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A diagnostic oligonucleotide microarray for simultaneous detection of grapevine viruses

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At least 58 viruses have been reported to infect grapevines causing economic damage globally. Conventional detection strategies based on serological assays, biological indexing and RT-PCR targeting one or few viruses in each assay are widely used. Grapevines are prone to contain mixed infections of several viruses, making the use of these techniques time-consuming. A 70-mer oligonucleotide microarray able to detect simultaneously a broad spectrum of known viruses as well as new viruses by cross-hybridization to highly conserved probes is reported in the present study. The array contains 570 unique probes designed against highly conserved and species-specific regions of 44 plant viral genomes. In addition probes designed against plant housekeeping genes are also included. By using a random primed RT-PCR amplification strategy of grapevine double stranded RNA-enriched samples, viral agents were detected in single and mixed infections. The microarray accuracy to detect 10 grapevine viruses was compared with RT-PCR yielding consistent results. For this purpose, grapevine samples containing single or mixed infections of Grapevine leafroll-associated virus-1, -2, -3, -4, -7, -9, Grapevine fanleaf virus, Grapevine rupestris stem pitting-associated virus, Grapevine virus A, and Grapevine virus B were used. Genomic libraries containing complete viral genomes were also used as part of the validation process. The specific probe hybridization pattern obtained from each virus makes this approach a powerful tool for high throughput plant certification purposes and also for virus discovery if the new viral genomic sequences have partial similarity with the microarray probes. Three Closteroviridae members (Grapevine leafroll-associated virus -4, -7 and -9) were detected for the first time in Chilean grapevines using the microarray.

1. Introduction

Plant pathogens cause significant damage to crops with at least 10% of global food production being lost due to diseases (Strange and Scott, 2005). Efficient and early detection of grapevine viral pathogens is critical to diminish losses by dissemination of infected material, the main cause of pathogen spread in grape producing countries. Viruses affect negatively plant vigor and longevity, as well as the quality and quantity of the yield. Grapevine is one of the oldest horticultural crops widely grown in temperate climates and represents a highly valuable agricultural commodity. There are more than 70 infectious agents including viruses, viroids and phytoplasmas that have been reported in grapevines. Among them, at least 58 are viral pathogens, some with extremely high incidences (Martelli and Boudon-Padieu, 2006).

Enzyme-linked immuno sorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) are the most common and widely used techniques for routine screening of pathogens. Nevertheless they have limitations such as the restricted number of viruses detectable in a single assay. Furthermore the user needs to have a preconceived idea of what virus to screen for, limiting the possibilities to detect new or divergent species. Infected grapevine samples are complex biological matrices which may contain several pathogens, making these techniques time-consuming and labor intensive. Serological based detection systems have to rely on the quality of the antisera available to detect different strains that in addition, are constantly evolving. Furthermore, some commercial antisera are strain-specific, limiting the detection to certain geographical regions.

PCR is a highly sensitive technique that revolutionized molecular biology and diagnostic methods. Multiplex primers can be

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included to detect more than one virus, however the design of compatible sets of primers and the difficulty in detecting correctly related viral sequences present in the same sample, make the number of detectable viruses in a single assay relatively low. In addition, the exact identity of the virus must be generally determined in other steps like RFLP or sequencing (Elnifro et al., 2000). Real-time RT-PCR presents some advantages over conventional RT-PCR and has been used for plant virus diagnostic methods in the last years (Osman et al., 2007).

Recently, several studies have reported to detect successfully human, environmental, fungal and plant pathogens using DNA microarrays. The explosive growth of pathogen genomic sequences available in public databases, makes this approach reliable for thousands of different viral species. The technique is based on the hybridization of labeled samples with thousands of unique immobilized probes. Important novel emerging infectious diseases like SARS coronavirus (Wang et al., 2003a), respiratory metapneumovirus (Chiu et al., 2007) and a beluga whale coronavirus (Mihindukulasuriya et al., 2008), among others, have been identified with this technique. Other examples include the detection of avian bornaviruses from cases of proventricular dilatation disease (Kistler et al., 2008), viruses from post-mortem brain tissue (Conejero-Goldberg et al., 2005), influenza viruses (Dawson et al., 2007), fungal pathogens (Leinberger et al., 2005) and environmental pathogens in addition to its use in bio-defense applications (Sergeev et al., 2004).

