Application of cDNA Macroarray for Simultaneous Detection of 12 Potato Viruses

T. Maoka, Potato Production and Protection Research Team, National Agricultural Research Center for Hokkaido Region, 1 Hitsujigaoka, Toyohira-ku, Sapporo, Hokkaido, 062-8555 Japan; **S. Sugiyama** and **Y. Maruta**, LAB Company, Ltd. 2-12 N27W6 Kita-ku Sapporo, Hokkaido, 001-0027 Japan; and **T. Hataya**, Laboratory of Pathogen-Plant Interactions, Plant Breeding Science, Division of Applied Bioscience, Research Faculty of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kita-ku, Sapporo, 060-8589 Japan

ABSTRACT

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A complementary DNA (cDNA) macroarray was developed for simultaneous detection of 12 different potato viruses. A suitable region in the viral genome for each was selected for *Alfalfa mosaic virus*, *Cucumber mosaic virus*, *Potato aucuba mosaic virus*, *Potato leafroll virus*, *Potato mop-top virus*, *Potato virus A*, *Potato virus M*, *Potato virus S*, *Potato virus X*, *Potato virus Y*, *Tomato ringspot virus*, and *Tomato spotted wilt virus*, and their respective cDNAs were cloned into plasmid vectors. Capture probes for each virus ranging from 290 to 577 bp were generated by polymerase chain reaction (PCR) and immobilized on a nylon membrane. Total RNAs were extracted from each of these virus infected-plants, and cDNAs were synthesized from the RNA extracts using a random 9-mer primer. Subsequently, PCR reactions were performed using one primer pair for each of the 12 viruses. During PCR, amplified cDNAs were labeled with biotin and used as a target for hybridization analyses on a macroarray membrane. Hybridization signals between capture probes for the 12 viruses and their respective target cDNAs were observed using chemiluminescent or colorimetric detection. In all viruses, hybridization signals with capture probes were detected only when homologous virus targets were examined, and no hybridization to healthy plant extract was observed, facilitating identification of each virus. The results by colorimetric detection agreed with those obtained using chemiluminescence. The macroarray method developed was 5×10^2 to 4×10^6 times more sensitive than enzyme-linked immunosorbent assay and 5 to 5×10^4 times more sensitive than reverse-transcription PCR, except for *Alfalfa mosaic virus*. Colorimetric detection and substantial reduction in cross-hybridization signals much improved the method compared with other array-based detection methods for practical use.

Thirty-six distinct viruses and a viroid have been reported as causal agents of potato (*Solanum tuberosum*) diseases around the world (29,32) (*Potato yellow mosaic virus* is excluded because it is synonymous with *Tomato yellow mosaic virus*). In Japan, 12 viruses—*Alfalfa mosaic virus* (AMV), *Cucumber mosaic virus* (CMV), *Potato aucuba mosaic virus* (PAMV), *Potato leafroll virus* (PLRV), *Potato mop-top virus* (PMTV), *Potato virus A* (PVA), *Potato virus M* (PVM), *Potato virus S* (PVS), *Potato virus X* (PVX), *Potato virus Y* (PVY), *Tomato ringspot virus* (ToRSV), and *Tomato spotted wilt virus* (TSWV)—have been identified as causal agents of virus diseases of potato (3,5,15–19,24–26,29,35). These pathogens are transmitted in seed tubers from generation to generation and cause severe reduction in yield and quality. Con-

trol strategies depend on the production of virus-free mother plants through tissue culture and the exclusion of infected seed potato tubers during plant quarantine and seed certification. Virus-detection methods are important factors for maintaining an economically viable certification program.

Molecularly based detection methods such as reverse-transcription polymerase chain reaction (RT-PCR) were established for some of the above viruses (13,26, 27,31). However, PCR conditions varied by target virus, and no method was available to detect the 12 viruses simultaneously. Recently, macroarrays have been developed to detect 11 potato viruses and a viroid (1,2). These viruses were detected by hybridization using synthesized oligonucleotide probes on membranes and chemically labeled targets prepared by the random primed amplified procedure. However, this macroarray was not completely satisfactory; there was cross-hybridization between viruses, making interpretation difficult (2). In addition, they required expensive tools such as an imaging system for chemiluminescence, preventing ubiquitous distribution of this technique. In order to solve these problems, we developed a simultaneous detection method for the 12 potato viruses using a complementary DNA (cDNA) macroarray. To enhance sensitivity and reduce cross-hybridization, virus-specific capture probes and biotin-labeled target amplification were used. In addition, a colorimetric detection method was employed for convenient use. The sensitivity of the method was compared with currently available diagnostic methods (i.e., enzyme-linked immunosorbent assay [ELISA] and RT-PCR) and the effi-

