Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Oligonucleotide-based microarray for detection of plant viruses employing sequence-independent amplification of targets

Veenita Grover, Margaret L. Pierce, Peter Hoyt, Fengqiu Zhang, Ulrich Melcher*

Department of Biochemistry & Molecular Biology, 246 NRC, Oklahoma State University, Stillwater, OK 74078, USA

A B S T R A C T

The potential of DNA microarrays for detection of plant viruses is hampered by underutilization of sequence-independent amplification methods for target nucleic acid enrichment. A microarray system is described for an unbiased detection of plant viruses using both short (30 nt) and long (50 and 70 nt) oligonucleotide probes. The assay involves amplification of target nucleic acid using random primers followed by in vitro transcription whose cRNA product is labeled chemically, fragmented and used as target for hybridization. Initial optimization tests with Turnip vein clearing virus and Cauliflower mosaic virus showed increased hybridization efficiency with shorter cDNA targets (100 bp) and longer probes (50 and 70 nt). The system was validated in pure and mixed samples by detection of three Tymovirus species: Asclepias asymptomatic virus, Kennedya yellow mosaic virus and Turnip yellow mosaic virus. The method could detect sequence variants with 70–75% or higher sequence identity, indicating the possible utility of the approach for virus discovery. Array performance comparison of long probes demonstrated the competence of 50-mers to provide a satisfactory balance between detection sensitivity and specificity. The work described is a significant step towards a method to assess, in one assay, the presence of a large diversity of relatives of known viruses of plants.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Development of sensitive and multiplexed detection tools capable of rapidly and economically identifying a broad spectrum of plant viruses is critical in epidemiological and ecological investigations, reacting to agricultural outbreaks and biodefense (Boonham et al., 2003; Wheelis et al., 2002). Common methods for plant virus detection include variations of the polymerase chain reaction (PCR), serological assays such as enzyme linked immunosorbent assays (ELISA) and immunofluorescent antibody tests and metagenomic approaches (Melcher et al., 2008; Menzel et al., 2002; Webster et al., 2004). PCR-based techniques have improved tremendously and are preferred often for definitive identification of the causative agent. These molecular techniques, whether protein or nucleic acid based, have limitations, including a requirement for prior knowledge or presumption regarding identities of viruses present in samples and detection restriction to a limited number of candidate viruses. Perhaps more importantly, most of these techniques lack the ability to detect novel viruses. For broad-spectrum identification of plant viruses, there is a need for complementary and comprehensive multi-targeted approaches for virus detection.

Microarrays, first developed to assay differential expression of mRNAs in different tissues or developmental stages (Schena et al., 1995), were recognized soon for their potential to identify pathogens. Arrays have been developed for the detection of animal and plant pathogens (Jaaskelainen and Maunula, 2006; Seifarth et al., 2003; Sengupta et al., 2003; Wilson et al., 2002), including a remarkable application of the technique in identification of the severe acute respiratory syndrome (SARS) virus as a member of the genus Coronavirus (Wang et al., 2002, 2003). Fewer arrays have been developed for the detection of plant viruses, the earliest of which were for the detection and discrimination between potato virus isolates (Boonham et al., 2003). Early arrays consisted of PCR products amplified from cDNA libraries (Boonham et al., 2003; Lee et al., 2003) and were improved later using high purity artificially synthesized oligonucleotides (Bystricka et al., 2005; Deyong et al., 2005). Oligonucleotide probes of 20–70 nt have been used successfully depending upon the desired level of detection specificity (Bystricka et al., 2005; Deyong et al., 2005; Pasquini et al., 2008).

In this article, 25–30-mer probes will be referred to as short oligonucleotide probes and 50–70-mer probes as long oligonucleotide probes. Literature data suggest that while long probes provide better detection sensitivity, only short probes allow efficient discrimination between closely related sequences (Chou et al., 2004; Letowski et al., 2004; Urakawa et al., 2003). Arrays with...
both types of probes targeting several different taxonomic groups of viruses should provide both high sensitivity as well as strong discrimination ability.

Target preparation methods and their resulting lengths influence the stability of duplex formation and hybridization signal intensity (Liu et al., 2007; Peplies et al., 2003; Peytavi et al., 2005; Southern et al., 1999). Secondary structure formation in longer targets can cause a decrease in hybridization efficiency by reducing the binding constant with probes by 10^5 to 10^6-fold, increasing false-negatives can cause a decrease in hybridization efficiency by reducing the stability of duplex formation and hybridization signal intensity (Liu et al., 2007; Peplies et al., 2003; Peytavi et al., 2005; Southern et al., 1999). Secondary structure hindrances, determination of an optimum target length and optimized technical conditions are critical to achieve an efficient and discriminating hybridization. A recent study (Liu et al., 2007) examined the effects of target length on hybridization efficiency using different length targets against Escherichia coli gene probes. Unfortunately, effects of both target and probe length on hybridization specificity and detection sensitivity in plant virus detection studies have not been investigated.

Inefficient hybridization can result also from low target nucleic acid concentrations. In case of microarrays for RNA viruses occurring at high concentrations, labeled cDNA targets can be generated by direct (Boonham et al., 2003; Lee et al., 2003) or indirect (Bystricka et al., 2005; Pasquini et al., 2008) incorporation of the label during reverse transcription reactions using random primers, without amplification. However, for viruses present in lower titers, target amplification is needed to increase the probability of virus detection. The use of group or genus-specific primers (Deyong et al., 2003; Sugiyama et al., 2008) for amplification of viral sequences is not suitable for detection of emerging unknown viruses. In addition, there are many groups of plant viruses for which no effective generic primers are available due to extreme nucleotide sequence variability of genomes. Thus, there is a significant need for the application of sequence-independent amplification methods for detection of plant viruses, especially when prior information about the identity of the virus(es) is not available. A method developed for non-specific amplification of DNA (Bohlander et al., 1992) was modified recently and used in a macroarray system for detection of plant RNA viruses (Agindotan and Perry, 2007).

