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Highly Sensitive Fluorescent-labeled Probes and Glass Slide Hybridization for the Detection of Plant RNA Viruses and a Viroid

Zhiyou DU[1,2], Bo JIN[3], Wenhong LIU[4], Liang CHEN[1], and Jishuang CHEN[2,1]*

[1] *College of Life Sciences, Zhejiang University, Hangzhou 310029, China;*
[2] *Institute of Bioengineering, Zhejiang Sci-Tech University, Hangzhou 310018, China;*
[3] *College of Life Sciences, Zhejiang Chinese Medical University, Hangzhou 310053, China;*
[4] *College of Bioengineering, Zhejiang Chinese Medical University, Hangzhou 310053, China*

Abstract In this study, a modified method of the conventional RNA dot-blot hybridization was established, by replacing ^{32}P labels with CY5 labels and replacing nylon membranes with positive-charged glass slides, for detecting plant RNA viruses and a viroid. The modified RNA dot-blot hybridization method was named glass slide hybridization. The optimum efficiency of RNA binding onto the surfaces of activated glass slide was achieved using aminosilane-coated glass slide as a solid matrix and 5×saline sodium citrate (SSC) as a spotting solution. Using a CY5-labeled DNA probe prepared through PCR amplification, the optimized glass slide hybridization could detect as little as 1.71 pg of tobacco mosaic virus (TMV) RNA. The sensitivity of the modified method was four times that of dot-blot hybridization on nylon membrane with a ^{32}P-labeled probe. The absence of false positive within the genus *Potyvirus* [potato virus A, potato virus Y (PVY) and zucchini yellow mosaic virus] showed that this method was highly specific. Furthermore, potato spindle tuber viroid (PSTVd) was also detected specifically. A test of 40 field potato samples showed that this method was equivalent to the conventional dot-blot hybridization for detecting PVY and PSTVd. To our knowledge, this is the first report of using dot-blot hybridization on glass slides with fluorescent-labeled probes for detecting plant RNA viruses and a viroid.

Key words glass slide hybridization; fluorescent labeling; dot-blot hybridization; virus; viroid

Plant diseases caused by viruses and related pathogenic agents bring about substantial losses in agricultural and horticultural crops. A majority of plant viruses of economic importance are RNA viruses or viroids of RNA characters. The proper diagnostic method for causative viruses and viroids is the first requirement for the control of such diseases. The main techniques applied to diagnose plant viruses include biological assays, electron microscopy, serological tests, viral double-stranded RNA analysis, nucleic acid hybridization, polymerase chain reaction (PCR) or reverse transcription (RT)-PCR, and other molecular means. Where large numbers of samples have to be handled, double antibody sandwich-enzyme-linked immunoabsorbent assay (DAS-ELISA) is the most widely used method. Viroids produce no specific protein, therefore the serological test, applied so widely to viruses, can not be used for their diagnosis. For this reason, nucleic acid hybridization on membrane [1–4] and RT-PCR [5–7] with some modifications were developed for the detection of viroids.

Most of the approaches to the application of nucleic acid hybridization for the diagnosis of plant viral diseases now involve the use of filter hybridization [8]. Nylon membrane is the most popular matrix for immobilizing nucleic acids [9]. When compared with nylon membrane, glass slides used for immobilization of nucleic acids in DNA microarray systems have unique merits, including covalent immobilization of DNA, durability to high temperature, high ionic strength, minimum hybridization

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* Corresponding author: Tel, 86-571-86843200; Fax, 86-571-86843196; E-mail, chenjs@zstu.edu.cn, mmlab@zju.edu.cn

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volume, and especially parallel hybridization capacities [10]. Presently, membrane hybridization with radioactive ^{32}P labels is still widely used, although some non-radioactive report systems have been developed for the detection of plant viruses or viroids [11]. DNA microarray as a newly-developed molecular means possesses potential advantages, including easy data analysis, high throughput, and parallelism. In the past few years, DNA microarray has been mostly applied to the diagnosis of human pathogens [12–15]. More recently, the technique has been used for the detection of plant viruses [16–19].

In this paper, we attempted to use the DNA microarray system to modify the conventional dot-blot hybridization by directly spotting extracted RNAs onto surfaces of activated glass slides, then carrying out dot-blot hybridization with CY5-labeled probes, for detection of plant RNA viruses and a viroid.

Materials and Methods

Viruses, viroid and their maintenance

Virus isolates and a viroid were separately maintained in indicator hosts in a greenhouse under 28 ºC with a 13 h photoperiod. Viral sources of tobacco mosaic virus (TMV), zucchini yellow mosaic virus (ZYMV), cucumber mosaic virus (CMV), and potato virus Y (PVY) have been described previously [20–22]. TMV and ZYMV were maintained on *Nicotiana tabacum* White Burley and zucchini squash, respectively; CMV and PVY were separately maintained on *Nicotiana glutinosa*. Potato spindle tuber viroid (PSTVd) was kindly provided by the General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China, Beijing, and maintained on *Lycopersicon esculentum*. TMV virions were extracted from TMV-infected tobacco leaf tissues using a previously described procedure [22].