Table 1. Isolates of potato viruses used

Virus ^a	Isolate	Propagation host	References or origin
AMV	No. 14	<i>Nicotiana benthamiana</i>	This report
CMV	IC ^b	<i>N. benthamiana</i>	30
PAMV	Hokudai	<i>N. occidentalis</i>	This report
PLRV	No. 264	<i>Solanum tuberosum</i>	This report
PMTV	Hiroshima	<i>N. benthamiana</i>	17
PVA	MAFF307028 ^c	<i>N. benthamiana</i>	24
PVM	17-2	<i>N. occidentalis</i>	This report
PVS	M	<i>N. occidentalis</i>	This report
PVX	O-IC249	<i>N. benthamiana</i>	This report
PVY	Y28 ^d	<i>N. benthamiana</i>	This report
ToRSV	MQ	<i>N. benthamiana</i>	29
TSWV	pt1	<i>N. benthamiana</i>	26

^a AMV = *Alfalfa mosaic virus*, CMV = *Cucumber mosaic virus*, PAMV = *Potato aucuba mosaic virus*, PLRV = *Potato leafroll virus*, PMTV = *Potato mop-top virus*, PVA = *Potato virus A*, PVM = *Potato virus M*, PVS = *Potato virus S*, PVX = *Potato virus X*, PVY = *Potato virus Y*, ToRSV = *Tomato ringspot virus*, TSWV = *Tomato spotted wilt virus*.

^b Subgroup I.

^c MAFF = from collection by the Genebank Project of the National Institute of Agrobiological Sciences (NIAS), Japan.

^d Strain O.

Corresponding author: T. Maoka
E-mail: maokat@affrc.go.jp

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cacy of the method was investigated using field samples.

MATERIALS AND METHODS

Virus and RNA extraction. All the viruses used in this study were obtained from potato plants in Japan (Table 1). Isolate IC of CMV belongs to subgroup I (30). PVY isolate Y28 belongs to the PVY^O strain. PLRV, PVX, and PVY were kindly provided by Mr. H. Fuwa, National Centre for Seeds and Seedlings, Japan, and TSWV by Dr. K. Matsuo, Nagasaki Agriculture and Forestry Experiment Station, Japan. These viruses were propagated in suitable hosts (Table 1) and stored at -80°C until use. Total RNA was extracted from 50 mg of frozen leaf tissue using TRIzol reagent (Invitrogen, Carlsbad, CA), dissolved in 50 µl of nuclease-free water (Promega Corp., Madison, WI), and stored at -80°C until use. Isolates of PVY^N and PVM strains from our laboratory collection were used to test primers and probes.

Capture probes and cDNA array construction. Reference sequences used for primer design to amplify cDNA of viral RNA genome were obtained from the National Center for Biotechnology Information (NCBI) database (6). The genomic sequences of each virus were aligned using DNASIS software (Hitachi Software Engineering, Yokohama, Japan). Regions of sequence identity conserved among aligned sequences were identified visually and used to design primers. To avoid self-annealing and cross-hybridization, gene-specific sequences that have no palindromes were selected using DNASIS (Hitachi Software Engineering) and BLAST (4) in NCBI, respectively. Primer sets that amplified fragments of approximately 300 to 600 bp were selected (Table 2). For PLRV, PVS, PVX, and PVY, the primer sets designed by Sato et al. (31) were used.

Each capture probe was prepared by PCR from the corresponding viral cDNA cloned into the plasmid vectors as described below. PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and used for spotting as capture probes.

In all, 5 ng of each capture probe in 0.2 µl of distilled water was spotted in quadruplicate onto Biotodyne plus (Pall, Port Washington, NY) nylon membranes using the high-throughput liquid handling systems with glass syringes, following the method of Sugiyama et al. (34). Membranes were incubated at 120°C for 30 min in a hybridization oven (Asahi Techno Glass, Chiba, Japan) to denature the cDNA capture probes on the solid phase. Probes were immobilized by irradiation (120 mJ/cm²) at 254 nm in a UV cross-linker (UVP, Upland, CA). In preliminary trials, blotting was made in a square on the membrane (65 by 25 mm; Fig. 1). For practical use, blotting was made in a line in the membrane (85 by 27 mm) and each virus name for the

capture probe was printed below the quadruplicate spots, with the individual lot number on the left of the membrane (Fig. 2).

