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Oligonucleotide-based microarray for detection of plant viruses employing sequence-independent amplification of targets

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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.

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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

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Table 1
Primer sequences used to amplify cDNA targets.

Primer	Sequence 5′–3′	Product size (bp)
TVCV100F	CAACCCAGGCGATGG	100
TVCV100R	AACTTTTCCCAGATCTTGTACTCTA	
TVCV300F	CACCAGAAAGACACCTGCGA	300
TVCV300R	GCAATGATGATGGTA	
TVCV1000F	CACCAGAAAGACACCTGCGA	1000
TVCV1000R	CTAGCCACTCTCCGG	
CaMV92F	ATGTCCACAAGGTCACT	92
CaMV92R	GAAATGCTTCGTCCAT	
CaMV307F	CGAGAAGCGAAGAGGAAAGAA	307
CaMV307R	TCTGAACACACGAAATGCTTC	

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-negative signals (Lima et al., 1992). To mitigate the effects of target 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., 2005; 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 $MgCl_2$, 0.35 μl of dNTPs/aa-dUTP

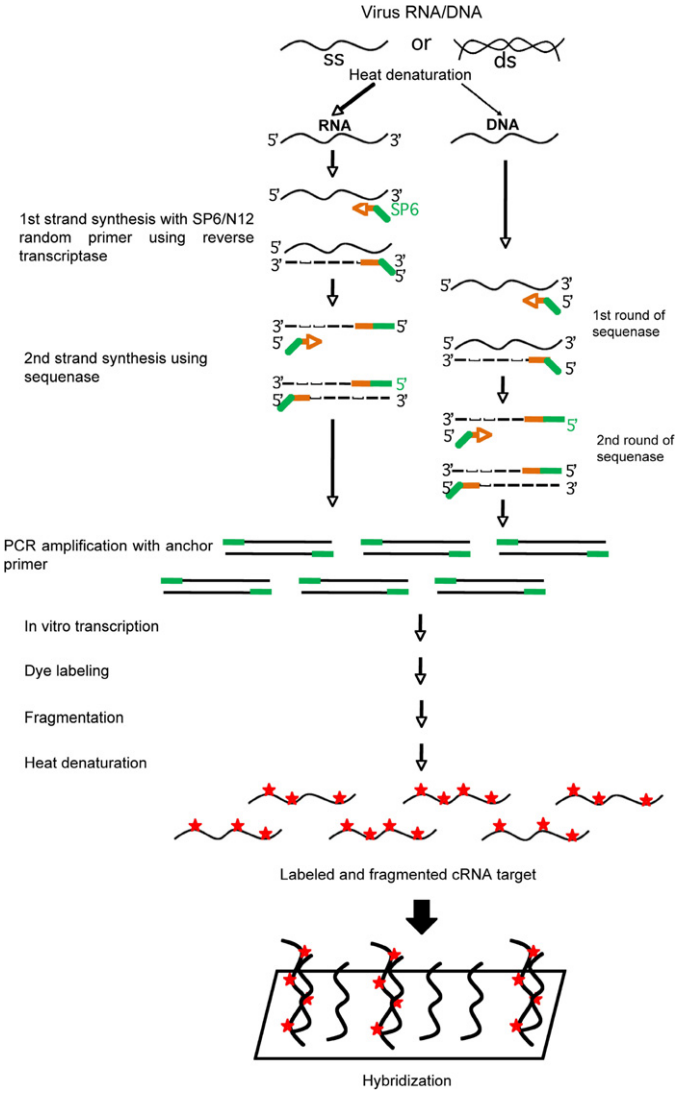

Fig. 1. Schematic outline of the strategy used to convert viral RNA and DNA into labeled cRNA for the microarray detection of viral sequences. The outline is abbreviated, with blocking and washing steps not depicted.

Table 2
Sequences of DNA oligonucleotide target-specific probes used in the microarray.

