Construction and Use of a Cloned cDNA Probe for the Detection of Plum Pox Virus in Plants

Christina Varveri, M. Ravelonandro, and J. Dunez

Station de Pathologie Végétale, Institut National de la Recherche Agronomique, Centre de Bordeaux, B.P. 131, 33140 Pont de la Maye, France.

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ABSTRACT

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Complementary DNAs to plum pox virus (PPV) were cloned into *Escherichia coli* plasmid pUC9. Clones containing about 1 kb of the 3′ region of PPV RNA were obtained. Subcloning of a 720-bp fragment lacking the 3′ poly-A region into pBR322 provided a recombinant plasmid named pPPV9A. This plasmid was labeled by nick translation and used in dot-blot hybridization assays. As little as 100 pg of purified virus was detected. This is equivalent to about 5 pg of PPV RNA. The enzyme-linked immunosorbent assay (ELISA) applied to the same purified virus solution allowed the detection of about 1 ng of virus per assay and was therefore 10 times less sensitive. When the respective sensitivities of the two methods were compared with crude sap samples, molecular hybridization was again more sensitive as far as actual amounts of virus or of infected tissues were concerned. The usefulness of this technique for routine detection work is discussed.

Plum pox virus (PPV), a member of the potyvirus group and the causal agent of Sharka disease on stone fruit trees (16), is of major agronomic importance. Until recently, detection of this virus relied mainly on biological or serological methods such as enzyme-linked immunosorbent assay (ELISA) or immunosorbent electron microscopy (ISEM) (3,15). Detection problems, mostly attributable to the uneven distribution and low concentration of the virus in the infected plants prompted us to look for more sensitive alternative detection techniques.

Molecular hybridization with recombinant cDNA probes has recently been shown to be a highly specific and sensitive method for the characterization (7,12,20,24) and detection (5,10,19,21) of viruses and viroids. Theoretically, the great advantage of this technique is that it detects the viral genome in all its forms, whether single- or double-stranded, encapsidated or not. This technique has been reported to provide a highly sensitive and very early detection of viral infection (25).

The PPV genome consists of a single-stranded RNA of 3.5×10^6 Da (13). We undertook the molecular cloning of DNA complementary to the 3′ extremity of the PPV RNA, then subcloned this region to eliminate the part corresponding to the poly-A tail located at the 3′ end of the genome RNA. The resulting probe was specific for PPV genome when used in dot-blot hybridization assays to detect viral RNA from purified preparations or crude sap samples.

MATERIALS AND METHODS

Viral strains. PPV strains have been classified in two serological groups (14). One strain representative of each group was used: strain D, an apricot isolate from southeastern France, and strain M, a peach isolate from northern Greece. These strains were propagated in *Pisum sativum* L. 'Express Genereux,' which shows systemic symptoms 10–12 days after inoculation.

Virus purification and RNA extraction. PPV was extracted by grinding plant tissue in citrate buffer (50 mM trisodium citrate, 1 mM ethylenediaminetetraacetate [EDTA], and 20 mM sodium diethyldithiocarbamate [DIECA], pH 8.2) containing 22% butanol. Two cycles of differential centrifugation at low and high speed (10,000 *g* for 15 min and 100,000 *g* for 1.5 hr) were followed by ultracentrifugation (200,000 *g* for 2 hr) through a linear 10–40% sucrose gradient in 10 mM trisodium citrate buffer, pH 8.3. The virus was collected and concentrated by high-speed centrifugation (150,000 *g* for 2.5 hr).

RNA was extracted by incubating the virus solution in 2% sodium dodecyl sulfate (SDS) at 60 C for 10 min. Disrupted virus was immediately layered onto 10–60% linear RNase-free sucrose gradients made in 40 mM Tris-Cl, pH 7.6, plus 50 mM NaCl, and centrifuged at 120,000 *g* for 15 hr. The RNA band was collected and precipitated with ethanol. After centrifugation, the RNA precipitates were resuspended in a small volume of sterile distilled water and concentrations were determined spectrophotometrically. The RNA was stored at −80 C.