Cloning and sequence analysis. Each viral cDNA was cloned into pT7Blue T (Novagen, Madison, WI) or pGEM-T Easy (Promega Corp.) vectors as described previously (21). Cloned cDNAs were sequenced with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Alignment of the nucleotide sequence data was performed using DNASIS (Hitachi Software Engineering). Nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB451173 to AB451184 (Table 3).

RT, labeling RT-PCR, hybridization, and detection. For first-strand cDNA synthesis, 1 µl of total RNA from plants was mixed with 1 µl (100 U/µl) of ReverTra Ace reverse transcriptase (TOYOBO, Osaka, Japan), 10 µl of nuclease-free water (Promega Corp.), 4 µl of 5× RT buffer (TOYOBO), 1 µl of 10 µM random 9-mer primer (TaKaRa, Otsu, Japan), 2 µl of 10 mM dNTPs, and 1 µl (40 U/µl) of a porcine liver ribonuclease inhibitor (TaKaRa). First-strand cDNA synthesis was carried out at 30°C for 10 min, 42°C for 20 min, and 99°C for 5 min.

For PCR amplification of cDNA, 2 µl of first-strand cDNA was mixed with 2 µl of 10× PCR Gold Buffer (Applied Biosystems), 1 µl of 25 mM MgCl₂, 1 µl of

dNTPs (4 mM each of dATP, dGTP, and dCTP and 2 mM dTTP), 2 µl of 1 mM biotin-16-dUTP (Roche, Mannheim, Germany), 1 µl each of 10 µM forward and reverse primers (Table 2), 0.125 µl (5 U/µl) of AmpliTaq Gold LD (Applied Biosystems), and 10.875 µl of distilled water. The PCR program consisted of one cycle of 94°C for 9 min; 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min; and final extension at 72°C for 7 min.

To prepare the labeled target cDNAs using first-strand cDNA synthesized from total RNA from each plant, 12 different PCR reactions each with one pair of primers were performed using the same primer sets used in capture probe amplification (Table 2). Two microliters each of biotin-labeled cDNA from each PCR reaction were mixed and purified by MinElute PCR Purification Kit (Qiagen, Germantown, MD). Eluted solution (10 µl) was mixed with 90 µl of distilled water, boiled for 5 min, and chilled rapidly on ice.

Before hybridization, membranes were preincubated in a plastic bag with 5 ml of PerfectHyb hybridization solution (TOYOBO) at 70°C for 1 h. The hybridization solution was discarded; then, hybridization was performed overnight in 5 ml of PerfectHyb hybridization solution with 100 µl of cDNA targets in a hybridization oven at 70°C. The membrane was washed three times in wash buffer I containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; pH 7.0) and 0.1% sodium dodecyl sulfate (SDS) at 70°C for 10 min and rinsed three times in wash buffer II

Table 2. Primer sequences used for reverse-transcription polymerase chain reaction and macroarray

Virus ^a	Direction ^b	Primer designation	Sequence (5' to 3')
AMV	F	AMV2F	CAAGAATTTCTCATGGCGCGTTT
	R	AMV1R	GGGTACATCAATGACGATCAAGA
CMV	F	CMV1F	AGAGAGTAGGTACAACGAAGGAGG
	R	CMV1R	ACGTTCTCGAAGGCATCTCTGGAA
PAMV	F	PAMV8P	ACCCAAGCGTTCCTATATACTC
	R	PAMV8M	GATTGCTGGCAATGATTTGACC
PLRV	F	PLRVCP9P ^c	CGCTCAAGAAGAACTGGAG
	R	PLRVCP1M ^c	CCGAATTCCTATTGGGGTTTGGCA
PMTV	F	PMTV1F	TCGACGGCGAGCGTGTCACTATTC
	R	PMTV4R	TCAAACACGGCTCAGCCAACTCCC
PVA	F	PVA2F	GAGAATGGAACYTCTCCAGACATT
	R	PVA3R	ATAGTACGGCACTTCGGTTACACC
PVM	F	PVM22F	GGTTTCGCACACATTTGCCGCGTGA
	R	PVM21R	TTCGAAGTTCACAGTGTCTCTCC
PVS	F	PVS7P ^c	TTCCCAACAGGCGCAGTG
	R	PVS2M ^c	CTAAACGGTCTGCCTTCAT
PVX	F	PVX1P ^c	TCCTATTCCAACCGGCATC
	R	PVX1M ^c	ATCTAGGTGGCAAAGTCG
PVY	F	PVYCP6P ^c	GGTCCAAAATGAGAATGCC
	R	PVYCP6M ^c	TCTGTGTACTGATGCCAC
ToRSV	F	ToR8F	CAACACCCTTTGTCTCTACTTATTTCA
	R	ToR11R	CTAAACCTAAAACCTACCCCGCGTCC
TSWV	F	TSWV13F	ATGTCTTCAAGTGTATGAGTCGATC
	R	TSWV15R	AGTGCCAGAACAGAGTCATCAATATT