Probe[a]	Target species/genus	Sequence 5′–3′	Probe Length (nt)
10000829	*Cauliflower mosaic virus*	GTCACTACGAATGGAATGTGGTCCCTTTCGGCTTAAAGCAAGCTCCATCCATATTCCAAAGACACATGGA	70
10000830	*Cauliflower mosaic virus*	TCCATGTGTCTTTGGAATATGGATGGAGCTTGCTTTAAGCCGAAAGGGACCACATTCCATTCGTAGTGAC	70
10003781	*Turnip vein clearing virus*	AAATTCTGGAACTCGACATTTCGAAGTACGATAAGTCACAAAACGAGTTCCATTGTGCTGTAGAGTACAA	70
10003782	*Turnip vein clearing virus*	TTGTACTCTACAGCACAATGGAACTCGTTTTGTGACTTATCGTACTTCGAAATGTCGAGTTCCAGAATTT	70
Caulimoa.4734	*Caulimovirus*	TGCCTTTTGGITTAAAGCAAGCGCC	28
Tobamo I–III.4557	*Tobamovirus*	CAGAATGAGTTTCATTGTGCWGTIGAGTAT	30
TV3781-21	*Turnip vein clearing virus*	GAGTTCCATTGTGCTGTAGAG	21
TV3781-24	*Turnip vein clearing virus*	GAGTTCCATTGTGCTGTAGAGTAC	24
TV3781-27	*Turnip vein clearing virus*	AACGAGTTCCATTGTGCTGTAGAGTAC	27
TV3781-30	*Turnip vein clearing virus*	CAAAACGAGTTCCATTGTGCTGTAGAGTAC	30
TV3781-50	*Turnip vein clearing virus*	GATAAGTCACAAAACGAGTTCCATTGTGCTGTAGAGTACAAGATCTGGGA	50
(T20)-TV3781-30	*Turnip vein clearing virus*	TTTTTTTTTTTTTTTTTTTCAAAACGAGTTCCATTGTGCTGTAGAGTAC	30
(TV3781-30)-T20	*Turnip vein clearing virus*	CAAAACGAGTTCCATTGTGCTGTAGAGTACTTTTTTTTTTTTTTTTTTTT	30
Acrypto2.66	*Alphacryptovirus*	GACTGCTCTACCTCAACTTTTTACTTACT	29
Acrypto2.66-T20	*Alphacryptovirus*	GACTGCTCTACCTCAACTTTTTACTTACTTTTTTTTTTTTTTTTTTTT	28
Furo1.773	*Furovirus*	CTATCCATAGTATTTATGATATTG	24
Furo1.773-T20	*Furovirus*	CTATCCATAGTATTTATGATATTGTTTTTTTTTTTTTTTTTTTT	24
Marafi.4636	*Marafivirus*	CCTGGAAAGCTTGCCAGACCCTCGCTCTCATGCACGATG	39
Marafi.4636-T20	*Marafivirus*	CCTGGAAAGCTTGCCAGACCCTCGCTCTCATGCACGATGTTTTTTTTTTTTTTTTTTTTT	39
AAV1-T05P	*Ambrosia asymptomatic virus 1*	GGTGAGGGGCCCACCTTCGACGCAAACACCGAGTTTTT	33
AAV1-T10P	*Ambrosia asymptomatic virus 1*	GGTGAGGGGCCCACCTTCGACGCAAACACCGAGTTTTTTTTTT	33
AAV1-T20P	*Ambrosia asymptomatic virus 1*	GGTGAGGGGCCCACCTTCGACGCAAACACCGAGTTTTTTTTTTTTTTTTTTTT	34
AAV1-T20M	*Ambrosia asymptomatic virus 1*	CTCGGTGTTTGCGTCGAAGGTGGGCCCCTCACCTTTTTTTTTTTTTTTTTTTT	34
Tymo.3202P (T)	Asclepias asymptomatic virus	AACATGAAAAATGGCTTCGATGGATTTTTTTTTTTTTTTTTTTT	44
Tymo.3202M (T)	Asclepias asymptomatic virus	TCCATCGAAGCCATTTTTCATGTTTTTTTTTTTTTTTTTTT	42
Tymo.3202M (NT)	Asclepias asymptomatic virus	TCCATCGAAGCCATTTTTCATG	22
Tymo.5391P(T)	Asclepias asymptomatic virus	ACTTACGACGACAACACTGACTATAACTTTTTTTTTTTTTTTTTTTT	47
Tymo.5391M(T)	Asclepias asymptomatic virus	GTTATAGTCAGTGTTGTCGTCGTAAGTTTTTTTTTTTTTTTTTTTT	46
Tymo.5391M(NT)	Asclepias asymptomatic virus	GTTATAGTCAGTGTTGTCGTCGTAAG	26
Tymo.544P (T)	Asclepias asymptomatic virus	CATGCACGACGCTCTCATGTATTTTTTTTTTTTTTTTTTT	41
Tymo.544M (T)	Asclepias asymptomatic virus	AATACATGAGAGCGTCGTGCATGTTTTTTTTTTTTTTTTTTT	43
Tymo.544M (NT)	Asclepias asymptomatic virus	AATACATGAGAGCGTCGTGCATG	23
Tymo.829P (T)	Asclepias asymptomatic virus	TCCTGGAATCCTGGGGCCCCCTTTTTTTTTTTTTTTTTTT	41
Tymo.829M (T)	Asclepias asymptomatic virus	GGGGGCCCCAGGATTCCAGGATTTTTTTTTTTTTTTTTTT	41
Tymo.829M (NT)	Asclepias asymptomatic virus	GGGGGCCCCAGGATTCCAGGA	21
Tymotp.3295P (T)	Asclepias asymptomatic virus	AACATGAAAAATGGCTTCGATGGAATTCTCTTTTTTTTTTTTTTTTTTTT	51
Tymotp.3295M (T)	Asclepias asymptomatic virus	GAGAATTCCATCGAAGCCATTTTTCATGTTTTTTTTTTTTTTTTTTTT	48
Tymotp.3295M (NT)	Asclepias asymptomatic virus	GAGAATTCCATCGAAGCCATTTTTCATG	28
Tymotp.4978P (T)	Asclepias asymptomatic virus	AACGACTATGCTCAGCTCTCCTCCAAAACCTTTTTTTTTTTTTTTTTTTT	51
Tymotp.4978M (T)	Asclepias asymptomatic virus	GGTTTTGGAGGAGAGCTGAGCATAGTCGTTTTTTTTTTTTTTTTTTTT	48
Tymotp.4978M (NT)	Asclepias asymptomatic virus	GGTTTTGGAGGAGAGCTGAGCATAGTCG	28
Tymotp.5007P (T)	Asclepias asymptomatic virus	CAAATCCACCATTGTCGCCAATGCTTCCCGTTTTTTTTTTTTTTTTTTTT	51
Tymotp.5007M (T)	Asclepias asymptomatic virus	CGGGAAGCATTGGCGACAATGGTGGATTTGTTTTTTTTTTTTTTTTTTTT	50
Tymotp.5007M (NT)	Asclepias asymptomatic virus	CGGGAAGCATTGGCGACAATGGTGGATTTG	30
Tymotp.5488P (T)	Asclepias asymptomatic virus	GGCACTTACGACGACAACACCGACTACAACTTTTTTTTTTTTTTTTTTTT	51
Tymotp.5488M (T)	Asclepias asymptomatic virus	GTTGTAGTCGGTGTTGTCGTCGTAAGTGCCTTTTTTTTTTTTTTTTTTTT	50
Tymotp.5488M (NT)	Asclepias asymptomatic virus	GTTGTAGTCGGTGTTGTCGTCGTAAGTGCC	30
Tymotp.5512P(T)	Asclepias asymptomatic virus	TACAACATTGCCGTGCTCTACTCTCAATACTTTTTTTTTTTTTTTTTTTT	51
Tymotp.5512M(T)	Asclepias asymptomatic virus	GTATTGAGAGTAGAGCACGGCAATGTTGTATTTTTTTTTTTTTTTTTTTT	50
Tymotp.5512M(NT)	Asclepias asymptomatic virus	GTATTGAGAGTAGAGCACGGCAATGTTGTA	30
Tymotp.5725P (T)	Asclepias asymptomatic virus	CCTCGCTCTGTTCGCCAAGCTGATGATCGCTTTTTTTTTTTTTTTTTTTT	51