Complementary DNA synthesis and molecular cloning. pUC9 (Pharmacia) oligo (dT) tailed at its Pst I site was used to prime complementary DNA (cDNA) synthesis on PPV strain D RNA template (11). Oligo (dC) tails were added to the cDNA 3′ end. After separation of the two strands through an alkaline sucrose gradient, the plasmid-cDNA construction was reannealed to oligo (dG) tailed pUC9. The oligo (dG) tail served as primer for the Klenow fragment of *Escherichia coli* DNA polymerase I to fill the single-stranded region of the circularized plasmid.

E. coli JM103 cells were used as transformation recipients. Recombinant clones with β-galactosidase-negative and ampicillin-resistant phenotype were selected. The presence of PPV-D cDNA inserts was checked by plasmid isolation followed by Bam HI and Hind III (Boehringer) digestions and electrophoresis on 1% agarose gel (6). After blotting the DNA onto a nitrocellulose membrane (26), the viral nature of the inserts was verified by hybridization with a single-stranded PPV cDNA probe (27) as described below.

PPV cDNA analysis and subcloning. Recombinant plasmids were amplified in *E. coli* cells and purified in CsCl-ethidium bromide gradients as described by Clewell and Helinski (8). The inserts were excised from recombinant plasmids by digestion with Bam H I and Hind III. The fragments were purified by non-denaturing electrophoresis in 6% polyacrylamide gels (18) and mapped by digestion (17) with various restriction enzymes from Boehringer. Taq I was used to cleave the insert near the viral 3′ end to eliminate the poly-A region. A Hind III-Taq I fragment was electrophoretically isolated (as described above) and subcloned into pBR322. The insert fragment and Pst I cut pBR322 were tailed by terminal transferase with oligo (dC) and oligo (dG), respectively, and annealed (28). *E. coli* HB101 cells were

transformed and selected for tetracycline resistance. Presence of the desired insert was verified by plasmid isolation and restriction enzyme digestion as described above.

Radioactive labeling and hybridization conditions. PPV cDNA was labeled upon reverse transcription (27) of the viral RNA by AMV reverse transcriptase (Genofit) in the presence of (α-^{32}P) dCTP (3,000 Ci/mM).This cDNA probe was used to hybridize with the digested fragments from the recombinant plasmid transferred to the nitrocellulose membrane (26). Prehybridization was carried out in polyethylene bags at 42 C for 4 hr in 50% formamide, 1 M NaCl, 5× Denhardt's solution (1× Denhardt's is 0.02% ficoll, 0.02% polyvinyl pyrrolidone, and 0.02% bovine serum albumin), 25 mM phosphate buffer, pH 6.5, and 250 μg/ml denatured salmon sperm DNA (1 ml buffer per 10 cm^2 membrane). For hybridization, this buffer was discarded and replaced by: 50% formamide, 1 M NaCl, 2× Denhardt's solution, 25 mM phosphate buffer, pH 6.5, 10% dextran sulfate, 1% SDS, 50 μg/ml denatured salmon sperm DNA, and a total of 1×10^6 cpm of denatured radioactive cDNA probe. Hybridization was carried out for 15 hr at 42 C. Membranes were then washed twice (5 min each) in a 2× SSC buffer (1× SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7), once in 2× SSC containing 0.1% SDS at 65 C for 1 hr, and finally, in 0.1× SSC for 30 min at room temperature. After washing, we autoradiographed the membrane at −80 C for 48 hr with X-ray films (Kodak) and intensifying screens.

Radioactive labeling of the PPV plasmid probe was performed by nick translation according to Rigby et al (23) using an Amersham nick-translation kit with the same radioactive marker as above. The specific radioactivity of the probe was usually about 3×10^8 cpm/μg. Unincorporated nucleotides were removed by ethanol/ammonium acetate precipitation. Hybridization conditions were as described above except that the hybridizations were done at 55 C with $1-2 \times 10^6$ cpm/ml of hybridization buffer.

Sample preparation for dot-blotting. Purified PPV preparations in 50 mM sodium citrate buffer, pH 8.3, were diluted in the same buffer and spotted on a nitrocellulose filter that had been soaked in 20× SSC. Viral RNA solutions were diluted in 20× SSC before spotting. One-gram samples of inoculated or healthy leaf tissue were ground with 2 ml of 50 mM sodium citrate, pH 8.3, and serially diluted (fivefold dilutions) in the same buffer. Aliquots of each dilution were applied as replicate 5-μl spots. After spotting, nitrocellulose membranes were air-dried and baked at 80 C for 2 hr under vacuum.