^a AMV = Alfalfa mosaic virus, CMV = Cucumber mosaic virus, PAMV = Potato aucuba mosaic virus, PLRV = Potato leafroll virus, PMTV = Potato mop-top virus, PVA = Potato virus A, PVM = Potato virus M, PVS = Potato virus S, PVX = Potato virus X, PVY = Potato virus Y, ToRSV = Tomato ring-spot virus, TSWV = Tomato spotted wilt virus.

^b F = forward and R = reverse.

^c Designed by Sato et al. (31).

(0.1× SSC and 0.1% SDS) at 70°C for 5 min. Hybridized cDNA targets were detected using the Phototope-Star Detection Kit for Nucleic Acids (New England Biolabs, Ipswich, MA) as follows. The membrane was washed with 4.5 ml of blocking buffer (5% SDS, 125 mM NaCl, 25 mM sodium phosphate, pH 7.2) at room temperature for 10 min and then in 15 ml of blocking buffer containing 10 µl of streptavidin for 7 min. After washing three times with wash buffer III (1:10 dilution of blocking buffer) for 5 min, the membrane was then incubated in 15 ml of blocking buffer containing 10 µl of biotinylated alkaline phosphatase for 7 min. The membrane was washed three times with wash buffer III for 5 min and two times with wash buffer IV (10 mM NaCl, 1 mM MgCl₂, 10 mM Tris-HCl, pH 9.5) for 5

min. The membrane was then incubated with the CDP-Star reagent for chemiluminescent detection. The image of each filter was obtained using the Fluor-S MultiImager system (Bio-Rad, Hercules, CA). Colorimetric detection was performed as follows: after washing with buffer IV, the membrane was incubated for 1 h in 10 ml of staining buffer (50 mM MgSO₄, 100 mM Tris buffer, pH 9.5) that contained 4 mg of nitroblue tetrazolium (NBT) and 1.9 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche), washed three times with distilled water, and dried in the dark. Signals were judged visually. A schematic outline of the procedure for macroarray detection of the 12 viruses for practical use is illustrated in Figure 3.

RT-PCR for agarose gel analysis. First-strand cDNA synthesis was carried

out as described above, with an exception as follows. Total RNA (1 µl) was mixed with 0.03 µl (35 U/µl) of AMV reverse-transcriptase XL (Life Sciences, St. Petersburg, FL), 6.37 µl of nuclease-free water (Promega Corp.), 1 µl of 10× reaction buffer (Life Sciences), 1 µl of 10 µM reverse primer (Table 2), 0.5 µl of 10 mM dNTPs, and 0.1 µl (40 U/µl) of a porcine liver ribonuclease inhibitor (TaKaRa). PCR was conducted as described, with an exception that biotin-16-dUTP was not used. RT-PCR product (5 µl) was separated on a 1% agarose gel in Tris-acetate-EDTA buffer (40 mM Tris-acetate and 1 mM EDTA), stained in ethidium bromide solution, and photographed under UV illumination.

ELISA. Double-antibody sandwich (DAS)-ELISA essentially followed the method of Clark and Adams (9), using commercially available antibodies and enzyme-conjugated antibodies at recommended dilutions; PMTV antibody and enzyme-conjugated antibody were provided by Neogen (Scotland, UK) and others by Agdia (Elkhart, IN). Assays were carried out according to Agdia's instructions. Samples were considered as positive when absorbance at 405 nm was at least three times greater than the mean absorbance of healthy controls after incubation for 60 to 90 min at room temperature.

Comparison of ELISA, RT-PCR, and macroarray. Sensitivity in virus detection was compared between ELISA, RT-PCR, and macroarray based on dilution end point. Leaf mass that corresponded to each of the detection thresholds indicated as dilution end point was calculated as follows. For ELISA, 50 mg of leaf samples frozen at -80°C (Table 1) were ground (defined as ×1). The original leaf extract (approximately 50 µl) was diluted with 200 µl of extraction buffer (Agdia) as ×5 and further diluted with extraction buffer to obtain a fivefold dilution series. Dilutions of 5⁻³ through 5⁻¹² were used, and each ELISA well received 100 µl of diluted samples. A 100-µl solution diluted to 5⁻³ to 5⁻¹² in a well was estimated to include 800 to 0.0004 µg of infected leaf tissue, respectively.

