Comparison of Techniques for Detection of Barley Yellow Dwarf Virus–PAV-IL

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ABSTRACT

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Detection of barley yellow dwarf virus (BYDV)-PAV-IL by an improved nucleic acid hybridization technique, using a nonradioactive probe with chromogenic and chemiluminescent substrates, was compared with detection by polymerase chain reaction (PCR), double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with polyclonal antibodies, and triple antibody sandwich ELISA with polyclonal and monoclonal antibodies. Each method was used to detect purified virus and virus in sap extracts from infected oat leaves. The detection limits for both ELISA procedures were 1 ng of purified BYDV-PAV-IL and the equivalent of 78 ng of infected tissue. Nucleic acid hybridization with either chemiluminescent or chromogenic substrates also detected as little as 1 ng of purified BYDV-PAV-IL, but it was slightly more sensitive at detecting virus in tissue extracts (25 ng of infected tissue). The most sensitive detection technique was PCR amplification, which could detect as little as 0.1 pg of RNA extracted from purified virus and detected viral RNA in the equivalent of 0.5 pg of infected leaf tissue.

Additional keywords: dot blot, luteovirus

Barley yellow dwarf viruses (BYDVs) are found worldwide in 54 countries from seven continents (18). The five strains of BYDV that have been detected in North America are divided into two subgroups based on biological and genomic properties (12,28). Strains related to BYDV-PAV, which are typically among the more damaging of the BYDV strains, have been found to be the most prevalent in more than 50% of the countries surveyed.

BYDVs are phloem-limited and are present at low concentrations in extracts of infected tissues (7). The sensitivity of a detection technique is therefore important for efficient detection of these viruses in field samples. Enzyme-linked immunosorbent assays (ELISA) are increasingly used to detect BYDVs, but other techniques, such as polymerase chain reaction (PCR) and nucleic acid hybridization, have been reported to be more sensitive and capable of distinguishing the different virus strains (2,11,14). Even though nucleic acid hybridization is considered a powerful technique for the detection of plant viruses (11,14,16), the hazards of using radioactive probes and the problems of nonspecific hybridization of probes with healthy samples, especially when crude

extracts are tested, have discouraged its widespread use.

In this paper, we present a direct comparison of the sensitivity of an improved nucleic acid hybridization technique using a nonradioactive probe with the sensitivities of ELISA and PCR for detecting BYDV-PAV-IL as purified virus or in extracts from infected plants. Variations of the techniques tested include use of chromogenic or chemiluminescent detection systems in the hybridization assay, double antibody sandwich (DAS) ELISA with polyclonal antibodies for virus capture and detection, and triple antibody sandwich (TAS) ELISA with monoclonal antibodies for virus detection.

MATERIALS AND METHODS

Virus preparation. The BYDV-PAV-IL strain and its aphid vector, *Rhopalosiphum padi* L., were maintained in Hudson barley (*Hordeum vulgare* L.) grown in the greenhouse. Virus was purified from Coast Black oats by the method described by D'Arcy et al. (9).

DAS- and TAS-ELISA. Leaf samples were collected from infested and noninfested greenhouse-grown Coast Black oats (*Avena sativa* L.) and tested fresh or after storage at -80°C for 1 to 2 months. For sap extractions, 0.5 g of leaf tissue was ground in liquid nitrogen, and the powder was resuspended in 10 ml of phosphate-buffered saline (PBS), pH 7.4. Leaf extracts were diluted 1:20 to 1:2,560 times and tested by DAS-ELISA and TAS-ELISA, as described by D'Arcy et al. (8), with the

modification that alkaline phosphatase conjugates were incubated 2 h at 37°C instead of overnight. Samples with absorbance at 405 nm greater than twice the mean absorbance of 24 control wells were considered positive.