Viral cDNA clones and primers

Clones separately inserted with cDNA fragments of CMV, potato virus A (PVA), PVY, ZYMV, and TMV have been previously described [20,21]. Full-length cDNA of PSTVd was amplified by RT-PCR using primers PSTVF (5′-GGGAAACCTGGAGCGAACTG-3′) and PSTVR (5′-CGAGGAAGGACACCCGAAGA-3′), and cloned to pGEM-T Easy Vector (Promega, Madison, USA) according to the manufacturer's instructions. The sequence data have been submitted to the GenBank databases under accession number AY360446. These clones were used as PCR templates for preparing fluorescent-labeled DNA probes. Virus- and viroid-specific primers for fluorescent labeling PCR were designed using the software Primer Premier 5.0 (Premier Biosoft International, Palo Alto, USA) and synthesized (**Table 1**).

Isolation of nucleic acid

Fresh leaf tissue (0.1 g each) was frozen in liquid nitrogen and normally ground with mortar and pestle. Total RNAs were extracted using Trizol reagent (Invitrogen, Carlsbad, USA), prior to precipitation of total RNA, supernatant was further extracted with an equal volume of

Table 1 List of primers used to prepare fluorescent-labeled DNA probes

Virus	Abbreviation	Accession No.	Sequence (5′→3′) *	Position	Product (bp)
CMV	CMV-L	AF533968	TGTGGGTGACAGTCCGTAAAG	566–584	277
	CMV-R		AGATGTGGGAATGCGTTGGT	841–822	
TMV	TMV-L	AY360447	TCACKACTCCATCKCAGTTYGTGTT	538–562	223
	TMV-R		GGGTCTARTACCGCATTGTACCTGT	760–736	
PVA	PVA-L	AY360381	TTTCTATGAGATCACTGCAACCACT	1–25	116
	PVA-R		TGACATTTCCGTCCAGTCCAA	116–96	
PVY	PVY-L	AY423428	ATACTCGRGCAACTCAATCACA	1–22	166
	PVY-R		CCATCCATCATAACCCAAACTC	166–145	
ZYMV	ZYMV-L	AY074809	CCAACGCTGCGACAAATAATG	493–513	287
	ZYMV-R		TGCCGTTCAGTGTCTTCGC	779–761	
PSTVd	PSTV-L	AY360446	GGGAAACCTGGAGCGAACTG	96–115	116
	PSTV-R		CGAGGAAGGACACCCGAAGA	192–211	

* K, G or T; R, A or G; Y, C or T.

chloroform. Extraction of TMV RNA from purified TMV virions was the same as described above. RNA concentration was determined by A_{260} measurement.

Nylon membrane hybridization with ^{32}P-labeled DNA probes

RNA samples were mixed with three volumes of the denaturing solution, which consisted of 66.7% (*V*/*V*) formamide, 9.8% (*V*/*V*) formaldehyde and 1.3×saline sodium citrate (SSC). The samples were incubated at 65 ºC for 5 min, immediately put on ice for 3 min, then made to a final concentration of 3×SSC. They were dispensed onto the ζ-probe nylon membranes as three replicates at 1 μl or 6 μl per dot (Bio-Rad Laboratories, Richmond, USA). RNAs were fixed on the membrane by baking at 80 ºC for 1 h. Hybridization was carried out according to a previously described procedure [23].

Glass slide hybridization with CY5-labeled DNA probes

Preparation of the printed slides RNA samples were mixed with three volumes of 4×SSC (pH 8.0). The mixture was incubated at 65 ºC for 5 min, quenched on ice for 3 min, then spotted onto surfaces of aminosilane slides (CEL Associates, Los Angeles, USA) as three or five replicates at 3 nl per spot with a manual Glass Slide Arrayer (VP478A; V&P Scientific, San Diego, USA). The printed slides were placed in a humid Petri dish and incubated at 65 ºC for 30 min. After baking for 1 h at 80 ºC, the slides were rinsed in 0.1% sodium dodecyl sulfate (SDS) for 2 min and subsequently cleaned by washing with diethyl pyrocarbonate-treated distilled water.