For RT-PCR and macroarray, leaf samples were thoroughly homogenized and 50 mg of homogenates was used to extract total RNA in 50 µl of nuclease-free water (Promega Corp.) as described above. The original RNA extract was designated as ×1 and diluted with nuclease-free water (Promega Corp.) to obtain a fivefold dilution series from 5⁻³ through 5⁻¹⁴. For RT-PCR and macroarray analyses, 1 µl of total RNA dilution was used. We estimated that 1 µl of the original total RNA derived from 1 mg of leaf tissue and that samples diluted to 5⁻³ to 5⁻¹⁴ contained 8 to 0.0000002 µg of leaf tissue, respectively.

Evaluation of macroarray. The efficacy of the macroarray was tested for practical use to detect viruses from potato sam-

Fig. 1. Macroarray detection of 12 viruses from single infected samples. **A**, Diagram of the spotting pattern of complementary DNA probes. Names of viruses are indicated above or below each blotting position. AMV = Alfalfa mosaic virus, CMV = Cucumber mosaic virus, PAMV = Potato aucuba mosaic virus, PLRV = Potato leafroll virus, PMTV = Potato mop-top virus, PVA = Potato virus A, PVM = Potato virus M, PVS = Potato virus S, PVX = Potato virus X, PVY = Potato virus Y, ToRSV = Tomato ringspot virus, TSWV = Tomato spotted wilt virus. **B**, Virus detection from single infected samples. Specific signals on the membrane were detected using chemiluminescence.

ples in the field. We collected leaf samples from farmers' fields planted with landrace potato in May 2007. Some of the landrace potato cultivars were introduced before 1870 (20), and farmers continued to produce seed potato by themselves without tissue culture and virus testing. From 19 fields in Yamanashi, Nagano, and Shizuoka, 95 potato plants were collected and stored at

–80°C. Of these, 11 samples were chosen using a random number generator and used for the macroarray test (Fig. 3). Results were compared with those from ELISA and RT-PCR using the same samples.

RESULTS

Primer design and cDNA amplification. Capture probe and target cDNA were

evaluated by RT-PCR for the 12 viruses using discriminatory primer sets. Primer sets that amplified a single band at the expected position without nonspecific bands from the target virus-infected sample were selected, and those that produced nonspecific bands from plants infected with other viruses and healthy plants were excluded. For example, it was confirmed

Fig. 2. Simultaneous macroarray detection of viruses present in single potato plants. Each virus name was printed below the quadruple spots, with the individual lot number on the left of the membrane. A healthy potato leaf was used as negative control (NC). Virus-specific complementary DNA (cDNA) was derived from each of single-infected samples of the 12 viruses, and biotin-labeled cDNAs were mixed and hybridized with a positive control (PC) membrane. AMV = Alfalfa mosaic virus, CMV = Cucumber mosaic virus, PAMV = *Potato aucuba mosaic virus*, PLRV = *Potato leafroll virus*, PMTV = *Potato mop-top virus*, PVA = *Potato virus A*, PVM = *Potato virus M*, PVS = *Potato virus S*, PVX = *Potato virus X*, PVY = *Potato virus Y*, ToRSV = *Tomato ringspot virus*, TSWV = *Tomato spotted wilt virus*.

that primers AMV2F and AMV1R amplified a 290-bp cDNA product only from an AMV-infected plant and not from any other plants infected with either of the 11 viruses or healthy plants (*data not shown*). In the same way, primer sets for CMV, PAMV, PLRV, PMTV, PVA, PVS, PVX, and PVY listed in Table 2 exclusively produced bands of their targets viruses (*data not shown*). Some of the initially designed primer sets for viruses such as ToRSV and TSWV produced single or multiple bands from healthy potato leaves by RT-PCR (*data not shown*). Consequently, primers were redesigned. For PVM strains (13), primer set PVM21R and PVM22F were selected from 22 sets of primers (*data not shown*). Amplified cDNAs for each virus ranging from 290 to 577 bp were used as capture probes (Table 3).

Hybridization of target cDNA to probes on macroarray. The specificity of capture probes (Table 3) in the macroarray assay was confirmed by hybridization. For example, for AMV, when an AMV-infected plant was used as template, PCR was performed using primers AMV2F and AMV1R, and biotin-labeled cDNA was hybridized on a macroarray, clear signals were observed in the positions of capture probes for AMV, and there was no signal for other viruses in chemiluminescent detection (Fig. 1B). These results indicate that the AMV cDNA fragment did not cross-hybridize to other virus probes. Also, when healthy plants were used as template, PCR products using primers AMV2F and AMV1R produced no signal on the macroarray membrane (*data not shown*). Similarly, the primer sets for CMV, PAMV,

PLRV, PMTV, PVA, PVM, PVS, PVX, PVY, ToRSV, and TSWV listed in Table 2 were confirmed in their hybridization specificity (Fig. 1B). Similar results were obtained by colorimetric detection (*data not shown*).