Dot blot hybridization. The cDNA used for nucleic acid hybridization was pPAV410, which represents a nearly full-length copy of BYDV-PAV-IL genome (11). Hybridization probes were prepared by random primer labeling and/or PCR amplification using either Biotin-14-dATP (Life Technologies, Gaithersburg, MD) or Biotin-14-dCTP (Tropix, Bedford, MA).

To prepare the samples for dot blot hybridization, 1 g of leaf was ground in liquid nitrogen and resuspended in 5 ml of PBS containing 1 to 3 mg of Mg-bentonite prepared by the method of Dunn and Hitchborn (10), with the bentonite-buffer mixture shaken for at least 3 h in the first and second steps before centrifugation. Each new Mg-bentonite preparation was tested in order to determine the optimal concentration of Mg-bentonite for extraction.

The leaf extract was stirred for 15 min at room temperature (RT) and centrifuged for 10 min at 14,000 rpm. One volume of supernatant was mixed with 3 volumes of disruption buffer (33% formamide, 12% formaldehyde, 20 mM 3-[N-morpholino]propane-sulfonic acid, 1 mM NaEDTA, pH 7.0), incubated for 5 min at 65°C , and cooled on ice for 2 min. Four volumes of 0.3 M sodium citrate buffer, pH 7, and 3.0 M NaCl (20 $\times$ SSC) were added, and the mixture was centrifuged at 14,000 rpm for 10 min. The supernatant was applied through a Hybri-Dot manifold (Gibco-BRL, Life Technologies, Gaithersburg, MD) to a positively charged nylon membrane (Amersham, Arlington Heights, IL) for chemiluminescent detection and to a nitrocellulose membrane for chromogenic detection, both prewetted in 10 $\times$ SSC. The volumes applied ranged from 200 to 1 μl in each well. Volumes higher than 200 μl were applied to the first membranes prepared, but the membranes became plugged and the chromogenic and chemiluminescent signals were not clear. For sample volumes below 10 μl , the volume was adjusted to 10 μl with 10 $\times$ SSC to produce a larger spot on the membrane. After sample application, wells were washed with 100 μl of 10 $\times$ SSC, and the membranes were allowed to dry for 10 min in a vacuum oven at 80°C and were UV crosslinked with a Stratagene Stratalinker.

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The prehybridization/hybridization solution contained 5× SSC, 0.1% N-lauroyl-sarcosine, 0.1% I-block (Tropix), and 0.02% sodium dodecyl sulfate (SDS). Membranes were prehybridized for 30 min at 68°C and hybridized overnight with 4 ng of probe per ml of hybridization solution in a roller bottle. Nylon membranes were washed three times with PBS, 0.5% SDS (twice for 5 min at RT and once for 30 min at 60°C), and finally for 5 min at RT with 2× PBS. The procedure for chemiluminescent detection was that of Martin and Olesen (21).

Nitrocellulose membranes were washed three times after hybridization with 0.1% SSC, 1% SDS (twice for 5 min at RT and once for 30 min at 60°C). The membranes were then washed once in 2× SSC for 5 min at RT and incubated with 3% BSA in 0.1 M Tris base, 150 mM NaCl, and 0.05% Tween 20, pH 7.5, (PBS-Tween) for 1 h at 60°C. The membranes were incubated with streptavidin alkaline phosphatase (BRL, Zymed, or Tropix) for 15 min and washed twice in TBS-Tween for 15 min at RT. The chromogenic signals were detected by incubation of membranes with chromogenic substrate BCIP/NBT (4-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) for approximately 2 h.

PCR amplification. RNA was extracted from purified BYDV-PAV-IL as described by Gibson et al. (13). Nucleic acids were extracted from infected and noninfected plants by grinding 0.5 g of leaf tissue in liquid nitrogen and resuspending the resulting powder in 5 ml of 0.1 M glycine buffer, pH 9.5, 0.1 M NaCl, and 10 mM EDTA. Purification of viral RNA from the plant extract and transcription of RNA from purified virus and plant extract were by the methods of Robertson et al. (23), with the modification that primers PAV3' and 6.7 kBam were used for PCR amplifications instead of Lu₁ and Lu₄. PCR products were analyzed by electrophoresis of 10 µl of the PCR reaction mixture on 1.4% agarose gels using Tris-acetate buffer, pH 8.0 (24).