The present study demonstrates the use of sequence-independent amplification starting from viral nucleic acid (VNA) (Melcher et al., 2008) or total RNA followed by in vitro transcription to generate cRNA targets for detection of plant viruses using microarrays. Though the method was validated using either VNA or total RNA as substrates, VNA has a twofold advantage for detection of encapsidated viruses. First, targets derived from VNA, isolated from virus-like particles, will contain lower proportions of host-derived nucleic acids reducing the background and improving target specificity and sensitivity of hybridization. Second, VNA, as the substrate for random amplification, targets both DNA and RNA plant viruses. This study describes the validation of an array constituting both short and long oligonucleotide probes using tymoviruses as model pathogens. Tymoviruses were chosen for the study because they are one of the most prominent viral genera present in non-cultivated plants of the Tallgrass Prairie Preserve of northeastern Oklahoma (Min et al., unpublished results; Muthukumar et al., 2009). Initial experiments were performed with Turnip vein clearing virus (TVCV, GenBank accession no. U03387) and Cauliflower mosaic virus (CaMV, GenBank accession no. M90541) to examine the effects of probe and target length variations on hybridization efficiency.

### 2. Materials and methods

#### 2.1. PCR amplification, agarose gel electrophoresis and purification of DNA targets

Different length DNA products for TVCV (100, 300 and 1000 bp) and CaMV (92 and 307 bp) were amplified from *E. coli* derived TVCV (Zhang et al., 1999) and CaMV (Armour et al., 1983) plasmids using specific primers (Table 1). Total reaction mixtures of 25 μl comprised of 16 μl of nuclease-free water, 2.5 μl of 10X *Taq* polymerase buffer, 2 μl of 25 mM MgCl₂, 0.35 μl of dNTPs/αdUTP

### Table 1

| Primer | Sequence 5′–3′ | Product size (bp) |
|--------|----------------|------------------|
| TVCV100F | CAACCCAGGCGATGG | 100 |
| TVCV100R | AACTTTCCAGCATGTGTCTCTCTTA | |
| TVCV300F | CACCAGAAGACACCTGCGA | 300 |
| TVCV300R | GCAATGATGATGTA | |
| TVCV1000F | CACCAGAAGACACCTGCGA | 1000 |
| TVCV1000R | CTAGCCACTCTCCGG | |
| CaMV92F | ATGCCACAAGGTCACT | 92 |
| CaMV92R | GAAATGGCTTCGTCAT | |
| CaMV307F | CGAGAAGCAGAGGAAGAAAGA | 307 |
| CaMV307R | TCTGAAACACCGAAATGCTTIC | |

Fig. 1. Schematic outline of the strategy used to convert viral RNA and DNA into labeled cRNA for the microarray detection of viral sequences. The outline is abbreviated, with blocking and washing steps not depicted.
Table 2
Sequences of DNA oligonucleotide target-specific probes used in the microarray.

| Probe* | Target species/genus | Sequence 5′ - 3′ | Probe Length (nt) |
|--------|----------------------|------------------|------------------|
| 10000829 | Cauliflower mosaic virus | GTACATCGAGATGGATGATGCTCTTCTCCGTCTTAAAGCAAGCTCCATATTTCCCAAGACACATGGA | 70 |
| 10000830 | Cauliflower mosaic virus | TCCATGTGTCTTTGGAATATGGATGGAGCTTGCTTTAAGCCGAAAGGGACCACATTCCATCTGAGCATCAC | 70 |
| 10003781 | Turnip vein clearing virus | AACGTGATCATTCTGCAGTAC | 24 |
| 10003782 | Turnip vein clearing virus | AAACCGATCATTCTGCAGTAC | 27 |
| 10003783 | Turnip vein clearing virus | CAAAGATCATTCTGAGCATCAC | 30 |
| 10003784 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003785 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003786 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003787 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003788 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003789 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003790 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003791 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003792 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003793 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003794 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003795 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003796 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003797 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003798 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003799 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003800 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003801 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003802 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003803 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003804 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003805 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003806 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003807 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003808 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003809 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003810 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003811 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003812 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003813 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003814 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003815 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003816 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003817 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003818 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003819 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003820 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003821 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003822 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| 10003823 | Turnip vein clearing virus | CAAACGGTATCATTCTGCAGTAC | 30 |
| 10003824 | Turnip vein clearing virus | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 30 |
| Probe | Target species/genus | Sequence 5′–3′ | Probe Length (nt) |
|-------|----------------------|----------------|------------------|
| Tymp7.5275M (T) | Asclepias asymptomatic virus | GCCATCAGGGATGCGAACGAGGTGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGTTTGGT
Table 2 (Continued)

| Probe | Target species/genus | Sequence 5'-3' |
|-------|----------------------|----------------|
| TYMV-20M-3314 | Turnip yellow mosaic virus | GCATGGCCTGGCCGACGGGTCGGGCTGGCG | 70 |
| TYMV-20M-3285 | Turnip yellow mosaic virus | TCGTCATAATTCTCGGCGATCCTCTMCAGGGCGAGTACCACTCCCAATCG | 50 |
| TYMV-20M-3314 | Turnip yellow mosaic virus | CGATTGGGAGTGGTACTCGCCCTGKAGAGGATCGCCGAGAATTATGACGAGCTCGAGGGCGGGGTCGGCG | 70 |
| TYMV4 | Turnip yellow mosaic virus | TYMV4 | |
| TYMV5 | Turnip yellow mosaic virus | TYMV5 | |
| TYMV-20M-5006 | Turnip yellow mosaic virus | CTCCACCCAGTTCGGCCCCCTCACATGCATGCGCCTAACCGGGGAACCCGGAACTTACGACGACAACACT | 70 |
| TYMV5 50M.5006 | Turnip yellow mosaic virus | TYMV5 | |
| TYMV-20M-3285 | Turnip yellow mosaic virus | CGTAAGTTCCGGGTTCCCCGGTTAGGCGCATGCATGTGAGGGGGCCGAAC | 50 |