Preparation of CY5-labeled probes CY5-labeled probes were prepared by PCR, incorporating CY5-dCTP (Amersham Biosciences, Piscataway, UK) into newly-synthesized DNA fragments. Twenty-five microliters of PCR mixture contained the following components: 1×PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$], 0.2 mM (each) of dATP, dTTP and dGTP, 0.02 mM dCTP, 0.01 mM CY5-dCTP, 0.25 μM (each) of forward and reverse primers, 0.05 U/μl r*Taq* DNA polymerase (TaKaRa, Dalian, China) and 0.05–0.5 ng/μl recombinant plasmid. PCR was carried out with one cycle of 94 ºC for 3 min, then 35 cycles of 94 ºC for 20 s, 56 ºC for 25 s, 72 ºC for 15–25 s, and an additional cycle of 72 ºC for 5 min. The PCR product was precipitated by the addition of two volumes of ethanol and 1/10 volume of 3 M ammonium acetate. The CY5-labeled DNA was then collected by centrifugation and dissolved in formamide hybridization solution [50% (*V*/*V*) formamide, 5×SSPE, 2×Denhardt's solution, 0.1% (*W*/*V*) SDS]. The concentrations of the probes were usually approximately 20–40 ng/μl.

Hybridization to printed slides To block the remaining binding sites, the printed slide was pre-hybridized with 12.5 μl of formamide hybridization solution containing 20 μg salmon sperm DNA in an array cassette (HybChamber, Gene Machines, San Carlos, USA) at 42 ºC for 30 min. The coverslip was then washed off by gently shaking the slide in 0.1×SSC, and the slide was dried by brief centrifugation. Prior to hybridization, the prepared probe was denatured at 94 ºC for 5 min, and immediately put on ice for 3 min. The denatured probe (12.5 μl) was pipetted onto the slide and a coverslip was placed over the probe. Hybridization was carried out in the array cassette at 42 ºC for 2 h. The slide was washed in pre-heated 2×SSC/0.1% SDS at 42 ºC for 5 min, then in pre-heated 0.5×SSC/0.1% SDS at 42 ºC for 5 min, and finally in 0.1×SSC at room temperature for 5 min. The slide was dried by brief centrifugation and stored in a light-proof slide box at room temperature.

Scanning and analysis The hybridized slides were scanned with a GenePix Personal 4100A scanner (Axon Instruments, Union, USA), and the resulting 16 bit TIFF images were analyzed using GenePix Pro 5.0 software (Axon Instruments). All slides were scanned using the same photo-multiplier tube gain of 650 for CY5. The background-subtracted median pixel intensities were used as a measure of overall spot intensity. The coefficient of variation (CV) of pixel intensity was calculated by dividing the standard deviation of the pixel intensity by the mean pixel intensity based on all of the pixels within a given spot. The measure was used to assess spot morphology homogeneity. Median pixel value was used to calculate the standard deviation and the mean of spot replicates.

Spot quality assessment

To obtain the optimum spot quality of RNA on surfaces of aminosilane glass slides, three types of solutions were tested as spotting solutions. One solution consisted of SSC alone, which was used as a spotting solution in DNA microarrays (DNA chips). Three concentrations (1×, 3×, and 5×) of the solution were evaluated. The second solution, named FF-SSC, consisted of formamide, formaldehyde and SSC, which was commonly used as a spotting solution in the conventional nucleic acid spot hybridization. The third solution, named DF-SSC, consisted of dimethyl sulfoxide (DMSO), formaldehyde and SSC. Three concentrations [10%, 20%, and 40% (*V*/*V*)] of formamide and DMSO in spotting solutions FF-SSC and DF-SSC were assayed. However, concentrations of

formaldehyde and SSC in these two solutions were constant, 7% (*V*/*V*) and 5×, respectively. Twofold serial dilutions of TMV RNA, prepared by diluting purified TMV RNA with total RNA from healthy tobacco leaves, were applied to each treatment of one of these three spotting solutions. Total RNA from healthy tobacco leaves was used as a negative control. Glass slide hybridization was carried out as described above.

Glass surface modification

Three types of surface-modified slides, including aminosilane, poly-*L*-lysine, and aldehyde slides (CEL Associates) were tested. Twofold serial dilutions of TMV RNA, prepared by diluting purified TMV RNA with 5×SSC, were spotted onto surfaces of these three types of slides. The printed slides were hybridized as described above.

Detection limits comparison

To compare the detection limit of glass slide hybridization with that of nylon membrane hybridization, twofold serial dilutions of TMV RNA were prepared by diluting purified TMV RNA with total RNA extracted from healthy tobacco leaves. The dilutions were spotted on aminosilane slides and nylon membranes at 3 nl and 1 μl per dot, respectively. Meanwhile, five independent RNA samples isolated from healthy tobacco leaves were added together to calculate cut-off values. CY5-labeled probes were prepared through incorporating CY5-dCTP into newly-synthesized DNA during either PCR amplification described as above or random-priming labeling reaction. The latter was carried out using a random primer labeling kit according to the manufacturer's instructions (TaKaRa) with a slight modification. The ^{32}P-dCTP was replaced with CY5-dCTP in the reaction recipes. Glass slide hybridization and nylon filter hybridization were carried out as described above. A criterion was set to access the detection limit of glass slide hybridization. The criterion was that a sample was regarded as positive if its fluorescent signal value exceeded the mean value of the healthy controls by three times standard deviations.