Table 2 (*Continued*)

Probe[a]	Target species/genus	Sequence 5′–3′	Probe Length (nt)
Tymotp.5725M (T)	Asclepias asymptomatic virus	GCGATCATCAGCTTGGCGAACAGAGCGAGGTTTTTTTTTTTTTTTTTTT	50
Tymotp.5725M (NT)	Asclepias asymptomatic virus	GCGATCATCAGCTTGGCGAACAGAGCGAGG	30
AsAV1_70P.1005	Asclepias asymptomatic virus	CCTCTTCACCTACACAAGAGCGGTTCGAACCCTCCGAACCTCCGACCCCGCCGGCTTCGTTCGAACCCAG	70
AsAV1_70M.1074	Asclepias asymptomatic virus	CTGGGTTCGAACGAAGCCGGCGGGGTCGGAGGTTCGGAGGGTTCGAACCGCTCTTGTGTAGGTGAAGAGG	70
AsAV1_50P.1010	Asclepias asymptomatic virus	TCACCTACACAAGAGCGGTTCGAACCCTCCGAACCTCCGACCCCGCCGGC	50
AsAV1_50M.1059	Asclepias asymptomatic virus	GCCGGCGGGGTCGGAGGTTCGGAGGGTTCGAACCGCTCTTGTGTAGGTGA	50
AsAV2_70P.3220	Asclepias asymptomatic virus	CTTCAATCAGAAACTGAGAGACTCTCGCAATTCATCGACCACTATTGTTGGTGGACGTACAGAGTCCCAT	70
AsAV2_70M.3289	Asclepias asymptomatic virus	ATGGGACTCTGTACGTCCACCAACAATAGTGGTCGATGAATTGCGAGAGTCTCTCAGTTTCTGATTGAAG	70
AsAV2_50P.3240	Asclepias asymptomatic virus	ACTCTCGCAATTCATCGACCACTATTGTTGGTGGACGTACAGAGTCCCAT	50
AsAV2_50M.3289	Asclepias asymptomatic virus	ATGGGACTCTGTACGTCCACCAACAATAGTGGTCGATGAATTGCGAGAGT	50
AsAV3 70P.4399	Asclepias asymptomatic virus	TCCACCATCGTCGCCAATGCTTCCCGGTCTGACCCAGATTGGAGACATACTGCCGTCAAGATATTCGCCA	70
AsAV3_70M.4468	Asclepias asymptomatic virus	TGGCGAATATCTTGACGGCAGTATGTCTCCAATCTGGGTCAGACCGGGAAGCATTGGCGACGATGGTGGA	70
AsAV3_50P.4407	Asclepias asymptomatic virus	CGTCGCCAATGCTTCCCGGTCTGACCCAGATTGGAGACATACTGCCGTCA	50
AsAV3_50M.4456	Asclepias asymptomatic virus	TGACGGCAGTATGTCTCCAATCTGGGTCAGACCGGGAAGCATTGGCGACG	50
AsAV4_70P.3129	Asclepias asymptomatic virus	TTCCGACCCTTCCATTTCTCTCATCATCCTCCTTGGAGACCCTCTCCAGGGAGAGTATCATTCCACTTCC	70
AsAV4_70M.3198	Asclepias asymptomatic virus	GGAAGTGGAATGATACTCTCCCTGGAGAGGGTCTCCAAGGAGGATGATGAGAGAAATGGAAGGGTCGGAA	70
AsAV4_50P.3149	Asclepias asymptomatic virus	TCATCATCCTCCTTGGAGACCCTCTCCAGGGAGAGTATCATTCCACTTCC	50
AsAV4_50M.3198	Asclepias asymptomatic virus	GGAAGTGGAATGATACTCTCCCTGGAGAGGGTCTCCAAGGAGGATGATGA	50
AsAV5_70P.4824	Asclepias asymptomatic virus	CTCCACCCAATTCGGACCCCTCACCTGCATGCGCCTTACTGGAGAGCCCGGCACTTACGACGACAACACT	70
AsAV5_70M.4893	Asclepias asymptomatic virus	AGTGTTGTCGTCGTAAGTGCCGGGCTCTCCAGTAAGGCGCATGCAGGTGAGGGGTCCGAATTGGGTGGAG	70
AsAV5_50P.4833	Asclepias asymptomatic virus	ATTCGGACCCCTCACCTGCATGCGCCTTACTGGAGAGCCCGGCACTTACG	50
AsAV5_50M.4882	Asclepias asymptomatic virus	CGTAAGTGCCGGGCTCTCCAGTAAGGCGCATGCAGGTGAGGGGTCCGAAT	50
KYMV1_70P.1012	*Kennedya yellow mosaic virus*	TCTCTTCACCTACACGCGAGCCGTCAGAACGCTCCGCGTCTCCGACCCCGCAGGCTTCGTTCGGACCCAA	70
KYMV1_70M.1081	*Kennedya yellow mosaic virus*	TTGGGTCCGAACGAAGCCTGCGGGGTCGGAGACGCGGAGCGTTCTGACGGCTCGCGTGTAGGTGAAGAGA	70
KYMV1_50P.1016	*Kennedya yellow mosaic virus*	TCACCTACACGCGAGCCGTCAGAACGCTCCGCGTCTCCGACCCCGCAGGC	50
KYMV1_50M.1065	*Kennedya yellow mosaic virus*	GCCTGCGGGGTCGGAGACGCGGAGCGTTCTGACGGCTCGCGTGTAGGTGA	50
KYMV2_70P.3425	*Kennedya yellow mosaic virus*	CTGCAGTCCGAGACCACCCGACTTCTCCCCTTCATTGATCACTACTGTTGGTGGACTTATCGTGTCCCCT	70