(a mixture of 10 mM dGTP, dATP, dCTP each, 5 mM dTTP and 5 mM aminoallyl dUTP), 2.5 U of Taq polymerase (Invitrogen, Carlsbad, CA, USA), 1 µl each of 0.4 mM forward and reverse primers and 2 µl of the template plasmid. Cycle parameters for the PCR amplification were as follows: 94 °C for 2 min followed by 30 cycles of 30 s at 94 °C, 40 s at 58 °C and 1 min at 72 °C with a final 10 min extension step at 72 °C. Synthesized PCR products were analyzed using 2% agarose gel electrophoresis in 40 mM Tris–acetate, 1 mM EDTA. DNA fragments of 307 bp or below were purified using QiAquick Nucleotide Removal Kit (Qiagen, Valencia, CA, USA) while QiAquick PCR Purification Kit (Qiagen) was used for 1000 bp products. Purified samples were dried and suspended in 8 µl of nuclease-free water. All oligonucleotides including primers and probes used in this study were synthesized commercially (Integrated DNA Technologies, Inc., Coralville, IA, USA and Midland Certified Reagent Co., Midland, TX, USA).

2.2. Plant materials, viruses and RNA synthesis

The three Tymovirus species used were *Kennedya yellow mosaic virus* (KYMV), *Turnip yellow mosaic virus* (TYMV) and a novel tymovirus (Min et al., unpublished results), designated in this paper as Asclepias asymptomatic virus (AsAV). The cDNAs for TYMV and KYMV were provided by Dr. Yannis Tzanetakis, University of Arkansas, USA. Briefly, the purified viral RNA of KYMV was extracted from virus particles prepared from an infected legume, *Kennedya rubicunda* (Dale and Gibbs, 1976), and total RNA was extracted from TYMV–infected *Brassica pekinensis* (Tzanetakis et al., 2007). The extracted RNAs were reverse-transcribed using random hexamers. *Asclepias viridis* infected with AsAV was collected from the Tallgrass Prairie Preserve, Oklahoma. Uninfected *A. viridis* tissue sample was provided by Dr. Richard S. Nelson, Samuel Roberts Noble Foundation, USA.

Virus-like particle isolation and subsequent VNA extraction from infected and uninfected *A. viridis* plant tissue samples were performed as previously described (Melcher et al., 2008). The strategy employed to convert viral RNA or DNA into a form suitable for hybridization to detect viral sequences is shown in Fig. 1. The sequence-independent amplification method described previously (Bohlander et al., 1992; Wang et al., 2002) was modified slightly to make amplified targets from mixed populations of single-stranded or double-stranded RNA and DNA. VNA obtained from plant tissues was reverse-transcribed using an SP6 anchor primer with twelve 3′-end random nucleotides (5′-ATTTAGGTGACACTATAGAAN12). The second strand cDNA synthesis was carried out using two rounds of Sequenase (USB, Cleveland, OH, USA), which also can synthesize cDNA from viral DNA genomes. The double-stranded cDNA was then PCR-amplified for 30 cycles using the SP6 anchor primer (5′-ATTTAGGTGACACTATAGAA) with Taq polymerase. To incorporate the SP6 promoter on the 5′-ends of TYMV and KYMV cDNAs obtained by reverse-transcription using random hexamers, another round of PCR amplification was performed using SP6-N12 random primer and SP6 primer. To prepare targets for labeling, aminoallyl UTP was incorporated into all three PCR-amplified cDNA samples by in vitro RNA transcription with SP6 RNA polymerase using the MEGAscript™ high yield transcription kit (Ambion, Austin, TX, USA) at 37 °C for 16 h. The synthesized cRNA mixture was treated with DNase to remove template cDNA, purified using a Mega Clear™ kit (Ambion), dried and suspended in 10 µl of nuclease-free water.

2.3. Design of oligonucleotide probes and printing

A collection of different oligonucleotide probes ranging from 25 to 70-mers were designed for this study (Table 2 and Supplementary Table 1). Conserved regions at a genus or subgenus
level were identified from most viral species and used for designing short degenerate probes for members of Tobamovirus, Caulimovirus, Potexvirus, Marafivirus, Alphacryptovirus and Furovirus genera. Degenerate probes were designed for genera, or if too complex, for subgenera, by aligning sequences and submitting the alignment to Primo Degenerate (Chang Biosciences, Castro Valley, CA, USA). The program finds probe sequences with the least degeneracy to pair with all probe members of the set. In the design, G–T mispairing was allowed and inosine was used in positions requiring all four bases.

Conserved 70-mer probes designed previously (Wang et al., 2002) were used as long probes for TVCV (10003781) and CaMV (10000830). Five probes of different lengths (TV3781-21, TV3781-24, TV3781-27, TV3781-30 and TV3781-50) were designed from within the conserved 70-mer TVCV probe (10003781). TVCV-specific spacer-probes were designed with a run of 20 consecutive thymidylates (T20) to provide separation of the hybridization sequence from the substrate. Spacers were located at 3′ (TV3781-30–T20) or 5′ (T20–TV3781-30) ends of the 30-mer probe. Three short 30-mer probes corresponding to Marafivirus (Maraf.4636), Alphacryptovirus (Acrypot2.66) and Furovirus (Furo1.773) were designed with and without a spacer at their 3′-ends. Short probes specific for Ambrosia asymptomatic virus 1, AAV1 (Melcher et al., 2008) were designed with no spacer or 5-, 10- and 20-mer thymidylate spacers at their 3′-ends. All the short TVCV-specific probes were plus-sense probes. Plus-sense probes represent the plus-sense viral sequence which will bind to the complementary minus-sense viral RNA of the incoming target sample. Conversely, minus-sense probes will bind to complementary plus-sense viral RNA. Ten plus-sense and ten minus-sense virus-specific short probes with terminal oligo-d(T) spacers were designed for the novel tymovirus, AsAV. In this study, the term “virus-specific” indicates that probe design was based on a specific virus sequence and that its hybridization will not necessarily discriminate against other closely related species. Hence, cross-hybridization to these probes by targets from related species of viruses was both expected and observed. The ten minus-sense virus-specific short probes for AsAV were designed both with and without spacers for comparison purposes. Five plus- and minus-sense pairs for each of the 50- and 70-mer virus-specific probes were designed for each of the three species: AsAV (Min et al., unpublished results), KYMV (GenBank accession no. D00637) and TYMV (GenBank accession no. X61367). The complete genome sequences of these species were aligned using Clustal W (Thompson et al., 1994). Regions of high sequence similarity for the three species were identified from alignments and used to design long oligonucleotide probes. The 50-mer probes were designed internal to the 70-mer probes for all three species.