Results

Effect of spotting solutions on spot quality

Three types of spotting solutions were compared in terms of overall fluorescent intensity and spot morphological homogeneity. Hybridization results showed that RNAs spotted in SSC solution yielded much higher signal intensities than those spotted in FF-SSC or DF-SSC [**Fig. 1(A,B)**]. Increasing concentrations of formamide and DMSO in FF-SSC and DF-SSC reduced signal intensities, indicating that high concentrations of these two denaturants were not beneficial to immobilizing RNA onto the surfaces of the aminosilane slides. In addition, SSC concentration affected hybridization signal intensity. RNA samples spotted in 5×SSC yielded significantly higher signal intensities than those spotted in 1×SSC or 3×SSC (P<0.0085). However, there was no significant difference between 1×SSC and 3×SSC in overall fluorescent intensities (P= 0.9133). Spot homogeneity is one critical criterion to evaluate spot quality. Inconsistent deposition of RNAs in many spots was observed [**Fig. 1(A)**]. In order to digitally analyze spot homogeneity, the CV of signal intensities was calculated for each spot produced by SSCs 1×, 3×, and 5×. The highest homogenous spots were yielded by using 5×SSC (lower CV values) as compared to 1×SSC and 3×SSC [**Fig. 1(C)**]. Taken together, 5×SSC was regarded as the optimum RNA spotting solution.

Effect of glass surface chemistries on efficiencies of RNA binding

Three types of glass slides with different chemical modifications (aminosilane, poly-*L*-lysine and aldehyde) were tested in terms of capabilities of binding RNA onto their surfaces. RNA samples immobilized onto aminosilane slides generally yielded stronger signals than those immobilized onto poly-*L*-lysine slides or aldehyde slides (**Fig. 2**). The overall signal intensities produced from aminosilane slides were significantly different from those produced from the other two types of slides (P=0.0001). **Fig. 2** showed that 7000.00 pg (2.33 μg/μl) of TMV RNA yielded much lower signal intensities on all the types of slides than corresponding 3500.00 pg (1.16 μg/μl) of TMV RNA did, showing that 3500.00 pg RNA almost reached the saturated quantity of RNA binding for these three types of slides. It was also found that the decreased signal intensity from 3500.00 pg to 7000.00 pg TMV RNA on the aminosilane slide was much less than those on the other two types of slides, showing that the former probably had a strong capability of binding high concentrations of RNA. Taken together, glass slides coated with aminosilane were more suitable for RNA binding than those coated with either poly-*L*-lysine or aldehyde.

Comparison of detection limits of glass slide hybridization and nylon membrane hybridization