There are few publications of plant virus detection using microarrays or macroarrays. Detection of different isolates of Cucumber mosaic virus (CMV) or Plum pox virus (PPV), four different cucurbit-infecting tobamoviruses and up to 11 viruses infecting cucumber or potato have been reported (Agindotan and Perry, 2008; Boonham et al., 2003; Bystricka et al., 2005; Deyong et al., 2005; Lee et al., 2003; Pasquini et al., 2008). Recently, the use of low-density arrays, a technique based on real-time RT-PCR (TaqMan) and antibody microarrays were reported for multiplex detection of up to 13 grapevine viruses (Abdullahi and Rott, 2009; Osman et al., 2008).

In the present report, a microarray system containing 570 unique viral oligonucleotides designed against 44 different grapevine viruses is described. Validation of the microarray was performed by the detection of 10 grapevine viruses in viral genomic libraries and grapevine samples with single and multiple infections.

2. Materials and methods

2.1. Viral source and nucleic acid isolation

Vitis vinifera plants naturally infected with GLRaV-1 (Grapevine leafroll associated virus-1), GLRaV-2 (Grapevine leafroll associated virus-2), GLRaV-3 (Grapevine leafroll associated virus-3), GLRaV-4 (Grapevine leafroll associated virus-4), GLRaV-7 (Grapevine leafroll associated virus-7), GLRaV-9 (Grapevine leafroll associated virus-9), GVA (Grapevine virus A), GVB (Grapevine virus B), GFLV (Grapevine fanleaf virus) and (Grapevine rupestris stem pitting-associated virus) as single or mixed infections were used. Samples were collected from different geographical regions of Chile and viral infections were confirmed by ELISA and RT-PCR. Total RNA was extracted from 0.1 g of fresh bark scrapings and grinded in liquid nitrogen (Chang et al., 1993). dsRNA was extracted from 1 to 2 g of fresh bark scrapings according to Valverde et al. (1990).

2.2. RT-PCR

Grapevine total RNA or dsRNA was heated at 75 °C or 95 °C respectively for 5 min in the presence of 50 ng of random hexamers (Invitrogen Carlsbad, CA, USA). The RT reaction was for 10 min at 25 °C and 50 min at 37 °C in a total volume of 25 μl with 200 U of SuperScript II (Invitrogen), 40 U of RNaseOUT (Invitrogen) and 1.6 mM dNTP mix. three μl of the product was used for the PCR in a total volume of 50 μl with 0.8 mM dNTP mix, 1.5 mM $MgCl_2$, 0.5 μM specific viral primers and 5 U of *Taq* DNA polymerase (Promega Corporation, Madison, WI, USA). Selected PCR products were purified and cloned for sequencing purposes in the pGEM-T easy vector (Promega).

2.3. Microarray oligonucleotide design and printing

70-mer oligonucleotides derived from 44 fully or partially sequenced plant virus genomes taken from GenBank (as of January, 2005) were designed. Genome sequences of local virus isolates were also considered for probe design. The probes were chosen against both, highly conserved regions within each viral family (after BLASTN (Altschul et al., 1997) alignment of all the viral sequences available for a given family) as well as specific genomic regions from each virus. The probes were synthesized (Illumina Inc, San Diego, CA, USA) in 384 well plates (Genetix Limited, New Milton, Hampshire, UK). The viral species considered belong to seven families (Bromoviridae, Bunyaviridae, Closteroviridae, Comoviridae, Flexiviridae, Tombusviridae and Tymoviridae), plus three viral species (Strawberry latent ringspot virus, Tobacco mild green mosaic virus and Tobacco mosaic virus) from unassigned families. The virus name for each probe refers to the reference genome used to design that probe and does not imply that the hybridization pattern for that probe will always be specific for the referred virus. Additionally, oligonucleotides targeting grapevine genes including polyubiquitin, ribulose-1, 5-bisphosphate carboxylase oxygenase, 23S, actin, β-tubulin, polygalacturonase, calmodulin and malate dehydrogenase were also included in the microarray.

A total of 570 oligonucleotides were suspended at 50 pmol/μl in 3× SSC together with 1 pmol/μl of Spike70R oligonucleotide and then printed in duplicates over poly-L-lysine (Sigma, St. Louis, MO, USA) pretreated microscope slides (Gold Seal, Portsmouth, NH, USA) using a DeRisi Linear Servo Motor Microarrayer as detailed elsewhere (Wang et al., 2002). Probes were designed from both, sense and antisense strands of viral genomes. From the 570 total probes printed, 334 were designed in this work and 236 were designed previously Wang et al. (2003a). The main goal of Wang et al. (2003a) was viral discovery, and therefore highly conserved probes were designed for each viral family. The additional 334 probes designed in this study included species-specific probes to determine the genus and species of the viral agent(s) as well as probes to detect viral species sequenced after 2003. Probe candidates were filtered to discard self-binding, highly redundant or plant sequence cross-reacting oligonucleotides. A list with the sequences of the probes printed used for this study is available as Supplementary Material (Table S1).