ELISA. The double-antibody sandwich method (3) was performed using an antiserum to the D viral strain. Optimized conditions implied a coating with an antibody concentration of 1 μg/ml and the use of the alkaline phosphatase conjugate diluted 1/2,000. Under these conditions, the threshold of detection (= mean of optical densities at 405 nm obtained from 10 wells with healthy samples plus three times their standard deviation) was 0.060.

RESULTS

Cloning of PPV-D cDNA. The Heidecker and Messing (11) cloning method was used to integrate PPV-D cDNA sequences in pUC9. Clones were screened by Southern hybridization using a single-stranded cDNA probe. Plasmid pPPV9, a recombinant plasmid carrying a PPV insert of approximately 1 kb, was selected. The insert was excised from the plasmid and mapped by restriction endonuclease digestions. The position of six restriction sites is shown in Figure 1.

Subcloning of the 3′ region of PPV deprived of its poly-A tail. Dot-blot hybridization experiments were carried out using healthy plant extracts and polyadenylated viral RNAs from tomato black ring virus (TBRV) as controls. Nick-translated pPPV9 was the probe. Nonspecific cross-reactions were observed, apparently attributable to the poly-A track existing in all these RNAs. To obtain a probe specific for PPV, we subcloned the PPV insert from pPPV9 after removing its poly-A-containing region. The biggest fragment (720 bp) remaining after endonuclease digestion was generated by Hind III-Taq I digestion. This fragment was subcloned in pBR322 as described in the Materials and Methods, thereby yielding plasmid pPPV9A.

Detection of PPV by dot-blot hybridization using pPPV9A as a probe. PPV strains form two serological groups described as D and M. Hybridization assays were performed against strains belonging to these two groups. Purified virus or nucleic acid was spotted onto a nitrocellulose membrane. As little as 100 pg of purified PPV-D was detected (Fig. 2). Because the virus particles contain about 5% RNA, this sensitivity represents the detection of 5 pg of RNA. When purified RNA was spotted, only 10 pg could be detected, which showed that isolation of RNA from particles is not more sensitive. Attempts to increase the sensitivity by denaturing purified RNA by heat treatment or using 6% formaldehyde (19) failed, probably because of the extreme fragility of PPV RNA (data not shown). When hybridization was performed against PPV-M, 1 ng of virus was the limit of detection; this is 10 times less sensitive than hybridization with the homologous strain. The probe gave no signal with the two polyadenylated TBRV RNAs or with the buffer (results not shown).

Sap extracted from healthy and infected pea and peach leaves was spotted directly on a nitrocellulose membrane. The highest dilutions of infected pea extracts in which PPV-D and PPV-M were detected were 1/625 and 1/125, respectively (Fig. 3A). The above assay was repeated with leaf samples that had been stored at −20 C. The responses were not significantly different from those obtained with fresh samples. Very low background appearing as faint spots could occasionally develop with sap from healthy plants. When purified PPV was diluted in crude sap before hybridization, the signal response was equally or more intense than after dilution in buffer, indicating that the presence of the plant extract may facilitate hybridization to some extent (Fig. 3B). Therefore, the lower hybridization obtained with the infected peach extracts probably resulted from low virus concentration in these samples and not from an interference with the hybridization procedure by plant material.

Preparations of purified virus were also assayed by ELISA to compare its sensitivity with that of the dot-blot hybridization. The limits of detection by ELISA performed with PPV-D antiserum were 1 ng of PPV-D and 10 ng of PPV-M per assay. Thus, the dot-blot hybridization that detected 100 pg and 1 ng per spot,

Fig. 1. Restriction endonuclease map of the pPPV9 clone. A = Alu I, E = Eco RI, R = Rsa I, and T = Taq I.