RESULTS

The sensitivities of the two serological methods tested—DAS-ELISA and TAS ELISA—for detection of BYDV-PAV-IL were similar. With either method, it was possible to detect as little as 1 ng of purified virus and virus in leaf extracts diluted as far as 1:640, which was equivalent to 78 ng of infected leaf tissue (Table 1). Besides having similar sensitivity, TAS presented lower absorbance variation than DAS-ELISA technique (Figs. 1 and 2).

The use of Mg-bentonite in the hybridization technique eliminated nonspecific hybridization of the probe with healthy plant samples and increased its sensitivity. The chemiluminescent and chromogenic substrates had very similar sensitivities for detection of BYDV-PAV-IL. The smallest quantity of purified virus that could be

detected varied from 0.5 to 2 ng, and it was possible to detect virus in extracts equivalent to between 25 and 50 ng of infected leaf tissue. Most tests made it possible to

detect 1 ng of purified virus and virus in 1 µl of plant extract, which was equivalent to 25 ng of infected leaf tissue (Table 1 and Fig. 3).

Table 1. Comparison of the sensitivities of detection techniques for barley yellow dwarf virus (BYDV)-PAV-IL

Technique	Detection endpoint	
	Infected tissue	Purified virus
DAS-ELISA ^a	78 ng	1 ng
TAS-ELISA ^a	78 ng	1 ng
Hybridization-chemiluminescent	25 ng	1 ng
Hybridization-chromogenic	25 ng	1 ng
Polymerase chain reaction	0.5 pg	0.1 pg ^b

^a Double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA); triple antibody sandwich (TAS) ELISA.

^b RNA extracted from purified virus and diluted in sterile water.

Fig. 1. Average of sample:control absorbance ratio obtained in 10 double antibody sandwich (DAS) and triple antibody sandwich (TAS) enzyme-linked immunosorbent assays (ELISA) for the detection of barley yellow dwarf virus (BYDV)-PAV-IL in infected leaf tissue.

Fig. 2. Average of sample:control absorbance ratio obtained in 10 double antibody sandwich (DAS) and triple antibody sandwich (TAS) enzyme-linked immunosorbent assays (ELISA) for the detection of purified barley yellow dwarf virus (BYDV)-PAV-IL.

cocktail, DAS-ELISA, and an amplification of the enzyme reaction in ELISA technique for the detection of PLRV. They could detect, respectively, 1 ng, 8 ng, and 50 to 100 pg of purified virus. Others authors found 2 ng of virus and 200 ng of infected leaf tissue, the limit for detection of PLRV by DAS-ELISA (1). For cherry leaf roll virus (CLRV) detection by DAS-ELISA, the authors found the limit at 40 ng of purified virus and 2.5 to 1.25 mg of infected leaf tissue (2).

Improvements in the nucleic acid hybridization technique developed in this study reduce some of the limitations cited for its use in routine virus diagnosis. The most significant improvement was the elimination of background, or nonspecific, reactions between the hybridization probe and components of the healthy plant extract (2,19,22). The extraction of plant sap with PBS containing Mg-bentonite not only eliminated this background, but also made the use of chromogenic substrate equally sensitive to use of chemiluminescent substrate for detecting BYDV-PAV-IL in infected plants. As chromogenic detection is faster, easier, and cheaper, it could become a good alternative for routine use in virus diagnosis.

Dunn and Hitchborn (10) found that Mg-bentonite absorbed ribosomes, 18S protein, and "green material" from plant extracts. Any of these could be the agent(s) responsible for nonspecific hybridization of cDNA probes, which makes it impossible to distinguish between healthy and infected plants. Indeed, the use of Mg-bentonite is easier and takes less time than the extraction and precipitation of plant nucleic acids described previously (4,14,22).