KYMV2_70M.3494	*Kennedya yellow mosaic virus*	AGGGGACACGATAAGTCCACCAACAGTAGTGATCAATGAAGGGGAGAAGTCGGGTGGTCTCGGACTGCAG	70
KYMV2_50P.3445	*Kennedya yellow mosaic virus*	ACTTCTCCCCTTCATTGATCACTACTGTTGGTGGACTTATCGTGTCCCCT	50
KYMV2_50M.3494	*Kennedya yellow mosaic virus*	AGGGGACACGATAAGTCCACCAACAGTAGTGATCAATGAAGGGGAGAAGT	50
KYMV3_70P.4594	*Kennedya yellow mosaic virus*	AACCCAAGCCACTCTCGTGGCCAACCACTCCCGTTCTGACCCCGACTGGCGCCACACAGCAGTCAAA	67
KYMV3_70M.4660	*Kennedya yellow mosaic virus*	TTTGACTGCTGTGTGGCGCCAGTCGGGGTCAGAACGGGAGTGGTTGGCCACGAGAGTGGCTTGGGTT	67
KYMV3_50P.4609	*Kennedya yellow mosaic virus*	CGTGGCCAACCACTCCCGTTCTGACCCCGACTGGCGCCACACAGCAGTCA	50
KYMV3_50M.4658	*Kennedya yellow mosaic virus*	TGACTGCTGTGTGGCGCCAGTCGGGGTCAGAACGGGAGTGGTTGGCCACG	50
KYMV4_70P.3334	*Kennedya yellow mosaic virus*	GGCAGACCCCTGTCTTGAACTGGTCATCATTCTCGGCGACCCTCTACAAGGCGAGTACCACTCCACTTCC	70
KYMV4_70M.3403	*Kennedya yellow mosaic virus*	GGAAGTGGAGTGGTACTCGCCTTGTAGAGGGTCGCCGAGAATGATGACCAGTTCAAGACAGGGGTCTGCC	70
KYMV4_50P.3354	*Kennedya yellow mosaic virus*	TGGTCATCATTCTCGGCGACCCTCTACAAGGCGAGTACCACTCCACTTCC	50
KYMV4_50M.3403	*Kennedya yellow mosaic virus*	GGAAGTGGAGTGGTACTCGCCTTGTAGAGGGTCGCCGAGAATGATGACCA	50
KYMV5_70P.5026	*Kennedya yellow mosaic virus*	TGCAACGCAGTTCGGCCCTCTGACCTGCATGCGCCTCACTGGCGAACCTGGCACCTACGACGACAACTCA	70
KYMV5_70M.5095	*Kennedya yellow mosaic virus*	TGAGTTGTCGTCGTAGGTGCCAGGTTCGCCAGTGAGGCGCATGCAGGTCAGAGGGCCGAACTGCGTTGCA	70
KYMV5_50P.5035	*Kennedya yellow mosaic virus*	GTTCGGCCCTCTGACCTGCATGCGCCTCACTGGCGAACCTGGCACCTACG	50
KYMV5_50M.5084	*Kennedya yellow mosaic virus*	CGTAGGTGCCAGGTTCGCCAGTGAGGCGCATGCAGGTCAGAGGGCCGAAC	50
TYMV1_70P.1033	*Turnip yellow mosaic virus*	CCTGTTCACCTATACCAGAGCAGTCCGCACACTCCGAACTTCAGACCCAGCAGCATTCGTAAGGATGCAC	70
TYMV1_70M.1102	*Turnip yellow mosaic virus*	GTGCATCCTTACGAATGCTGCTGGGTCTGAAGTTCGGAGTGTGCGGACTGCTCTGGTATAGGTGAACAGG	70
TYMV1_50P.1038	*Turnip yellow mosaic virus*	TCACCTATACCAGAGCAGTCCGCACACTCCGAACTTCAGACCCAGCAGCA	50
TYMV1_50M.1087	*Turnip yellow mosaic virus*	TGCTGCTGGGTCTGAAGTTCGGAGTGTGCGGACTGCTCTGGTATAGGTGA	50
TYMV2_70P.3356	*Turnip yellow mosaic virus*	CTTCCCTCTGAAACTCTCAGGCTGCTACCATACATCGACATGTACTGCTGGTGGAGTTACCGCATTCCTC	70
TYMV2_70M.3425	*Turnip yellow mosaic virus*	GAGGAATGCGGTAACTCCACCAGCAGTACATGTCGATGTATGGTAGCAGCCTGAGAGTTTCAGAGGGAAG	70
TYMV2_50P.3376	*Turnip yellow mosaic virus*	GCTGCTACCATACATCGACATGTACTGCTGGTGGAGTTACCGCATTCCTC	50
TYMV2_50M.3425	*Turnip yellow mosaic virus*	GAGGAATGCGGTAACTCCACCAGCAGTACATGTCGATGTATGGTAGCAGC	50
TYMV3_70P.4520	*Turnip yellow mosaic virus*	TCCACCATAGTGGCCAACGCTTCACGCTCCGACCCAGACTGGCGACACACCACCGTCAAGATCTTCGCGA	70
TYMV3_70M.4589	*Turnip yellow mosaic virus*	TCGCGAAGATCTTGACGGTGGTGTGTCGCCAGTCTGGGTCGGAGCGTGAAGCGTTGGCCACTATGGTGGA	70
TYMV3_50P.4528	*Turnip yellow mosaic virus*	AGTGGCCAACGCTTCACGCTCCGACCCAGACTGGCGACACACCACCGTCA	50
TYMV3_50M.4577	*Turnip yellow mosaic virus*	TGACGGTGGTGTGTCGCCAGTCTGGGTCGGAGCGTGAAGCGTTGGCCACT	50
TYMV4_70P.3265	*Turnip yellow mosaic virus*	CGCCGACCCCGCCCTCGAGCTCGTCATAATTCTCGGCGATCCTCTMCAGGGCGAGTACCACTCCCAATCG	70