Each oligonucleotide was suspended at a concentration of 20 μM in 3 × SSC (Invitrogen, 1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0). An Omni Grid™ DNA microarray printer (Gene Machines, San Carlos, CA, USA) with Stealth SM3B pins (TeleChem International, Inc., Sunnyvale, CA, USA) was used to print arrays on polycarboxamide-coated slides (Carbo StationTM Nisshinbo Industries, Inc., Tokyo, Japan). Each probe was printed as four replicates in different areas of the array, to give both adequate replication and location randomization. A Cy3-labeled oligonucleotide was printed on the slides once in each block to provide positional information on the array. The printed oligonucleotide spots had an average diameter of 100–110 μm and 250 μm center to center spacing. The humidity was maintained around 57% during printing.

The printed arrays were subjected to UV irradiation (0.6 J/cm2) (Kimura et al., 2004) using a UV Stratalinker™ 1800 (Stratagene, La Jolla, CA, USA). The arrays were treated then with a blocking solution (3% bovine serum albumin, 0.1 M Tris–HCl pH 7.5, 0.2 M NaCl and 0.1% sarsosyl) for 30 min, washed in TE buffer (10 mM Tris–HCl pH 7.2, 1 mM EDTA) for 20 min, rinsed briefly with gently flowing nanopure water and dried using a slide centrifuge (TeleChem International, Inc.). All of these steps were performed at room temperature. During the course of this study, several versions of the array were fabricated incorporating new oligonucleotides at different stages.

2.4. Sample labeling, hybridization and image analysis

The purified cDNA samples for TVCV and CaMV as well as in vitro transcribed cRNA samples for tymoviruses containing aminomethyl moieties were coupled to NHS-ester derivatized fluorescent dye. The cDNA or cRNA samples dissolved in nuclease-free water were denatured at 90 °C for 2 min followed by snap cooling on ice. The denatured targets were mixed with 3 μl of 0.1 M sodium bicarbonate and 2 μl (14 nanomoles) of alexa647 dye (Invitrogen) suspended in anhydrous dimethylsulfoxide (EMD Chemicals, Inc., Gibbstown, NJ, USA). While protected from light, the coupling reaction proceeded for 1.5 h at room temperature. The labeled cRNA was purified using the Mega Clear Kit while QIAquick Nucleotide Removal Kit and Qiaquick PCR kit were used for purification of 100–300 bp and 1000 bp labeled cDNA samples, respectively. The fluorescently labeled cRNA was treated with a fragmentation buffer (Ambion) as per manufacturer's instructions to produce shorter RNA products of 60–200 bp. Non-specific target interactions were blocked by addition of 0.08 μg oligo-d(A20) / μg target, prior to hybridization against probes with thymidylate spacers. The targets were dried, resuspended in 10 μl of water, denatured at 95 °C for 5 min and snap–cooled on ice for 30 s. After addition of 20 μl of pre-heated Unihyb hybridization buffer (TeleChem International, Inc.), the targets were applied to the slide by flowing underneath a 25 mm × 40 mm lifter slip (Erie Scientific Company, Portsmouth, NH, USA). The slide was placed in a sealed hybridization cassette plate (Corning Life Sciences, Lowell, MA, USA). The available slots in the hybridization cassettes were filled with 10 μl of 3.5 × SSC to maintain humidity during the reaction. DNA targets were hybridized at 42 °C for 16–18 h and cRNA targets were hybridized at 46 and 60 °C for 2 h. After hybridization, the arrays were washed sequentially once in 2× SDS, 2× SSC and once in 1× SSC (Sengupta et al., 2003). The slides were dried and scanned using a Scan Array™ Express scanner (Packard Bioscience, Meriden, CT, USA). Array image acquisition and signal analysis were performed using GenePix Pro 4.0 software (Molecular Devices, Sunnyvale, CA, USA). Data analysis was performed essentially as previously described (Sengupta et al., 2003).

3. Results

3.1. Effect of target and probe length variation on hybridization signal intensity

To test the effects of probe and target length variation on hybridization efficiency, DNA targets of different lengths were hybridized to arrays containing different length oligonucleotide probes specific for the targets (Grover et al., 2007). Hybridizations of three TVCV cDNA targets (100, 300, 1000 bp) and two CaMV cDNA targets (92, 307 bp) were examined against short conserved degenerate 30- and 25-mer probes (Tobamo I–III 4557, Caulimovirus.4734) and long virus-specific 70-mer probes (10003781, 1000830) (Table 2). In all experiments, the fluorescence value of an oligonucleotide was required to be at least twenty times above the average background signal to be considered positive. Short degenerate probes did not show detectable signals with any of the target lengths for either of the two species (Fig. 2). On the other hand, the longer 10003781 TVCV probe produced a positive hybridization signal with the TVCV target but not with the CaMV target.
observed hybridization inefficiency of probes with longer targets may thus be due to formation of secondary structures in longer DNA targets, which is a well documented factor affecting probe binding for both DNA and RNA molecules (Lima et al., 1992; Liu et al., 2007; Peplies et al., 2003; Southern et al., 1999). The superior hybridization of TV3781-50 relative to shorter probes could be due to its substantially higher calculated melting temperature \(T_{m} \) or to its extra length. The extra length could circumvent possibly limited accessibility of short surface-bound DNA probes to targets.