The reduction of the prehybridization to 30 min shortens the time required to complete the nucleic acid hybridization protocol. In most other protocols for chemiluminescent and chromogenic detection of plant viruses, prehybridization times vary from 2 to 4 h (2,5,11,22). Habili et al. (14) used 4 to 16 h for prehybridization. In our experiments, no differences in results were observed when the prehybridization time was increased beyond 30 min.

The sensitivity of the hybridization technique varied when alkaline phosphatase from different sources was used. Variation in detection sensitivity with different enzymes and substrates has been noted by others as well (19,20). This indicates that the real sensitivity of the nucleic hybridization technique depends on the optimal ratio of enzyme to substrate. In our study, the detection limits of the hybridization assays varied from 0.5 to 2 ng of purified virus and from 12.5 to 50 ng of virus in infected leaf tissue.

Different sensitivities for dot blot hybridization have been found by several authors. Using nonradioactive probes, Borja and Ponz (2) detected CLRV in 160

µg of infected leaf tissue and in less than 1 ng of purified viral RNA with a chromogenic assay. They also detected virus in 30 µg of infected leaf tissue and 250 pg of purified viral RNA with a chemiluminescent assay. Using the same technique, Fouly et al. (11) detected 1 ng of purified BYDV-PAV-IL. Radioactive probe was used by Borja and Ponz (2) to detect CLRV. They detected 26 ng of purified virus and found that the sensitivity of nitrocellulose membrane was about eight times greater than that of nylon membrane, detecting down to 0.3 mg of virus in infected leaf tissue.

When the chemiluminescent substrate was used with large-volume samples (320 µl, data not shown) applied onto a positively charged nylon membrane, the chemiluminescent signal obtained was very light, suggesting some inhibition of hybridization. Similar inhibition was observed by Chu et al. (3) and by Borja and Ponz (2) when they applied larger amounts of samples onto a nylon membrane in chemiluminescent assays.

PCR, as reported previously by many others (2,15,17,23,27), is a very sensitive technique for plant virus detection and was the most sensitive of the techniques tested in this study. PCR allowed detection of 0.1 pg of purified BYDV-PAV-IL RNA and of virus in 0.5 pg of infected leaf tissue. When the primers Lu₁ and Lu₄ (23) were used, the PCR products often showed very long smears on agarose gels (data not shown), making interpretation of results difficult. The use of primers specific for the 3'-end of BYDV-PAV-IL RNA, PAV3' and 6.7 kBam, increased the sensitivity of the assay and produced more clearly defined bands.

The sensitivity of PCR for detection of plant virus can be different for different virus and plant materials, but it is always higher than other methods. Borja and Ponz (2) detected CLRV in 5 pg of infected tobacco leaves, 5 ng of infected walnut buds, and 5 µg of infected walnut twigs. Hadidi et al. (15) estimated that the theoretical minimum amount of purified PLRV RNA required for detection by RT-PCR amplification and polyacrylamide gel electrophoresis analysis was 1 fg.

Since PCR is a very sensitive technique and requires only a small quantity of infected tissue, it has been considered a useful alternative to ELISA for plant virus detection (2). The results of this study indicate that PCR certainly is the most sensitive method for detection of BYDV-PAV-IL. However, the less sensitive ELISA techniques may still be better suited for work with large numbers of samples, as in routine indexing or epidemiological studies. The simplicity of the ELISA techniques, the relatively small amount of labor required, and the high quality, readily available reagents employed make their use more economical and practical when

many samples need to be processed at the same time. For molecular biology research, where small quantities of material normally are processed, or for analyses of material from germplasm repositories, where it is important to detect small concentrations of contaminating virus, PCR and cDNA hybridization techniques are probably the methods of choice for plant virus detection.

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