Table 2 (*Continued*)

Probe[a]	Target species/genus	Sequence 5′–3′	Probe Length (nt)
TYMV4.70M.3334	*Turnip yellow mosaic virus*	CGATTGGCAGTGGTACTCGCCCTGKAGAGGATCGCCGAGAATTATGACGAGCTCGAGGGCGGGGTCGGCG	70
TYMV4.50P.3285	*Turnip yellow mosaic virus*	TCGTCATAATTCTCGGCGATCCTCTMCAGGGCGAGTACCACTCCCAATCG	50
TYMV4.50M.3334	*Turnip yellow mosaic virus*	CGATTGGCAGTGGTACTCGCCCTGKAGAGGATCGCCGAGAATTATGACGA	50
TYMV5.70P.4948	*Turnip yellow mosaic virus*	CTCCACCCAGTTCGGCCCCCTCACATGCATGCGCCTAACCGGGGAACCCGGAACTTACGACGACAACACT	70
TYMV5.70M.5017	*Turnip yellow mosaic virus*	AGTGTTGTCGTCGTAAGTTCCGGGTTCCCCGGTTAGGCGCATGCATGTGAGGGGCCCGAACTGGGTGGAG	70
TYMV5.50P.4957	*Turnip yellow mosaic virus*	GTTCGGCCCCCTCACATGCATGCGCCTAACCGGGGAACCCGGAACTTACG	50
TYMV5 50M.5006	*Turnip yellow mosaic virus*	CGTAAGTTCCGGGTTCCCCGGTTAGGCGCATGCATGTGAGGGGCCGAAC	50

The table lists all the probes used in the study and described in the article.

[a] 70-mer probes for *Turnip vein clearing virus* and *Cauliflower mosaic virus* are probes described by Wang et al. (2003); M—minus-sense probe, P—plus-sense probe, (T)—tailed/spacer probe, (NT)—non-tailed/non-spacer probe. Probes not indicated as P or M are plus-sense probes.