The specificity among genera or families was also determined. No cross-hybridization was observed between viruses belonging to the same genera or families; in the case of AMV and CMV, both belonging to the family *Bromoviridae*, target cDNA amplified from an AMV-infected plant using primers AMV2F and AMV1R did not react to CMV probe, and vice versa. Also, cross hybridization was not detected between PAMV and PVX (the genus *Potexvirus* in the family *Flexiviridae*), PVM and PVS (the genus *Carlavirus* in the family *Flexiviridae*), or PVA and PVY (the genus *Potyvirus* in the family *Potyviridae*) (Fig. 1). However, in a preliminary study, nonspecific signals to other viruses were observed in the macroarray and were attributed to the labeling PCR using KOD Dash DNA polymerase (TOYOBO) (*data not shown*). Consequently, AmpliTaq Gold LD (Applied Biosystems) was selected to solve the problem from several thermostable DNA polymerase and used for the labeling PCR in this study, with a modification from the previous report (34).

Reactivity between PVY^O and PVY^N was confirmed using our laboratory collection. The probe for PVY^O hybridized not only to the isolate of the same strain but also to an isolate belonging to PVY^N, although the probe had weaker hybridization signals to PVY^N than to the homologous virus isolate (*data not shown*).

Sensitivity and evaluation of the macroarray. For all viruses except AMV, the sensitivity of the macroarray assay was higher than RT-PCR, and RT-PCR was more sensitive than ELISA (Table 4). For example, the threshold for PLRV detection was 160, 0.0026, and 0.0001 µg of leaf tissue for ELISA, RT-PCR, and macroarray, respectively. The macroarray method was 5×10^2 (PVM) to 4×10^6 (PAMV) times more sensitive than ELISA, and 5 (PVX) to 5×10^4 (CMV) times compared with RT-PCR (except AMV; Table 4). RT-PCR detected a smaller amount of AMV than macroarray in two independent experiments. The lower sensitivity of macroarray for AMV may be ascribed to the secondary structure of the capture probe and the labeled cDNA, resulting in poor hybridization.

Our previous study revealed that only PVS, PVX, and PVY were occasionally detected from samples because farmers use certified seed potato lines for commercial cultivation in Japan (22). Therefore, we sought landrace potato cultivars which had not been cleaned up by tissue culture. One to five viruses were simultaneously detected from 11 Japanese landrace potato

Table 3. Target regions and size of capture probes used in the macroarray

Virus ^a	Target region	Sequence ^b	Size (bp) ^c
AMV	Coat protein on RNA 3	AB451173	290
CMV	2a Protein on RNA 2	AB451174	434
PAMV	26-kDa protein	AB451175	366
PLRV	Coat protein	AB451176	470
PMTV	Read-through protein on RNA 2	AB451177	471
PVA	Coat protein and 3' untranslated region (UTR)	AB451178	460
PVM	23-kDa protein	AB451179	395
PVS	Coat and 11-kDa proteins	AB451180	417
PVX	Coat protein	AB451181	337
PVY	Coat protein	AB451182	577
ToRSV	Coat protein and 3' UTR on RNA 2	AB451183	538
TSWV	NSs protein on S RNA segment	AB451184	293

^a AMV = Alfalfa mosaic virus, CMV = Cucumber mosaic virus, PAMV = Potato aucuba mosaic virus, PLRV = Potato leafroll virus, PMTV = Potato mop-top virus, PVA = Potato virus A, PVM = Potato virus M, PVS = Potato virus S, PVX = Potato virus X, PVY = Potato virus Y, ToRSV = Tomato ring-spot virus, TSWV = Tomato spotted wilt virus.

^b DDBJ/EMBL/GenBank accession number.

^c Primer regions included.

Fig. 3. Schematic outline of steps in macroarray detection of the 12 viruses for practical use. Washing steps not depicted.

samples (Table 5), of which 6 were symptomless. Among the samples, macroarray detected one virus from Murasaki-imo (sample no. 33), two viruses from Misakubo-shiro (no. 77), and more than three viruses from other samples (Fig. 2). They were identified as PLRV, PVA, PVM, PVS, PVX, and PVY and present in various combinations. AMV, CMV, PAMV, PMTV, ToRSV, and TSWV were not detected. Five viruses (PLRV, PVA, PVM, PVS, and PVY) were detected simultaneously from Fujinonegata (no. 4) and Ochiai-imo (no. 13) (Fig. 2). The macroarray signals obtained by colorimetric detection were distinct, and virus infection could be judged as positive or negative without difficulty.