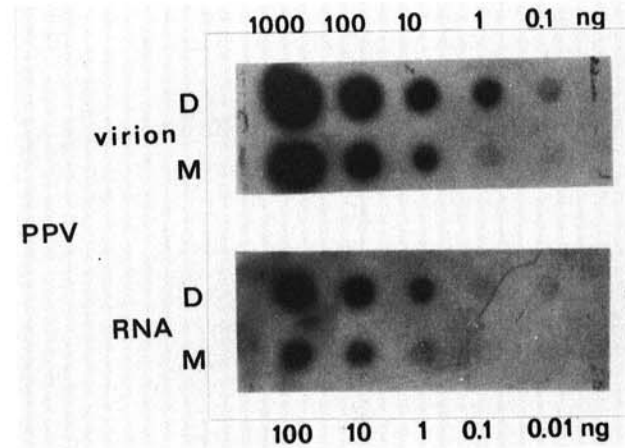

Fig. 2. Detection of PPV-D and PPV-M virions and RNAs by hybridization with cloned cDNA to PPV-D (pPPV9A). Samples of plum pox virus (PPV) RNA were diluted in 20× SSC (NaCl + sodium citrate) and those of PPV virion in 50 mM sodium citrate buffer.

respectively, was 10 times more sensitive than ELISA. It is interesting to note that, in terms of strain specificity, the results of molecular hybridization correlate well those of ELISA.

To compare the two diagnostic methods under realistic conditions, both tests were conducted with the same infected or healthy pea and peach samples. Table 1 shows the minimal quantity of plant tissue that was necessary for the detection of PPV by ELISA and dot-blot hybridization. In all cases, regardless of the virus strain or host plant, molecular hybridization allowed detection of the virus in three times less infected plant tissue.

DISCUSSION

The results presented here show that hybridization of a cloned PPV-cDNA probe to immobilized sap samples may be a useful method for the detection of PPV in plants. With the method described, the limit of detection for purified PPV-D virions was 100 pg, which is equivalent to 5 pg of viral RNA. This level of sensitivity is similar to those published for other viruses (19). Use of intact virus does not inhibit hybridization of the probe to the viral RNA. In fact, the results were better when intact virions rather than purified viral RNA were spotted. The high-salt conditions used for spotting, the high baking temperature, and the presence of SDS/formamide in the hybridization buffer probably all contributed by disrupting the virus particles, which made the viral RNA more accessible for hybridization. Isolation and purification of viral RNA from virions could decrease the proportion of the intact viral RNA and therefore limit hybridization. On the other hand, the use of intact virus would protect RNA from degradation by plant nucleases.

TABLE 1. Comparison of detection sensitivities[a] of plum pox virus by ELISA (antibodies to D strain) and molecular hybridization (pPPV9A probe)

Origin of infected material	Minimum quantity of infected plant material (μg) necessary for detection of PPV	
	ELISA[b]	Molecular hybridization[c]
PPV-D pea	12	4
PPV-M pea	60	20
PPV-D peach	75	25
PPV-M peach	75	25

[a] Both techniques were performed with the same samples.
[b] ELISA: 1 g of sample was ground in 4 ml of buffer, serially diluted, and applied as 250 μl.
[c] Molecular hybridization: 1-g sample was ground in 2 ml of buffer, serially diluted, and applied as 5-μl spots.

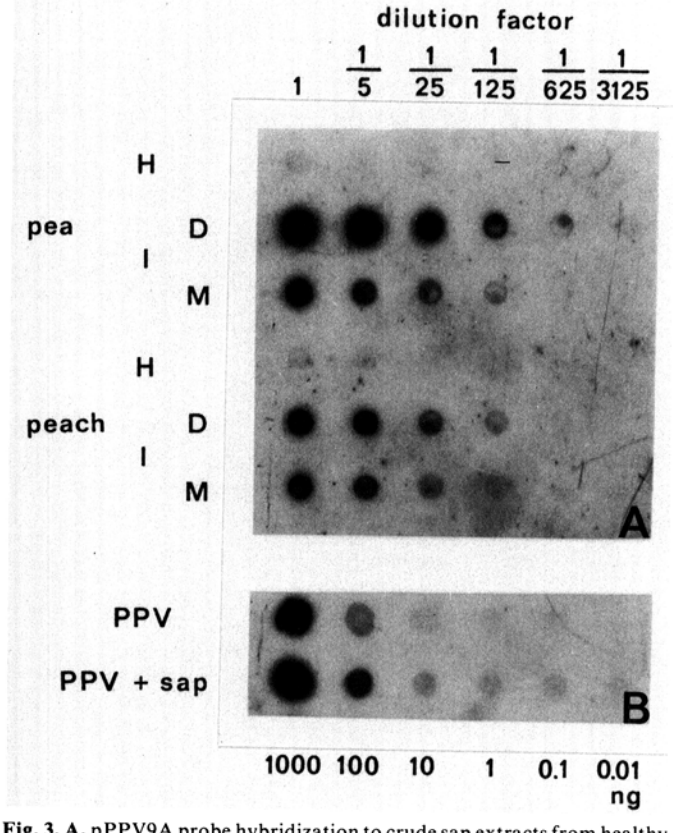

Fig. 3. A, pPPV9A probe hybridization to crude sap extracts from healthy (H) or infected (I) pea and peach (D or M strain). **B,** Effect of sap from healthy peach leaves on the hybridization obtained with purified PPV.