(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 48 °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, l 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′-ATTTAGGTGACACTATAGAAN$_{12}$). 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-N$_{12}$ 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 (T_{20}) to provide separation of the hybridization sequence from the substrate. Spacers were located at 3′ (TV3781-30-T_{20}) or 5′ (T_{20}-TV3781-30) ends of the 30-mer probe. Three short 30-mer probes corresponding to *Marafivirus* (Marafi.4636), *Alphacryptovirus* (Acrypto2.66) 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. XI6378). 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 polycarbodiimide-coated slides (Carbo Station™ 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/cm^2) (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% sarcosyl) 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 aminoallyl 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(A_{20})/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 oligonucelotide 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, Caulimoa.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,

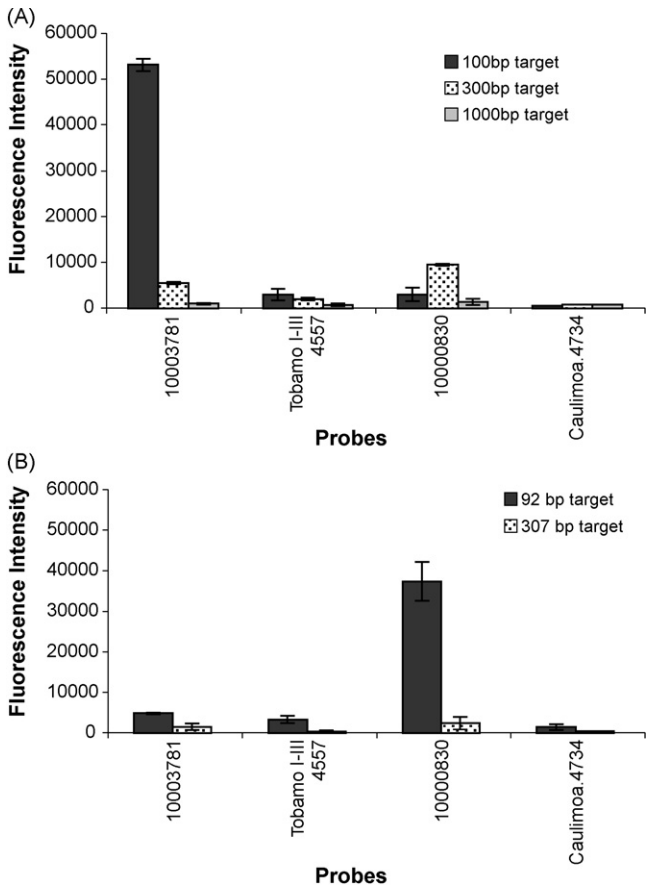

Fig. 2. TVCV and CaMV cDNA hybridization fluorescence intensity as a function of target and probe length. Signal patterns observed after microarray hybridization of (A) TVCV cDNA targets of three different lengths and (B) CaMV cDNA targets of two different lengths to a set of short and long TVCV- and CaMV-specific oligonucleotide probes. The long probes (10003781 and 10000830) are TVCV- and CaMV-specific perfect match 70-mer probes. Tobamo I–III 4557 and Caulimoa.4734 are short degenerate probes for TVCV and CaMV with five and two nucleotide mismatches, respectively. Error bars represent the standard deviations for analyzed probe replicates.

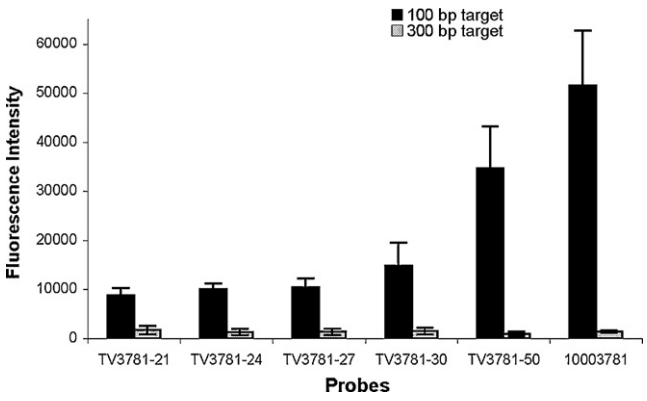

Fig. 3. Signal patterns after hybridization of 100 and 300 bp cDNA targets of TVCV to six different length probes ranging from 21 to 70-mers for the same target. All six probes are sequence-specific perfect match probes. The suffix numbers of probes specify their length, for e.g. TV3781-21 is a 21 nt long probe. 10003781 is the long TVCV-specific 70-mer probe. Error bars represent the standard deviations for analyzed probe replicates.

while the opposite was true for the CaMV probe 1000830, indicating the expected specificity had occurred. Positive signals were strongest with shorter TVCV and CaMV targets (100 and 92 bp, respectively).