Detection limits of glass slide hybridization and nylon

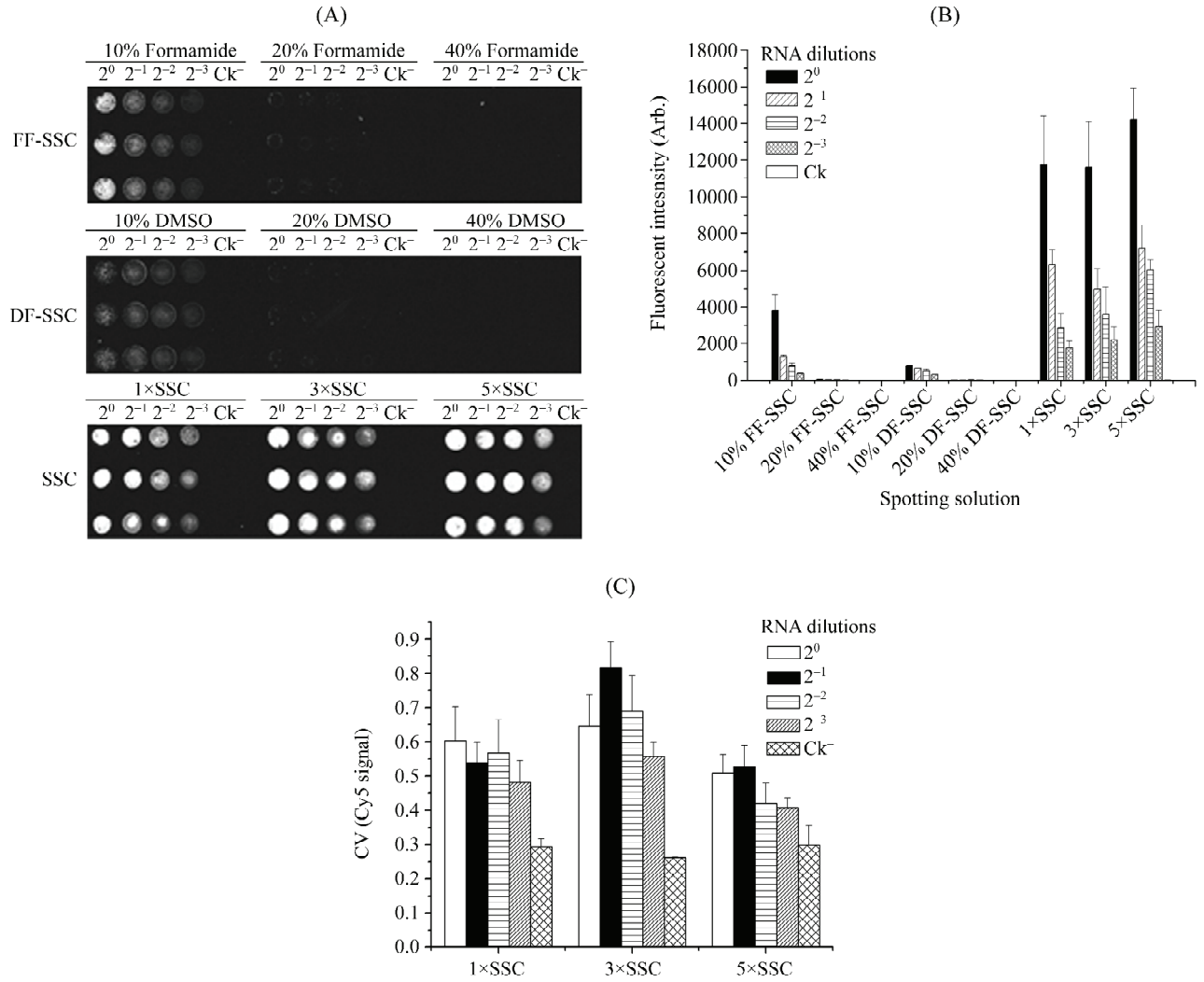

Fig. 1 Spot quality assessment of different spotting solutions

(A) Images of twofold serial dilutions of tobacco mosaic virus RNA spotted in three different solutions after hybridization with the CY5-labeled cDNA probe. The three concentrations [10%, 20%, and 40% (*V*/*V*)] of the denaturants [formamide and dimethyl sulfoxide (DMSO)] in formamide, formaldehyde and saline sodium citrate (FF-SSC) and DMSO, formaldehyde and SSC (DF-SSC) were tested. Three concentrations (1×, 3×, and 5×) of SSC in the solution SSC alone were tested. The concentrations of SSC and formaldehyde in FF-SSC and DF-aSSC were constant, 5× and 7% (*V*/*V*), respectively. (B) The average and standard deviation of the background-subtracted CY5 signals obtained from the spots of the tobacco mosaic virus RNA dilutions for each spotting solution shown in (A). Arb., arbitrary unit. (C) The average coefficient of variation (CV) and standard deviations of the signal intensities obtained for CY5 based on all of the spots for the SSC spotting solution.

filter hybridization were compared by detecting TMV RNA in serial dilutions. Nylon membrane hybridization could detect 6.83 pg of TMV RNA [**Fig. 3(A)**]. CY5-labeled DNA probes synthesized through either PCR amplification or random-priming labeling reaction were used to probe TMV RNA in its serial dilutions, as shown in **Fig. 3(B,C)**. The five healthy RNA samples gave a signal value of 13.46±3.87 [Mean (F635 median–B635)±standard deviation (SD)], when hybridized with the PCR labeling probe [**Fig. 3(D)**]. They gave a signal value of 34.13±16.26, when hybridized with the random-priming labeling probe [**Fig. 3(E)**]. According to these two signal values and the criterion described above, the cut-off values for the PCR labeling probe and the random-priming labeling probe were calculated to be 25.07 and 82.91, respectively. By comparing the cut-off values with the corresponding signal values of TMV RNA dilutions, the detection limit of glass slide hybridization with the PCR labeling probe was 1.71

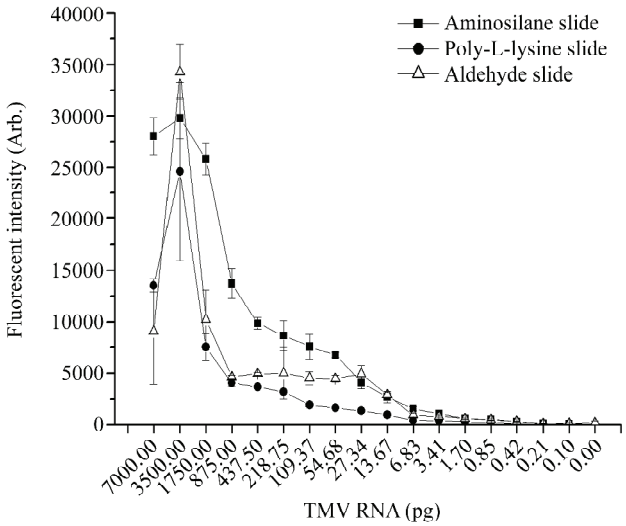

Fig. 2 Effect of different types of activated glass slides on RNA binding efficiency

The average and standard deviation of the background-subtracted CY5 signals obtained from spots of tobacco mosaic virus (TMV) RNA dilutions for each slide. Arb., arbitrary unit.