3.2. Spacer effect

To test the theory of limited accessibility of shorter probes hindering hybridization efficiency, a spacer molecule was introduced to increase the distance between the DNA probe sequence and the slide surface. To find an optimum spacer length, oligo-d(T) spacers of different lengths (5, 10 and 20-mers) were attached on an AAV1-specific probe. The probes were hybridized to the complementary target synthesized after virus purification from Ambrosia psilostachya (Melcher et al., 2008). The results showed that 20-mer spacer length produced the strongest and most specific hybridization signals (data not shown). Further experiments were performed using the selected 20-mer oligo-d(T) spacer. TVCV cDNA targets of 100 and 300 bp were hybridized to probes TV3781-30, T20-TV3781-30 and TV3781-30-T20, providing no spacer, a 5′-end T20 spacer and a 3′-end T20 spacer, respectively. No effect was observed on the hybridization efficiency of the longer target (300 bp) when hybridized to TVCV-specific spacer-probes, whereas the hybridization efficiency of the shorter target (100 bp) improved with spacer-probes (Fig. 4). The signal intensity of the shorter target with probe TV3781-30-T20 was 6.2-fold higher than that of the same probe without the spacer, whereas the signal for probe T20-TV3781-30 increased only 2.5-fold relative to the non-spacer probe, indicating that spacers were optimal when placed on the 3′-end.

Although the calculated \(T_{m}\) values for T V3781-30-T20 and T20-TV3781-30 are the same, and slightly higher (\(<3 \ {}^{\circ}\)C) than that of TV3781-30, there was a significant difference among the hybridization efficiencies of these three probes, suggesting that the increased hybridization efficiency was due not to an effect of \(T_{m}\), but to the increased length of the probe. To ensure that the increase in intensities was not due to specific hybridization of targets to the spacer, hybridization intensities of targets to three target-irrelevant probes (Marafi.4636, Acrypto2.66 and Furol.773) were compared with and without 3′-end spacers. Regardless of the presence or absence of spacers, these probes produced intensities less
3.3. Detection of tymoviruses singly and in mixtures

To validate the DNA array with material from plant samples, the array was tested for sequence-specific detection using Tymovirus species: AsAV, KYMV and TYMV. Labeled and fragmented cRNA targets of pure and mixed samples were hybridized on separate arrays.

3.3.1. Hybridization with short oligonucleotide probes

To test the hybridization method using short oligonucleotide probes, cRNA targets derived from an AsAV-infected and uninfected control A. viridis were hybridized to an array containing a set of ten AsAV-specific probe pairs along with other unrelated viral probes. The cRNA target from an uninfected plant did not hybridize with any of the viral probes on the array, including 25-70-mers, validating the design of the array and the hybridization protocol (Fig. 5A). Labeled AsAV target demonstrated highly specific hybridization with short AsAV-specific probes (Fig. 5B). All minus-sense probes hybridized with strong signals to the target while plus-sense probes did not hybridize or hybridized poorly. Hybridization with long oligonucleotide probes (described in Section 3.3.2) also showed such preferential hybridization to minus-sense probes. The poor hybridization performance of plus-sense probes (discussed below) caused us to focus on the minus-sense probes in what follows.

As in Fig. 4, short minus-sense probes without spacers displayed weaker hybridization signals than corresponding probes with spacers (Fig. 5C), confirming the importance of spacers for short oligomers. A possible disadvantage of using an oligonucleotide spacer is the potential base pairing between the spacer and the target molecule. A 20-mer oligo-d(A) was added to the fragmented cRNA target just prior to hybridization to bind to the complementary oligo-d(T) spacer and prevent any random pairing between targets and spacers. The false positive signals observed in earlier hybridizations were lowered to near background levels, resulting in a decline in non-specific hybridizations without a loss in signals for specific hybridizations (data not shown).

3.3.2. Hybridization with long oligonucleotide probes

Long oligonucleotide probes are becoming employed widely in arrays for pathogen detection studies (Agindotan and Perry, 2008; Pasquini et al., 2008; Wang et al., 2003). The study used two types of long probes, 50 and 70-mers, to compare their array performance against cRNA targets derived from three Tymovirus species (AsAV, KYMV and TYMV) and an uninfected A. viridis using a hybridization temperature of 60 °C (TeleChem International). Fig. 6A shows the compiled results from five individual hybridizations. The cRNA target from an uninfected plant did not hybridize with any of the viral probes on the array. As shown in Fig. 5B for AsAV target hybridized with short probes, cRNA targets for all three species when hybridized to an array containing longer probes also demonstrated a lack of hybridization to non-tymoviral probes on the array (data not shown). For AsAV and TYMV targets, all specific long probes hybridized strongly to their respective viral targets. In contrast, only three out of five KYMV probe pairs (50 and 70-mers) were able to detect the target species. The other two probe-pairs (KYMV2-50M/KYMV2-70M and KYMV4-50M/KYMV4-70M) produced signals below the detection threshold and did not qualify for use in detecting KYMV.
Fig. 6. Hybridization results of AsAV-, TYMV- and KYMV-infected samples as single infections or mixture. Uninfected A. viridis sample was a negative control target. The figure shows a composite overview of signal patterns in the form of a heat map for five individual hybridization reactions performed at (A) 60 °C and (B) 46 °C. Each column represents the signal intensities of the fifteen 50- or 70-mer species-specific oligonucleotide probes hybridized to the incoming viral targets.

As predicted, cross-hybridizations to probes with targets derived from heterologous species were observed, reflecting the successful representation of conserved regions within the Tymovirus genus on the array (Fig. 6A). Cross-hybridization signals resulted from probe sequence identities ranging from 60 to 88% and increased approximately linearly with sequence identity values. For example, the AsAV5, KYMV5 and TYMV5 probe group has the highest (>78%) sequence identities of all probe pairs, and produced the strongest cross-hybridizations with viral targets. In general, 50-mer probes with less than 75% overall sequence identity and 70-mer probes with less than 70% overall sequence identity with non-target sequences were virus species-specific under the described hybridization conditions. Cross-hybridizations of targets from heterologous species were more intense with 70-mer probes than with 50-mer probes, which was expected since shorter probes provide greater discrimination between hybridizing nucleic acids. In total, ~34% (9/26) of the heterologous 50-mer probes and ~46% (12/26) of the heterologous 70-mer probes gave a hybridization signal greater than 35% of the strongest signal for that probe. The observed cross-hybridizations did not hinder the identification of individual target species in the respective infected samples since multiple homologous probes hybridized with their targets with stronger signals.