To test whether poor hybridization of targets to shorter probes was due to degeneracy present in the short probes, the 100 and 300 bp TVCV targets were hybridized to an array containing six virus-specific probes with lengths ranging from 21 nt (TV3781-21) to 70 nt (10003781) (Table 2). Once again, the longer target (300 bp) produced hybridization signals close to background with all of the six different length probes (Fig. 3). Shorter targets (100 bp) did not produce strong hybridizations with shorter probes but the hybridization efficiency improved approximately fivefold as the probe length increased from 21 to 70 nt. To explain the consistent higher hybridization efficiency patterns observed with longer targets, the relationship between the targets' hybridization efficiencies and overall Gibbs free energies (ΔG), which consider the occurrences of secondary structures in the DNA targets, was evaluated. As calculated using the DNA mfold server (Zuker, 2003), the negative values of ΔG for 300 and 1000 bp targets were twice and seven times lower than for the 100 bp target, respectively. Furthermore, both of the longer targets contain an additional sequence capable of forming a hairpin whose loop could pair in pseudo-knot fashion with sequence complementary to the probe used. The 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, 66 °C vs. 53–58 °C) 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 AAVl-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, T_{20}-TV3781-30 and TV3781-30-T_{20}, providing no spacer, a 5′-end T_{20} spacer and a 3′-end T_{20} 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-T_{20} was 6.2-fold higher than that of the same probe without the spacer, whereas the signal for probe T_{20}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-T_{20} and T_{20}-TV3781-30 are the same, and slightly higher (<3 °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 were not due to non-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

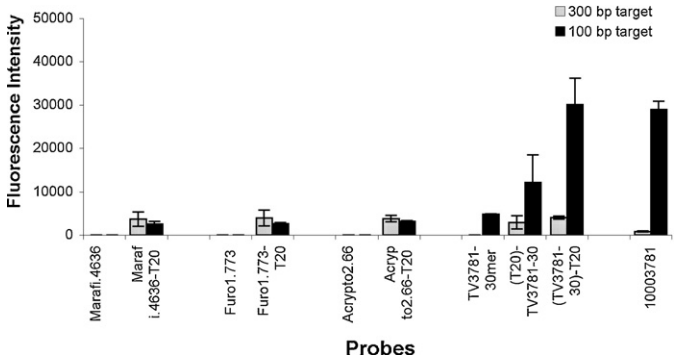

Fig. 4. Impact of spacers on hybridization efficiency of shorter probes. Hybridization of 100 and 300 bp TVCV cDNA targets to probes TV3781, T_{20}-TV3781-30 and TV3781-30-T_{20} provided with no spacer, 5′-end T_{20} spacer and 3′-end T_{20} spacer, respectively. 10003781 is the long conserved TVCV-specific 70-mer probe. Hybridization of incoming targets to three target-irrelevant control probes (Marafi.4636, Acrypto2.66 and Furo1.773) with and without 3′-end spacers was also monitored to test for non-specific pairing of targets to spacers.

than 20% of target-specific probes with spacers at either ends. Together the results suggested that the hybridization efficiency of short probes could be improved to produce detectable and specific signals by addition of oligo-d(T) spacers at 3′-ends. These findings were in agreement with similar previous reports using spacers with different slide chemistries (Chou et al., 2004; Peplies et al., 2003; Southern et al., 1999).

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).

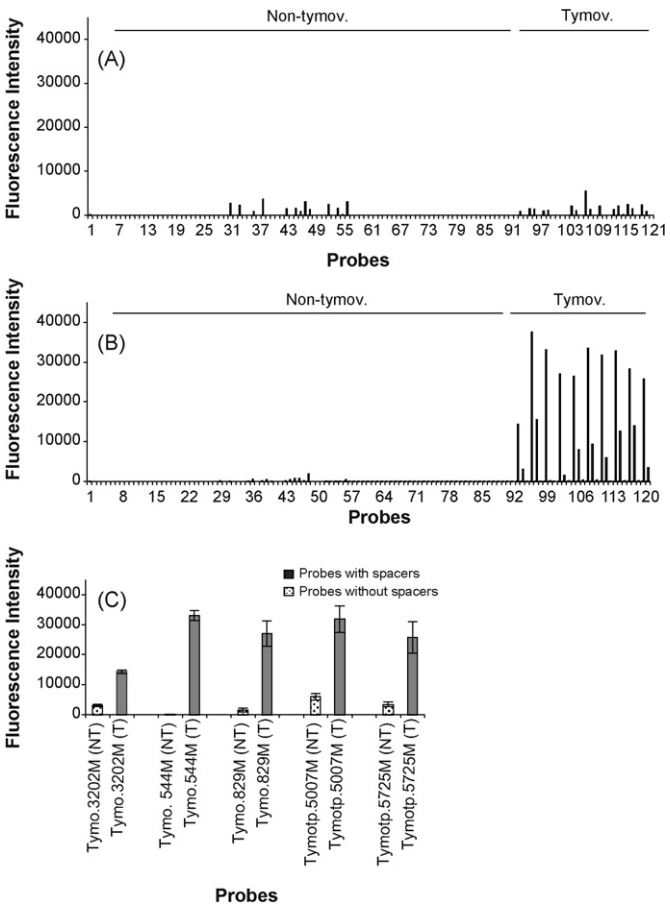

Fig. 5. Signal patterns after microarray hybridization of labeled cRNA from (A) uninfected *A. viridis* used as a negative control and (B) AsAV infected *A. viridis* at 46 °C to a set of short target-specific and non-target probes (Table 2). Probe numbers 1–91 in both A and B are the non-tymoviral probes on the array ranging from 25 to 70-mers, whereas probe numbers 92–121 are the specific tymoviral probes. There are ten tymoviral probe sets in triplets, P(T), M(T), and M(NT) as shown in Table 2. P, M, T and NT stand for positive-sense, minus-sense, tailed/spacer and non-tailed/non-spacer-probes, respectively. The results for each triplet are presented in the same order, P(T), M(T), and M(NT), with M(T) probe showing the strongest signal in each set. The insert (C) shows the hybridization signal comparison between the AsAV-specific short probes with and without spacers. Only five of the ten with vs. without spacer probe comparisons are shown. Error bars represent the standard deviations for analyzed probe replicates.

