., 1991).
Fig. 3. Sau3AI restriction analysis of PCR products from BYDV field samples. Lanes 1 to 3, Sau3AI-cut pUC19 DNA markers, and uncut and digested PAV PCR product. Lanes 4 to 8, digested PCR product from BYDV field isolates; lane 9, digested PCR product from healthy oat leaves.

Fig. 4. Southern blot of 530 bp PCR product from BYDV serotypes, healthy oat control and BYDV-infected field isolates. The concentration of the PCR product differed among the BYDV samples, but each of the five blots contained an identical amount of any given sample. Blots probed with RMV and SGV were exposed five times as long as other blots. Lanes 1 to 5, BYDV serotypes MAV, PAV, RMV, RPV and SGV; lane 6, healthy plant control; lanes 7 to 11, field isolates 1 to 5.

Typically, the background of host-specific PCR products was more obvious in samples from luteovirus-free plants than in samples from luteovirus-infected plants. This is particularly striking with the PCR products shown in Fig. 2. The reasons for this are not clear, but may be related to differences in rates of primer depletion in reactions containing luteovirus cDNA target molecules compared to those in reactions not containing specific targets.

We were also interested in the degree of nucleic acid similarity among the BYDV serotypes, based upon hybridization of the 530 bp PCR products. To generate radioactive probes (Schowalter & Sommer, 1989) that included only the approximately 530 bp fragment for each of the BYDV serotypes, 30 to 50 μl of each PCR reaction was electrophoresed in a 2% low melting point agarose gel (Sea Plaque; FMC BioProducts), the DNA band was cut out and purified (Maniatis et al., 1982), and the dried pellet was resuspended in 20 μl TE. A 20 μl PCR mix included 5 μl of the gel-purified DNA, 50 μCi \([\alpha-32P]dCTP\) (3000 Ci/μmol), 0.2 mM each of dATP, dGTP, and dTTP, 5 pmol primers Lu 1 and Lu 4, 1 U Taq DNA polymerase and PCR buffer (Cetus). The temperature/time regime was as previously described except that the first 20 min annealing step was omitted and the number of cycles was reduced to 30. After DNA amplification, the layer of oil was removed by emulsifying with chloroform followed by brief centrifugation. The radioactive probe was isolated by placing the aqueous phase in a Millipore filter (10000 NMWL Filter Unit), adding 100 μl distilled water and centrifuging at 6000 r.p.m. until 10 to 50 μl remained on the filter. The cycle was repeated twice more to remove small fragments and free \([\alpha-32P]dCTP\). The remaining 10 μl of concentrated probe solution was then diluted with 100 μl distilled water. Initially, 10 to 20 μl of unpurified PCR product of each of the five BYDV serotypes and five BYDV field isolates was run on a 2% agarose gel, stained to ascertain the presence of the 530 bp band and then blotted to Zeta-Probe blotting membranes according to the supplier's instructions (Bio-Rad). Nucleic acids were hybridized using 25 μl of the radioactive probe preparations under high stringency (0.5 M-NaH₂PO₄, 1 mM-EDTA, 7% SDS at 65 °C; washes in 40 mM-NaH₂PO₄ at 65 °C).
As expected, Southern blots revealed that each of the 530 bp PCR products of the NY-BYDV serotypes hybridized most strongly to itself (Fig. 4). BYDV serotypes PAV, MAV, SGV and RPV showed limited cross-hybridization with at least one other serotype. Only DNA derived from serotype RMV did not cross-hybridize with PCR products from any of the other serotypes. Samples of the field isolates hybridized strongly with both the serotype MAV and PAV probes, but altogether their hybridization behaviour was most similar to that of serotype PAV samples. Probes derived from serotypes SGV and RPV also hybridized weakly to samples of serotypes MAV and PAV, and all field isolates. These results agree with the current placement of serotypes MAV, PAV and SGV into one cluster, but do not reveal any similarities between serotypes RMV and RPV. Other studies have not found any relationship between RMV and other BYDV serotypes (D’Arcy et al., 1989; Fattouh et al., 1990).

Luteovirus group-specific primers were derived from sequences conserved among sequenced isolates of BYDV-PAV, BWYV and PLRV. Despite their relatively small size, Lu 1 (a 15-mer) and Lu 4 (a 14-mer) amplified relatively low levels of host-specific PCR products under our reaction conditions. The primer pair did, however, specifically amplify a 530 bp DNA fragment from cDNA of total nucleic acid extracts from plants infected with each of five NY-BYDV serotypes, or several Nebraska BYDV, BWYV or PLRV isolates. All seven viruses examined here could be differentiated on the basis of restriction fragment length polymorphisms. Differences were found between some of the BYDV field isolates which probably would have been overlooked by methods of diagnosis in current usage.

Current methods of detecting and identifying luteoviruses depend on multiple tests with aphid species, antisera or cDNA probes. The use of the PCR with a single luteovirus primer pair described here allows diagnosis of five BYDV serotypes, and BWYV and PLRV, by one rapid and simple, yet sensitive, assay. This technique clearly takes advantage of both conserved and variable portions of a genomic region. The DNA fragment produced by the PCR includes much of the coat protein gene, which encodes those properties that determine antigenicity and perhaps aphid vector specificity. This PCR method yields results which correlate well with previous insect transmission and serological studies. Owing to its simplicity, the PCR provides an attractive alternative to other diagnostic techniques. The 530 bp cDNA fragments can provide a ready source of initial sequence information for luteoviruses from which cDNAs have not yet been made and cloned. These sequence data could then be used to design virus-specific PCR primers if desired. The primers and procedure described here will be useful in the diagnosis of luteovirus diseases and in epidemiological studies.

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References


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