To test for simultaneous detection of multiple viruses in a single sample, cDNAs of AsAV, TYMV and KYMV were mixed prior to in vitro transcription and the labeled cRNA mixture was tested on the array (Fig. 6A). The results showed that the presence of multiple viruses did not interfere with the detection of any single virus in the sample. Probe pairs 1–4 of AsAV and TYMV as well as 1 and 3 of KYMV, which achieved high signal intensities with mixed species targets, were virus species-specific in single hybridizations, as cross-species hybridizations were absent or weak (Fig. 6A). The signatures of all three viral species were readily detected by 14 out of 15 probe pairs in the mixture. These results demonstrate that the array approach can reliably detect multiple viruses present in individual plants, and has a potential for screening of viral species in environmental samples.

3.4. Influence of temperature on signal intensities of long oligonucleotide probes

Hybridizations of the uninfected control target and all three viral targets were repeated at 46 °C instead of 60 °C to test the effect of temperature on hybridization. The uninfected sample did not hybridize with any viral probe on the array (Fig. 6B). The decrease in hybridization temperature was accompanied by a decrease in signal intensities of target-specific long oligonucleotide probes. The temperature decrease did not result in positive hybridization to KYMV2 and KYMV4 probes, false negatives at 60 °C. However, a variation in sensitivity of hybridization between 50-mers vs. 70-mers was observed at 46 °C. The average ratio of mean median intensities for 70 to 50-mer probes rose to 1.6 for 86% (13/15) of the probe pairs when hybridized at 46 °C compared to an average of 1.1 when hybridized at 60 °C. Two of the probe pairs AsAV2 50 M vs. AsAV2 70 M and TYMV1 50 M vs. TYMV1 70 M, hybridized to their targets at 46 °C, with almost equally strong signals. Concurrent with a decrease in the hybridization temperature, the percentage of heterologous probes producing hybridization signals greater than 35% of the strongest signal for that probe also increased from 34% (9/26) to 46% (11/26) in the case of 50-mer probes and from 46% (12/26) to 57% (15/26) in the case of 70-mer probes. Thus, comparison of hybridizations performed at two different temperatures showed that hybridization performed at 60 °C produced more sensitive and specific detection signals.

4. Discussion

One aspect of this work was to investigate and optimize parameters that could influence the hybridization efficiency of
oligonucleotide probes using polycarbodiimide slide chemistry for microarray detection of plant viruses. The oligonucleotide probes attached to polycarbodiimide-coated slides are bound most likely via thiamine bases forming covalent bonds in the presence of UV irradiation (Kimura et al., 2004). Thus, it is possible that immobi-
lized DNA containing thymine bases on polycarbodiimide-coated
slides may limit oligonucleotide accessibility to the DNA target.
This risk is higher presumably for smaller immobilized probes
than longer probes. The use of terminal thymidylate spacers pro-
duced an improvement in the hybridization efficiency of shorter
probes. Although the exact mechanism is not proven, the suggested
hypothesis is that the spacers are extending these probe sequences
away from the slide surface, making the probes accessible for
interaction with the target. Another benefit of oligothymidyli-
tate spacers is that the spacer itself decreases the possibility that a
thymidine internal to the virus sequence will be used for attach-
ment.

The observation that targets hybridized preferentially to
probes of one polarity was highly reproducible. Investiga-
tions elsewhere (David Wang, personal communication) have
indicated similar observations with double stranded fluores-
cent targets, whereas tests using single-stranded fluorescent
targets of both polarities produced signals with appropriate
complementary oligonucleotides. The reason for such extreme
strand preference for target-probe hybridization in presence
of a double-stranded fluorescent target remain to be eluci-
dated.

Target length is also an important parameter in hybridization
studies (Liu et al., 2007; Pepilies et al., 2003; Peytavi et al., 2005;
Southern et al., 1999). Shorter fragments of around 100 bp tar-
et length produced stronger hybridization signals on the array
than longer targets for both TVCV and CaMV species. The observa-
tions above suggest that stronger signals could be due to secondary
structure formation in the longer target strands making the tar-
get regions inaccessible to probes. The present results were in
agreement with a recent study using E. coli 16S RNA gene probes
which showed enhanced hybridization with PCR amplicons of less
than 150 bp and fragmented rRNA between 20 and 100 nt (Liu
et al., 2007). In summary, these results contributed to the estab-
lishment of efficient probe design and target synthesis strategy
to improve the sensitivity and specificity of virus detection for
the microarray format. The method described herein provides a
viable procedure for nucleic acid amplification and hybridiza-
tion that should be effective in detecting most plant RNA or
dNA viruses as long as the virus has representative sequence
information available. In it, viral nucleic acid concentrations for
hybridization are increased by preliminary particle enrichment and
by synthesis of in vitro transcribed cRNA containing aminonucle-
olieties. In previous reports using microarrays to detect plant
viruses, labeling of targets produced using random primers was
either achieved by incorporation of labeled nucleotides during
reverse transcription of the total RNA (Boonham et al., 2003; Lee
et al., 2003) or using indirect fluorochrome labeling (Bystricka
et al., 2005; Pasquini et al., 2008). These procedures did not
include a PCR amplification step that could increase the sensi-
tivity of this technique. Direct incorporation of the fluorophore
at the reverse transcription step can result in a lower amount of
DNA obtained than by indirect labeling, due to poor incorporation
of fluorophore-labeled nucleotides into DNA during polymeriza-
tion. Combining sequence-independent target amplification and
in vitro transcription with indirect labeling ensures a highly effi-
cient label incorporation as well as sufficient target yield of the
final cRNA product. A fragmentation step was added to decrease
the formation of possible secondary structures in labeled cRNA
target molecules and increase the diffusion rate of the target
molecules.