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

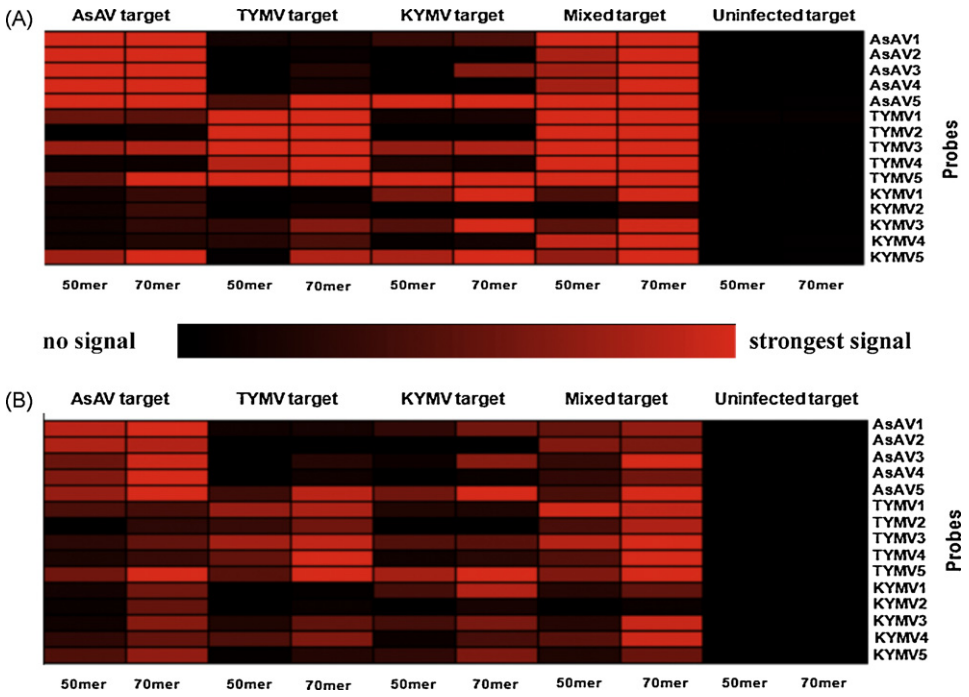

Fig. 6. Hybridization results of AsAV-, TYMV- and KYMV-infected samples as single infections or mixture. Uninfected *A. viridis* sample was a negative control target. The figure shows a composite overview of signal patterns in the form of a heat map for five individual hybridization reactions performed 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 positives. These two probe pairs were not considered in further analyses. The average ratio of mean median intensities for 70 to 50-mer probes was about 1.1 for 13/15 of the probe pairs, indicating the ability of 50-mer probes to produce as strong signals as 70-mer probes under optimal hybridization conditions.

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 an 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 immobilized 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 produced 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 oligothymidylate spacers is that the spacer itself decreases the possibility that a thymidine internal to the virus sequence will be used for attachment.

The observation that targets hybridized preferentially to probes of one polarity was highly reproducible. Investigations elsewhere (David Wang, personal communication) have indicated similar observations with double stranded fluorescent 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 remains to be elucidated.

Target length is also an important parameter in hybridization studies (Liu et al., 2007; Peplies et al., 2003; Peytavi et al., 2005; Southern et al., 1999). Shorter fragments of around 100 bp target length produced stronger hybridization signals on the array than longer targets for both TVCV and CaMV species. The observations above suggest that stronger signals could be due to secondary structure formation in the longer target strands making the target regions inaccessible to probes. The present results were in agreement with a recent study using *E. coli* 16S rRNA 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 establishment 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 hybridization 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 aminoallyl moieties. 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 sensitivity 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 polymerization. Combining sequence-independent target amplification and in vitro transcription with indirect labeling ensures a highly efficient 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 discrimination 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 taxonomic 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 conditions. 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 signal intensities at higher hybridization temperatures (50 and 63 °C) for both 50- and 70-mer probes. One explanation for this discrepancy 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 attractive 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 undertaken. 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 interpretation of results simple and reliable. Second, all three individual species used in this report were readily detected by hybridizations to the appropriate oligonucleotides without any non-specific hybridization to unrelated viral probes. Virus-specific hybridizations 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, signature sequences of all three viral species were detected readily in the mixed viral target validating the feasibility of our microarray for simultaneous detection of multiple viruses in a single plant sample.

Although most of the oligonucleotide probes performed as predicted, 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 microarray method with the potential to detect a broad group of plant viruses. Such a hybridization approach can facilitate the development of a powerful multi-viral detection system of considerably expansive application for identification of both known and related uncharacterized emerging viruses.

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Appendix A. Supplementary data

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

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