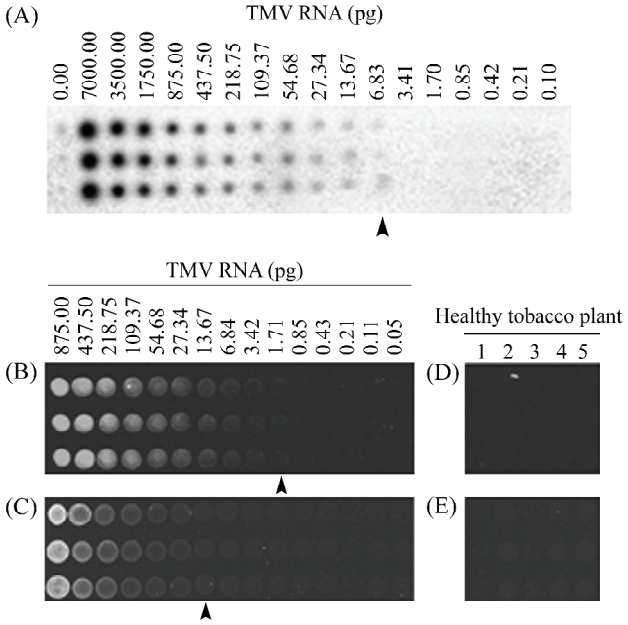

Fig. 3 Detection limits of two hybridization methods for detecting tobacco mosaic virus (TMV) RNA in serial dilutions

(A) Nylon membrane hybridization with a ^{32}P-labeled DNA probe. (B, D) Glass slide hybridization with a CY5-labeled DNA probe prepared through polymerase chain reaction (PCR) amplification. (C, E) Glass slide hybridization with a CY5-labeled DNA probe prepared through random-priming labeling reaction. In both assays, twofold serial dilutions of TMV RNA were prepared by diluting purified TMV RNA with total RNA from healthy tobacco leaf. Five healthy RNA samples were used to calculate the cut-off values for positiveness (D, E). Detection limits of both methods are indicated with arrowheads. Quantities of TMV RNA used in (A) and (B) are indicated on the upper of panel (A) and (B), respectively. The five healthy RNA samples are indicated on the upper of panel (D).

pg of TMV RNA, four times as sensitive as nylon filter hybridization [**Fig. 3(B)**]. However, the detection limit of glass slide hybridization with the random-priming labeling probe was 13.67 pg of TMV RNA, half as sensitive as nylon filter hybridization [**Fig. 3(C)**].

Specificity of glass slide hybridization

To examine the specificity of glass slide hybridization, RNA samples, extracted from leaves infected singly with TMV [12 days post-inoculation (dpi)], Q6-CMV (12 dpi), N6-CMV (6 dpi), PVY (60 dpi), ZYMV (30 dpi), and PSTVd (12 dpi), were spotted onto surfaces of aminosilane slides. The printed slides were hybridized individually with six CY5-labeled probes: p-TMV, p-CMV, p-PVA, p-PVY, p-ZYMV, and p-PSTVd, being specific for TMV, CMV, PVA, PVY, ZYMV, and PSTVd, respectively. The hybridization results are shown in **Table 2**. A criterion was set to evaluate the specificity of glass slide hybridization. The criterion was that a sample was regarded as positive if its signal value produced by its targeted probe exceeded the mean of the healthy control plus three times SD, and was regarded as false positive if its fluorescence value produced by an untargeted probe exceeded the mean of the healthy control plus three times SD. Results showed that the RNA samples hybridized with their target probes produced reasonably strong hybridization signals. No false positive was found, even within the genus *Potyvirus* (PVA, PVY, and ZYMV). In most cases, fluorescence values between the samples and the untargeted probes were less than 10, but some were in the range of 20 to 50. This case was regarded as the fluctuation of negative background according to the criterion described above. Both CMV isolates (Q6, N6) presented rather strong hybridization signals with the CMV-specific probe (p-CMV). As well as the plant viruses, glass slide hybridization detected PSTVd specifically. Taken together, glass slide hybridization possesses rather high specificity.

Detection of PVY and PSTVd from field potato samples

The ultimate validation of any diagnostic method involves testing samples of unknown identity. In this

Table 2 Detection of CMV, TMV, PVY, ZYMV and PSTVd in single infection by the glass slide hybridization