The sensitivities of dot-blot hybridization and ELISA could be easily compared using purified viral preparations. The limits of detection were 100 pg and 1 ng, respectively, per assay, which demonstrated that hybridization was 10 times more sensitive than ELISA. When applied to infected crude sap extracts, molecular hybridization appeared to be more sensitive than ELISA as far as actual amounts of plant tissue were concerned. Dilution end points of infected crude extracts were 1/625 and 1/2,000 respectively for hybridization and ELISA. Assuming a virus concentration of 20 μg/g of infected leaves, these diluted extracts contained 80 pg and 1 ng of virus per assay, respectively; this fits in with the detection limits of the two methods as observed with purified virus suspensions. When dealing with virus concentration, allowing for the different sample volumes (250 μl for ELISA and 5 μl for dot-blot), ELISA detected PPV down to 4 ng/ml, and dot-blot, down to 20 ng/ml. Correspondingly, a low virus concentration (in the range of 5 ng/ml) can be detected by ELISA and not by hybridization. Baulcombe et al in 1984 (5) also found molecular hybridization to be a bit less sensitive than ELISA for the detection of potato virus X. ELISA detection of 1 ng of PPV per assay is unusual for this type of virus and results from the optimization of the test (*unpublished*). It is likely that with some slight modifications in the hybridization procedure and sample preparation, dot sensitivity could be increased by one to five orders of magnitude. Thus, it appears that hybridization and ELISA have the same range of sensitivities when applied to elongated virus particles, hybridization being more sensitive with spherical virus particles. When comparing sensitivities of the two techniques applied to field samples, the following questions should be addressed: Should samples containing as low as 20 ng of virus or less per gram of infected tissue be considered? How common are they in a natural situation, such as in a mass-indexing procedure? Furthermore, it is difficult to compare ELISA and molecular hybridization precisely because, in addition to the viral nucleoprotein itself, these methods detect the soluble coat protein and the various forms of nonencapsidated viral RNA. Large-scale application of molecular hybridization is now required to confirm results from the laboratory and its reliability in field indexing.

The serological variations among strains found previously were also observed by hybridization with the pPPV9A probe. Similar results are reported with other potyviruses (1,2,22). The good correlation between serological properties and molecular hybridization was probable because the cloned insert represents nearly the entire virus coat protein gene, which is probably localized near the RNA 3′ end as reported for other potyviruses such as tobacco etch virus (4) and pepper mottle virus (9). The disadvantage of the high strain specificity of the pPPV9A probe in routine detection could be overcome by the construction of other probes, which would include sequences from D and M groups. Sequencing of the viral genome is now in progress in our laboratory that should allow the identification of regions common to the different strains. This will provide another approach to the construction of probes with broad detection spectra. Such probes would be especially useful in routine detection work.

LITERATURE CITED

1. Abu-Samah, N., and Randles, J. W. 1981. A comparison of the mucleotide sequence homologies of three isolates of bean yellow mosaic virus and their relationship to other potyviruses. Virology 110:436-444.