While long probes are reported to be superior in sensitivity,
short oligonucleotide probes are suitable for efficient discrimina-
tion between closely related species (Chou et al., 2004; Letowski
et al., 2004; Urakawa et al., 2003). Hence, a potential exists for the
utilization of longer probes for detection of viruses at higher taxo-
nomic levels like genus or family level, along with shorter probes for
discrimination between closely related viral species or strains. The
study successfully validated the use of both long and short probes
(with spacers) under the described hybridization method and con-
ditions. Comparison was made also between the two types of long
probes (50-mers vs. 70-mers) under two different hybridization
temperatures using three Tymovirus species. An augmentation in
hybridization signals occurred with an increase in hybridization
temperature (60 °C). It could be explained by the destabilization
of secondary structures within target molecules, increasing their
accessibility to probes. These results disagree with an earlier report
(Chou et al., 2004) that observed a reduction in hybridization sig-
nal intensities at higher hybridization temperatures (50 and 63 °C)
for both 50- and 70-mer probes. One explanation for this discrep-
ancy could be the excellent signal-to-noise ratio provided by the
polycarbodiimide slide chemistry (Kimura et al., 2004). The results
demonstrate the use of 50-mer oligonucleotide probes as an attrac-
tive choice, especially for plant virus detection studies given the
inherent nucleotide variability in genomes of most plant viruses.
The 50-mer probes can produce an ideal balance between probe
sensitivity and specificity making the assay specific enough, but
not too specific to overlook closely related viral species.

Because the emphasis of this report is on the description
of methods, a broader testing of many viral strains was not under-
taken. However, several features of this microarray are particularly
promising with regards to its ultimate use as a simple, accurate
hybridization method for detection of a broad group of viruses.
First, the reproducible absence of false hybridization by targets
prepared from uninfected plant samples made the interpreta-
tion of results simple and reliable. Second, all three individual
species used in this report were readily detected by hybridiza-
tions to the appropriate oligonucleotides without any non-specific
hybridization to unrelated viral probes. Virus-specific hybridiza-
tions produced strong signals for multiple virus-specific probes
providing explicit interpretations. Since the probe design for all
long oligonucleotide probes was focused on regions conserved
among the three species, it was not surprising to observe some
cross-hybridizations between heterologous species. Indeed, they
demonstrated the ability of the array to detect and differentiate
between closely related uncharacterized plant viruses. Third, sig-
nature sequences of all three viral species were detected readily
in the mixed viral target validating the feasibility of our microar-
ray for simultaneous detection of multiple viruses in a single plant
sample.

Although most of the oligonucleotide probes performed as pre-
dicted, some probes worked better than others. Since it has already
been reported that oligonucleotide probes binding to different
regions of a genome yield different signal intensities (Li and Stormo,
2001; Lockhart et al., 1996), the ability of an oligonucleotide probe
to yield a good hybridization signal is unpredictable just on the basis
of sequence information alone. Thus, multiple probes per species
should be used in oligonucleotide array designs to obtain reliable
information because seldom do they all prove effective (Agindotan
and Perry, 2008).

In conclusion, the report illustrates a significant step forward in
plant virus diagnostics by detailing for the first time, a microar-
ray method with the potential to detect a broad group of plant
viruses. Such a hybridization approach can facilitate the develop-
ment of a powerful multi-viral detection system of considerably
expansive application for identification of both known and related
uncharacterized emerging viruses.
Acknowledgements

This research was supported by the National Science Foundation-EPSCoR award EPS-0447262 and the Oklahoma Agricultural Experiment Station whose Director has approved the manuscript for publication. The authors would like to thank Dr. Yannis Tzanetakis and Dr. Richard S. Nelson for providing the virus infected and uninfected cDNAs and plant tissue samples. They express their appreciation to Dr. Michael W. Palmer and his team for assistance in specimen collection from the Tallgrass Prairie Preserve and Vijay Muthukumar for his help in sample processing. The assistance of OSU Microarray Core Facility and the OSU Recombinant DNA/Protein Resource Facility is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.vijem.2009.08.023.