Probes	Position on the glass slide [Mean (F635 median– B635)±standard deviation][**]						
	Q6-CMV	N6-CMV	TMV	PVY	ZYMV	PSTVd	Healthy control
p-CMV	2582.0±353.4[*]	3943.4±789.2[*]	8.4±2.8	5.8±4.2	9.4±23.5	2.7±7.3	5.4±3.2
p-TMV	10.0±7.7	15.5±11.2	34342.6±409.0[*]	2.0±5.6	12.4±11.2	10.2±4.3	8.2±2.5
p-PVY	18.4±15.1	7.6±7.5	12.6±6.1	444.0±51.1[*]	23.0±10.8	10.8±19.3	12.3±6.8
p-ZYMV	17.4±26.9	5.4±4.1	7.2±14.2	3.0±4.7	2052.0±4.3.4[*]	14.8±12.4	7.2±5.6
p- PSTVd	17.6±14.9	44.8±37.1	32.6±37.5	44.2±13.0	16.8v16.4	17199.0±2312.4[*]	20.0±15.2
p-PVA	7.4±7.0	6.4±12.2	0.4±4.5	8.3±3.7	9.4±23.5	2.6±7.3	6.5±5.6

[*] indicates the fluorescent values exceeds the mean of the healthy control plus three standard deviations, and regarded as positive. [**] indicates these figures (on the upper in grids) are the hybridization images, and these numbers (on the lower in grids) are the signal values corresponding to their hybridization images.

assay, we analyzed 40 potato leaf samples by using both glass slide hybridization and nylon filter hybridization. Each RNA sample was spotted onto aminosilane slides and nylon membranes at 3 nl and 6 μl per dot, respectively. The printed glass slides were hybridized separately with CY5-labeled probes specific for PVY and PSTVd, and the prepared nylon membranes were hybridized separately with ^{32}P-labeled probes specific for PVY and PSTVd. The hybridization results are shown in **Fig. 4**. Using the criterion for assessing the detection limit and specificity, the cut-off values of glass slide hybridization for PVY and PSTVd were calculated to be 64.8 and 53.7, respectively. By comparing the cut-off values with the fluorescent signal values of 40 samples tested, eight samples were found infected with PVY and PSTVd by glass slide hybridization [**Fig. 4(A,B)**]. The result of PVY detection was consistent with that of nylon membrane hybridization [**Fig. 4 (C)**]. However, there was slight discrimination about PSTVd detection between glass slide hybridization and nylon filter hybridization [**Fig. 4(B,D)**]. The samples IV-3 and IV-4 presented a faint hybridization signal by nylon filter hybridization, but they were regarded as negative by glass slide hybridization. Further investigation was carried out by inoculating the crude saps of these two ambiguous samples on *L. esculentum* plants. However, PSTVd-induced disease symptoms were not observed on the inoculated plants (data not shown). Taken together, glass slide hybridization had a similar efficiency to nylon filter hybridization for the detection of PVY and PSTVd

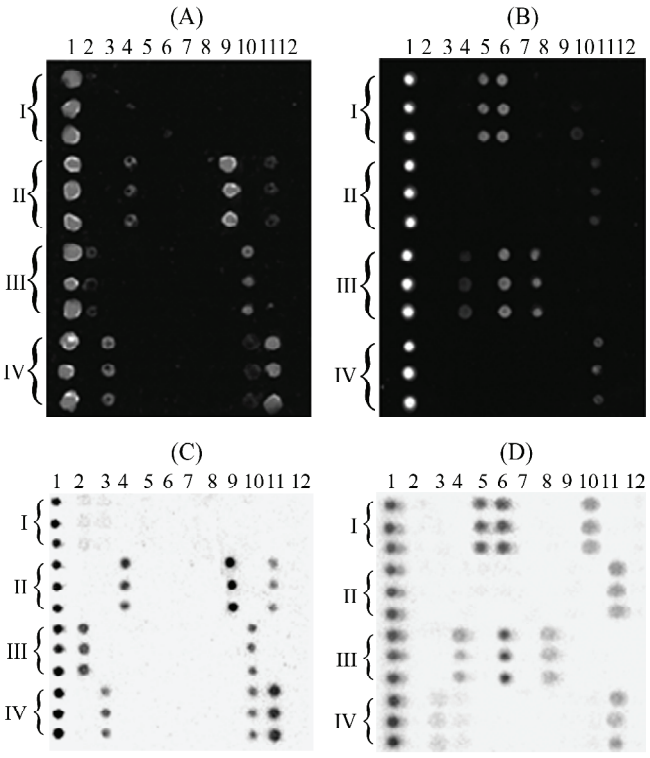

Fig. 4 Detection of potato virus Y (PVY) and potato spindle tuber viroid (PSTVd) in field potato samples by two hybridization methods

(A, B) Glass slide hybridization. (C, D) Nylon filter hybridization. (A, C) PVY detection. (B, D) PSTVd detection. I–IV: three replicative spots each sample; column 1 (A, C), PVY-infected potato as positive control; column 1 (B, D), PSTVd-infected potato as positive control; column 12, healthy potato as negative control; columns 2–11 (I–IV), field potato samples.

from field samples.

Discussion

Initially, nucleic acid hybridization was used to obtain hybrids between RNAs and DNAs in solution [24]. The method is now an important component of many techniques of molecular biology. Membrane hybridization methods exploit the high specificity of molecular hybridization for detecting rare molecules in complex mixtures. Membrane dot-blot hybridization was first applied to PSTVd detection [4]. After that, the method with some modifications was successfully applied to detection of plant RNA and DNA viruses.