2. Abu-Samah, N., and Randles, J. W. 1983. A comparison of Australian bean yellow mosaic virus isolates using molecular hybridization analysis. Ann. Appl. Biol. 103:97-107.
3. Adams, A. N. 1978. The detection of plum pox virus in *Prunus* species by enzyme linked immunosorbent assay (ELISA). Ann. Appl. Biol. 90:215-221.
4. Allison, R. F., Sorenson, J. C., Kelly, M. E., Armstrong, F. B., and Dougherty, W. G. 1985. Sequence determination of the capsid protein gene and flanking regions of tobacco etch virus: Evidence for synthesis and processing of a polyprotein in potyvirus genome expression. Proc. Nat. Acad. Sci. USA 82:3969-3972.
5. Baulcombe, D., Flavell, R. B., Boulton, R. E., and Jellis, G. J. 1984. The sensitivity and specificity of a rapid nucleic acid hybridization method for the detection of potato virus X in crude sap samples. Plant Pathol. 33:361-370.
6. Birnboim, H. C., and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.
7. Carrington, J. C., and Morris, T. J. 1984. Complementary DNA cloning and analysis of carnation mottle virus RNA. Virology 139:22-31.
8. Clewell, D. B., and Helinski, D. R. 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: Purification and induced conversion to an open circular DNA form. Proc. Nat. Acad. Sci. USA 62:1159-1166.
9. Dougherty, W. G., Allison, R. F., Parks, T. D., Johnston, R. E., Field, M.I., and Armstrong, F.B. 1985. Nucleotide sequence at the 3′ terminus of pepper mottle virus genomic RNA: Evidence for an alternative probe of potyvirus capsid protein gene organization. Virology 146:282-291.
10. French, R. C., Simon, S. H., and Derrich, K. S. 1984. Detection of sugarcane mosaic virus by dot-blot hybridization. (Abstr.) Phytopathology 74:859.
11. Heidecker, U., and Messing, J. 1983. Sequence analysis of zein cDNAs obtained by an efficient mRNA cloning method. Nucleic Acids Res. 11:4891-4906.
12. Hellman, G. M., Shahabuddin, M., Shaw, J. G., and Rhoads, R. E. 1983. Molecular cloning of DNA complementary to tobacco vein mottling virus RNA Virology 128:210-220.
13. Kerlan, C., and Dunez, J. 1976. Some properties of plum pox virus and its nucleic acid and protein components. Acta Hortic. 67:185-192.
14. Kerlan, C., and Dunez, J. 1979. Differenciation biologique et serologique de souches du virus de la Sharka. Ann. Phytopathol. 11(2):241-250.
15. Kerlan, C., Mille, B., and Dunez, J. 1981. Immunosorbent electron microscopy for detecting apple chlorotic leaf spot and plum pox viruses. Phytopathology 71:400-404.
16. Lansac, M., Bernhard, R., Massonié, G., Maison, P., Kerlan, C., Dunez J., and Morvan, G. 1979. La Sharka: Connaissances actuelles. Pages 432-468 in: IIIe Journal ACTA Les Maladies des Plantes, Paris.
17. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
18. Maniatis, T., Jeffrey, A., and Van de Sande, H. 1975. Chain length determination of small double and single-stranded DNA molecules by polyacrylamide gel electrophoresis. Biochemistry 14:3787-3794.
19. Maule, A. J., Hull, R., and Donson, J. 1983. The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues. J. Virol. Methods 6:215-224.
20. Ohno, T., Takamutsu, N., Meshi, T., and Okada, Y. 1983. Hop stunt viroid: Molecular cloning and nucleotide sequence of the complete cDNA copy. Nucleic Acids Res. 11:6181-6197.
21. Owens, R. A., and Diener, T. O. 1981. Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. Science 213:670-672.
22. Reddick, B. B., and Barnett, 0. W. 1983. A comparison of three potyviruses by direct hybridization analysis. Phytopathology 73:1506-1510.
23. Rigby, P. W. I., Dieckmann, M., Rhodes, C., and Berg, P. 1977. Labeling deoxyribonucleic acid to high specific activity by nick translation with DNA polymerase I. J. Mol. Biol. 133:237-251.
24. Rosner, A., and Bar-Joseph, M. 1984. Diversity of Citrus Tristeza Virus strains indicated by hybridization with cloned cDNA sequences. Virology 139:189-193.
25. Sela, I., Reichman, M., and Weissbach, A. 1984. Comparison of dot molecular hybridization and enzyme-linked immunosorbent assay for detecting tobacco mosaic virus in plant tissues and protoplasts. Phytopathology 74:385-389.
26. Southern E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Molec. Biol. 98:503-517.
27. Varveri, C., Bretout, C., and Dunez, J. 1986. Synthesis and characterization of a DNA complementary of plum pox virus RNA to be used as a specific detection probe. Acta Hortic. 193:173-182.
28. Villa-Komaroff, L., Efstradiadis, A., Broamme, S., Lomedico, R., Tizards, R., Nabe, S. P., Chick, W. L., and Gilbert, W. 1978. A bacterial clone synthesizing proinsulin. Proc. Nat. Acad. Sci. USA. 75:3721-3727.