Sensitivity was compared among ELISA, RT-PCR, and macroarray using the six viruses detected by macroarray to demonstrate the efficacy of the method (Table 5). The three methods agreed well in their ability to detect the viruses, except PLRV and PVA. The macroarray was the only method detecting PLRV from Ochiai-imo (no. 13) and Misakubo-aka (no. 84).

ELISA failed to find PLRV and PVA from Fujinonegata (no. 4) (Table 5).

DISCUSSION

There are prerequisites to the development of virus-detection methods that are acceptable for practical use, such as scale-up potential and convenience that does not require special expertise or clean, well-equipped laboratories. Sensitivity is also an important factor. Commonly used techniques for the detection of plant viruses include ELISA and RT-PCR or PCR (14,23,37,38). RT-PCR has proven to be more sensitive than ELISA (1,12,28). Macroarray assays for plant viruses have recently been developed (1,2,33). Agindotan and Perry (1,2) reported that sensitivity of their macroarray for 11 potato viruses and a viroid was comparable with that of DAS-ELISA but was lower than that for RT-PCR. Sugiyama et al. (33) reported that RT-PCR was more sensitive than macroarray for four lily viruses. Our cDNA macroarray method was generally more sensitive than RT-PCR and was always

more sensitive than ELISA. Individual PCR using primers specific to each virus amplified target viruses exclusively and enhanced the sensitivity of the macroarray.

Simultaneous detection of plant pathogens using macroarray has been reported in many articles (1,2,10,33,36,39,40). For simultaneous detection of plant viruses using macroarray, cross- or nonspecific-hybridizations complicated interpretation, hindering practical use. Agindotan and Perry (1,2) used universal primers to amplify any RNAs of potato pathogens. In addition, to reduce the risk of overlooking potential pathogens, they included additional sets of oligonucleotide capture probes. Their methods incited nonspecific hybridization, even though it detected a wider range of pathogens. In our macroarray detection, a single capture probe was selected for each virus and blotted onto the macroarray. In addition, 12 PCR reactions with biotin-labeling were performed using one primer pair for each of the 12 viruses, and AmpliTaq Gold LD (Applied Biosystems) was selected for PCR. Because of those modifications, no cross-hybridization was observed in our examination. This made interpretation simpler than other macroarray assays (2). In this report, we demonstrated that five viruses were simultaneously detected by macroarray. This is the first example of simultaneous detection using macroarray from field samples.

Intraspecies variation has been reported to exist in some of the viruses tested in this study (7,11). Strains PVY^O and PVY^N were reported to be distinguishable serologically (7,8). We could detect both strains of PVY using the macroarray, although the signal for PVY^N was relatively weak. Genetic diversity within Japanese isolates of PVM was reported by Hataya and Maoka (13). Following their results, we designed 22 sets of primers from all regions of genomic sequence of PVM (data not shown) and, of them, only PVM21R and PVM22F, which amplified

Table 4. Comparison in sensitivity between enzyme-linked immunosorbent assay (ELISA), reverse-transcription polymerase chain reaction (RT-PCR), and macroarray as estimated by dilution end-point (Dilution) and the amount of corresponding leave tissue (Estimate)

Virus ^a	ELISA		RT-PCR		Macroarray	
	Dilution	Estimate (µg)	Dilution	Estimate (µg)	Dilution	Estimate (µg)
AMV	5 ⁻⁸	0.26	5 ⁻¹²	0.000004	5 ⁻¹⁰	0.0001
CMV	5 ⁻⁸	0.26	5 ⁻⁷	0.0128	5 ⁻¹⁴	0.0000002
PAMV	5 ⁻⁴	160.00	5 ⁻⁹	0.0005	5 ⁻¹²	0.000004
PLRV	5 ⁻⁴	160.00	5 ⁻⁸	0.0026	5 ⁻¹⁰	0.0001
PMTV	5 ⁻⁴	160.00	5 ⁻⁵	0.3200	5 ⁻⁹	0.0005
PVA	5 ⁻⁴	160.00	5 ⁻⁴	1.6000	5 ⁻⁹	0.0005
PVM	5 ⁻⁵	32.00	5 ⁻³	8.0000	5 ⁻⁶	0.0640
PVS	5 ⁻⁵	32.00	5 ⁻⁶	0.0640	5 ⁻⁹	0.0005
PVX	5 ⁻⁶	6.40	5 ⁻⁸	0.0026	5 ⁻⁹	0.0005
PVY	5 ⁻⁴	160.00	5 ⁻⁷	0.0128	5 ⁻¹⁰	0.0001
ToRSV	5 ⁻⁵	32.00	5 ⁻⁶	0.0640	5 ⁻¹⁰	0.0001
TSWV	5 ⁻⁶	6.40	5 ⁻⁷	0.0128	5 ⁻¹⁰	0.0001