References

Agindotan, B., Perry, K.L., 2007. Macroarray detection of plant RNA viruses using randomly primed and amplified complementary DNAs from infected plants. Phytopathology 97, 119–127.
Agindotan, B., Perry, K.L., 2008. Macroarray detection of eleven potato-infecting Tobamoviruses. J. Virol. Methods 153, 81–92.
Armour, S.L., Melcher, U., Priore, T.P., Lyttle, D.J., Eisenberg, R.C., 1983. Helper component for aphid transmission encoded by region II of Cauliflower mosaic virus DNA. Virology 129, 25–30.
Bohnard, S.K., Espinosa, R., Le Beau, M.M., Rowley, J.D., Diao, M.O., 1992. A method for the rapid sequence-independent amplification of microdissected chromosomal material. Genomics 13, 1322–1324.
Boonham, N., Walsh, K., Smith, P., Madigan, K., Graham, I., Barker, I., 2003. Detection of potato viruses using microarray technology: towards a generic method for plant viral disease diagnosis. J. Virol. Methods 108, 181–187.
Bystricka, D., Lenz, O., Mraz, I., Pihorova, L., Knoch, S., Sip, M., 2005. Oligonucleotide-based microarray: a new improvement in microarray detection of plant viruses. J. Virol. Methods 128, 176–182.
Chou, C.-C, Chen, C.-H., Lee, T.-T., Peck, K., 2004. Optimization of probe length and the number of probes per gene for optimal microarray analysis of gene expression. Nucleic Acid Res. 32, e99.
Dale, J., Gibbs, A., 1976. Kennetha yellow mosaic virus: another Tymovirus. Aust. J. Biol. Sci. 29, 397–403.
Deyong, Z., Willingham, P., Heinze, C., Adam, G., Pflunder, M., Frey, B., Frey, J.E., 2005. Differentiation of Cucumber mosaic virus isolates by hybridization to oligonucleotides in a microarray format. J. Virol. Methods 123, 101–108.
Grover, V., Pierce, M.L., Melcher, U., 2007. Microarray hybridization for detection of plant viruses from natural settings. Phytopathology 97, 543.
Jaaskelainen, A.J., Maunula, L., 2006. Applicability of microarray technique for the detection of norovirus and astroviruses. J. Virol. Methods 126, 210–216.
Kimura, N., Oda, R., Inaki, Y., Suzuki, O., 2004. Attachment of oligonucleotide probes to poly carbodiimide-coated glass for microarray applications. Nucleic Acids Res. 32, e68.
Lee, G.P., Min, B.E., Kim, CS., Choi, SJH., Harn, CH., Kim, SU., Ryu, KH., 2003. Plant virus DNA chip hybridization for detection and differentiation of four cucurbit-infecting Tobamoviruses. J. Virol. Methods 110, 19–24.
Letowski, J., Brousseau, R., Masson, L., 2004. Designing better probes: effect of probe size, mismatch position and number on hybridization in DNA oligonucleotide microarrays. J. Microbiol. Methods 61, 269–278.
Li, F., Stormo, G.D., 2001. Selection of optimal DNA oligos for gene expression arrays. Bioinformatics 17, 1067–1076.
Lima, W.F., Monia, B.P., Ecker, D.J., Freier, S.M., 1992. Implication of RNA structure on antisense oligonucleotide hybridization kinetics. Biochemistry 31, 12055–12061.
Liu, W.-T., Guo, H., Wu, J.-H., 2007. Effects of target length on the hybridization efficiency and specificity of RNA-based oligonucleotide microarrays. Appl. Environ. Microbiol. 73, 73–82.
Lockhart, D.J., Dong, H., Byrne, M.C., Follette, M.T., Gallo, M.V., Chee, M.S., Muttam, M., Wang, C., Kobayashi, M., Horton, B., Brown, E.L., 1996. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat. Biotechnol. 14, 1675–1680.
Melcher, U., Muthukumar, V., Wiley, G.B., Min, B.E., Palmer, M.W., Verchoz-Lubicz, J., Ali, A., Nelson, R.S., Roe, B.A., Thapa, V., Pierce, M.L., 2008. Evidence for novel viruses by analysis of nucleic acids in virus-like particle fractions from Ambrosia psilostachya. J. Virol. Methods 152, 49–55.
Menzel, W., Jeelkman, W., Maiss, E., 2002. Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. J. Virol. Methods 99, 81–92.
Min, B.-E., Feldman, T., Graham, W., Muthukumar, V., Roe, B.A., Palmer, M.W., Ali, A., Roosinck, M.J., Melcher, U., Nelson, R.S., unpublished results. Physiological and ecological analysis of a novel tymovirus in Asclepias viridis (milkweed) from the Tallgrass Prairie Preserve of Oklahoma.
Muthukumar, V., Melcher, U., Pierce, M., Wiley, G.B., Roe, B.A., Palmer, M.W., Thapa, V., Ali, A., Ding, T., 2009. Non-cultivated plants of the Tallgrass Prairie Preserve of northeastern Oklahoma frequently contain virus-like sequences in particulate fractions. Virus Res. 141, 165–173.
Pasquini, G., Barba, M., Hadidi, A., Faggioni, F., Negri, R., Sobol, I., Tiberini, A., Caglayan, K., Mazayd, H., Anfoka, G., Chanim, M., Zeidman, D., Czosnek, H., 2008. Oligonucleotide microarray-based detection and genotyping of Plum pox virus. J. Virol. Methods 147, 118–126.
Plesij, J., Glockner, F.O., Amann, R., 2003. Optimization strategies for DNA microarray-based detection of bacteria with 16s rRNA-targeting oligonucleotide probes. Appl. Environ. Microbiol. 69, 1397–1407.
Perini, R., Tang, L.-Y., Raymond, E.R., Roisini, K., Bissonnette, L., Boissinot, M., Picard, F.J., Huletsky, A., Ouellette, M., Bergeron, M.G., 2005. Correlation between DNA microarray hybridization efficiency and the position of short capture probe on the target nucleic acid. BioTechniques 39, 89–96.
Schenoa, M., Shalon, D., Davis, R.W., Brown, P.O., 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270, 467–470.
Seefarth, W., Spiess, B., Zeilfelder, U., Speth, C., Hehlmann, R., Leib-Mosch, C., 2003. Assessment of retroviral activity using a universal retrovirus chip. J. Virol. Methods 112, 79–91.
Sengupta, S., Onodera, K., Lai, A., Melcher, U., 2003. Molecular detection and identification of Influenza viruses by oligonucleotide microarray hybridization. J. Clin. Microbiol. 41, 4542–4550.
Southern, E., Mir, K., Shechepinov, M., 1999. Molecular interactions on microarrays. Nat. Genet. 21, 5–9.
Sugiyama, S., Masuta, C., Sekiguchi, H., Uehara, T., Shimura, H., Murata, Y., 2008. A simple, sensitive, specific detection of mixed infection of multiple plant viruses using microarray and microtube hybridization. J. Virol. Methods 153, 241–244.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
Tzanetakis, I.E., Halgren, A., Mosier, N., Martin, R.R., 2007. Identification and characterization of Raspberry mottle virus, a novel member of the Closteroviridae. Virus Res. 127, 26–33.
Urakawa, H., Fantroussi, S.E., Smidt, H., Smoot, J.C., Tribou, E.H., Kelly, J.J., Noble, P.A., Stahl, D.A., 2003. Optimization of single-base-pair mismatch discrimination in oligonucleotide microarrays. Appl. Environ. Microbiol. 69, 2848–2856.
Wang, D., Coscoy, L., Zylberberg, M., Avila, P.C., Boushey, H.A., Ganem, D., DeRisi, J.L., 2002. Microarray-based detection and genotyping of viral pathogens. Proc. Natl. Acad. Sci. U.S.A. 99, 15687–15692.
Wang, D., Coscoy, L., Zylberberg, M., Wang, C., Kobayashi, M., Norton, H., Brown, E.L., 1996. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat. Biotechnol. 14, 163 (2010) 57–67.