In the developed glass slide hybridization procedure, one critical factor is the amount of RNA attached to glass slides available for hybridization. It was determined to a great extent by glass slide modification chemistries and spotting solutions. In conventional nucleic acid spot hybridization procedures, positive-charged nylon membranes were usually used to bind RNA through electrostatic interaction. In glass slide cDNA microarray systems, activated glass slides are usually positive-charged, such as aminosilane slides and poly-*L*-lysine slides. The aminosilane slide was shown to have a stronger capability of binding cDNA than the poly-*L*-lysine slide or the aldehyde slide [25]. In theory, RNA can be immobilized onto surfaces of positive-charged glass slides under electrostatic interaction. In our test, the aminosilane slide could efficiently immobilize RNA, and had higher RNA binding efficiency than the poly-*L*-lysine slide or the aldehyde slide (**Fig. 2**). In glass slide DNA microarray systems, 3×SSC and 50% DMSO are the most common spotting solutions [26]. In our present work, we tested SSC alone, SSC-based DMSO spotting solution (DF-SSC), and SSC-based formamide spotting solution (FF-SSC). It was found that the presence of the denaturants (formamide and DMSO) in DF-SSC and FF-SSC was not beneficial for binding RNA onto the surfaces of aminosilane slides, and the binding efficiency of RNA molecules decreased with the increasing concentration of DMSO or formamide [**Fig. 1 (A,B)**]. It seems that the two denaturants blocked the interaction of RNA molecules with aminosilane in some way. In physical terms, formamide and DMSO both increase the viscosities of the spotting solutions of FF-SSC and DF-SSC, and reduce the rates of evaporation, depending on their concentrations. The SSC solution evaporates quickly, even 5×SSC. However, high concentrations of formamide and DMSO (20% and 40%) make evaporation of FF-SSC and DF-SSC difficult in a short period. Unfortunately, RNA molecules are inclined to be degraded when they are exposed in liquid solutions for a long time. The decreased binding efficiencies of RNA molecules with the increasing concentrations of formamide and DMSO were most likely due to RNA degradation.

Random-priming labeling reaction is used widely for preparation of ^{32}P-labeled DNA probes in conventional nucleic acid spot hybridization. The CY5-labeled DNA probe prepared by the labeling method detected 13.67 pg TMV RNA [**Fig. 3(C)**], which was one-half the sensitivity of the ^{32}P-labeled DNA probe [**Fig. 3(A)**]. However, the detection sensitivity of the established glass slide hybridization increased by eight times using the CY5-labeled DNA probe prepared by the PCR method [**Fig. 3(B)**], when compared with the CY5-labeled DNA probe prepared by the random-priming labeling method. Furthermore, the five healthy RNA samples hybridized with the former yielded significantly lower signal intensities than those hybridized with the latter [**Fig. 3(D,E)**]. Both the high sensitivity and the low signal intensities from the healthy RNA samples showed that using the PCR labeling method to yield CY5-labeled probes was more suitable for the glass slide hybridization system. In our conditional tests, similar results were obtained when using CY3-labeled cDNA probes.

ELISA is popular in routine diagnostic laboratories due to its easy operation and interpretation. However, the ELISA test, in the case of viruses, is limited to viral coat proteins; it can not be applied to viroid diagnosis because viroids produce no specific protein. Dot-blot hybridization has been used widely for detecting viroids. This is a highly sensitive method as compared with polyacrylamide gel electrophoresis, and requires less time to finish a diagnosis than bioassay. Glass slide hybridization had an equivalent efficiency to nylon membrane hybridization for detecting PSTVd from field samples [**Fig. 4(B,D)**]. Furthermore, it takes less than 8 h to finish a whole diagnosis using glass slide hybridization, which is more time-saving than nylon membrane hybridization. As well as viroid detection, glass slide hybridization has three further advantages, when compared with conventional RNA spot hybridization. First, it is easier to judge positive or negative by digital analysis. Second, a glass slide would potentially allow testing a very large number of samples on one slide, and is able to spot the same samples to multiple slides with high-speed robots, enabling multiple viruses to be tested. Finally, glass slide hybridization does not involve dangerous chemicals or procedures. However, it does have a significant disadvantage. It demands a relatively high purity of RNA

samples, which would be quite laborious and is the main barrier for detecting a large number of samples. In contrast, an obvious advantage of conventional nucleic acid spot hybridization is that little RNA sample preparation is required and a crude sap can be applied to nylon membrane, making it very suitable for testing many samples. In general, glass slide hybridization, using CY5-labeled probes to detect target RNA molecules immobilized on surfaces of aminosilane slides, is a feasible method for the detection of plant RNA viruses and viroids in a large number of plant samples.

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Edited by
Minghua XU