^a AMV = Alfalfa mosaic virus, CMV = Cucumber mosaic virus, PAMV = Potato aucuba mosaic virus, PLRV = Potato leafroll virus, PMTV = Potato mop-top virus, PVA = Potato virus A, PVM = Potato virus M, PVS = Potato virus S, PVX = Potato virus X, PVY = Potato virus Y, ToRSV = Tomato ringspot virus, TSWV = Tomato spotted wilt virus.

Table 5. Detection of potato viruses from field samples by enzyme-linked immunosorbent assay (E), reverse-transcription polymerase chain reaction (R), and macroarray (M)

No. ^b	Cultivar	Symp. ^c	Virus ^a																	
			PLRV			PVA			PVM			PVS			PVX			PVY		
			E	R	M	E	R	M	E	R	M	E	R	M	E	R	M	E	R	M
3	Fujinonegata	sl	—	—	—	+	+	+	+	+	+	+	+	+	—	—	—	+	+	+
4	Fujinonegata	m	—	+	+	—	+	+	+	+	+	+	+	+	—	—	—	+	+	+
13	Ochiai-imo	m	—	—	+	+	+	+	+	+	+	+	+	+	—	—	—	+	+	+
17	Seinaiji-shiro	sl	+	+	+	—	—	—	+	+	+	+	+	+	—	—	—	+	+	+
27	Hiratani-imo	sl	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+
30	Hiratani-imo	sl	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+
33	Murasaki-imo	sl	—	—	—	—	—	—	+	+	+	—	—	—	—	—	—	—	—	—
59	Shimoguri-aka	m	+	+	+	—	—	—	+	+	+	+	+	+	—	—	—	+	+	+
77	Misakubo-shiro	m	—	—	—	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—
84	Misakubo-aka	m	—	—	+	—	—	—	+	+	+	+	+	+	—	—	—	—	—	—
93	Zairai-murasaki	sl	—	—	—	—	—	—	+	+	+	+	+	+	—	—	—	+	+	+

^a Viruses detectable by any of the three methods are listed. PLRV = Potato leafroll virus, PVA = Potato virus A, PVM = Potato virus M, PVS = Potato virus S, PVX = Potato virus X, PVY = Potato virus Y.

^b Sample number.

^c Abbreviations for symptoms: sl = symptomless, m = weak mottling, — = reaction negative, and + = reaction positive.

the 23-kDa protein region, detected all isolates tested by RT-PCR. Selection of a suitable primer set and probe was the key to obtaining strong signals of PVM in the field trial (Fig. 2). A similar difficulty in PVM detection was reported by Agindotan and Perry (2).

There are several limitations of the macroarray technique. One is common to PCR-based techniques, including macroarray. Any virus variant with a genome sequence mutation may not be amplified or detected. The risk of overlooking potential pathogens by our macroarray may be higher than the previous one (2). Another limitation is scale-up potential. Although macroarray is not as effective as RT-PCR or ELISA in terms of experimental scale-up, our macroarray is equivalent to other array-based techniques (2,33). Macroarray is not designed to examine a large number of samples but is suitable for the simultaneous detection of many different viruses.

Our macroarray is also improved in convenience. For certification and field survey, the macroarray method should be simple and provide clear-cut results. The macroarray method consists of common techniques such as RT-PCR and hybridization. Colorimetric detection requires no expensive equipment (e.g., imaging system for fluorescence or chemiluminescence in micro- or macroarray detections) and enables visual interpretation. Virus names printed on macroarray membranes helped prompt visual interpretation. In addition, the cDNA macroarray is commercially available (LAB Company, Sapporo, Japan). These advantages should accelerate ubiquitous distribution of the technique.

In conclusion, we established the technique for sensitive and simultaneous detection of the 12 viruses from potato. The efficacy of the method for practical use was proved by the test with field samples. This technique has a potential for practical use such as breeding, quarantine, foundation of seed potato production, and field survey as well as application to other plant viruses.

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