2.4. Sample amplification and labeling

dsRNA obtained from infected grapevines was denatured for 5 min at 95 °C and the RT reaction was carried out in a volume of 25 μl containing 20 pmol of primer EErnd (5′-GTAAGGTGCACGTAGTTGNNNNNNNNN-3′), 200 U of SuperScript II (Invitrogen), 40 U of RNaseOUT, 2.4 mM dNTP mix and 5 μl of 5× RT buffer. The reaction profile was 10 min at 25 °C, 60 min at 42 °C and 30 min at 50 °C. This was followed by 40 cycles of PCR amplification using primer EEadp (5′-GTAAGGTGCACGTAGTTG-′3) and the profile: 30 s at 94 °C, 30 s at 40 °C, 30 s at 50 °C, 60 s at 72 °C. Additional 20 PCR cycles with primer EEadp were used to incorporate aminoallyl-dUTP (Fermentas, Ontario, Canada) to the samples. Purified products were labeled with Cy3 mono NHS ester (GE

Healthcare, Buckinghamshire, UK) according to the manufacturer instructions. Simultaneously, 0.5 pmol of Spike70F oligonucleotide was labeled with Cy5 mono NHS ester, and mixed with the sample previous to hybridization. Alternatively viral genomic libraries in pGEM-T Easy (Promega) vector were labeled with Cy3 after random priming with DNA Pol I (New England Biolabs, Ipswich, MA, USA) to incorporate aminoallyl-dUTP to the samples.

2.5. Microarray hybridization, scanning and data analysis

Oligonucleotide microarrays were hybridized for 12 h at 65 °C and washed as detailed elsewhere (Bowtell and Sambrook, 2003). Arrays were imaged with a Perkin Elmer ScanArray Gx instrument containing a 532 nm laser for Cy3 and a 635 nm laser for Cy5 and analyzed using ScanArray Express version 3 (Perkin Elmer, Waltham, MA, USA) or GenePix version 3 (Molecular Devices, Sunnyvale, CA, USA) software. Normalized background-subtracted Cy3 pixel intensity was analyzed by hierarchical cluster using centroid linkage method with Cluster software version 3.0 (Eisen et al., 1998). Clustogram visualization was done with Java TreeView software version 1.1 (Saldanha, 2004) to plot selected microarray probes as horizontal stripes showing the Cy3 intensity as a red linear scale. Black stripes corresponded to probes with Cy3 intensity below the threshold. A spot was considered positive only if the Cy3 intensity of duplicate probes printed was above the threshold. The number of positive spots required to assume the presence a given virus was determined arbitrarily by clustogram examination after iteration of several hybridization events for each viral species. All microarray viral-positive samples were further confirmed by another technique such as RT-PCR and sequencing or ELISA.

3. Results

3.1. 70-mer oligonucleotide design

A combined approach was used in the 70-mer oligonucleotides design. First, the most highly conserved sequences within each viral family were chosen to maximize the spectrum of detectable viruses including new viruses that may cross-react with conserved probes if the viral family of the novel member is represented. This was done after BLASTN alignment of all the viral species of a given family using total or partial genomic sequences available as described previously (Wang et al., 2002). The second approach was aimed at designing more species-specific sequences not necessarily conserved at the family level. The goal was to discriminate the viral agent at the genus or species level when possible, depending on the amount of sequences available for each virus. In this case the BLASTN alignment considered all the sequences available for each viral species to identify conserved regions among different isolates of the same virus. The feasibility of using long unmodified oligonucleotides as detection probes has been widely demonstrated (Bozdech et al., 2003; Chou et al., 2004; Hughes et al., 2001). Initial validation of the probes performance was done by hybridizing GLRaV-3 or GFLV Cy3 labeled genomic libraries and uninfected samples as described below.

3.2. Random amplification of dsRNA obtained from grapevine tissue

It has been reported widely that viruses tend to be distributed unevenly in plant tissues and that some viruses exist in very low concentration in the host, especially in the summer season (Dovas and Katis, 2003; Lenz et al., 2008). Also, as might be expected, when total RNA are extracted from infected grapevines the ratio between host and viral RNA is large, making it very difficult to detect some viruses by hybridization methods since the background of host

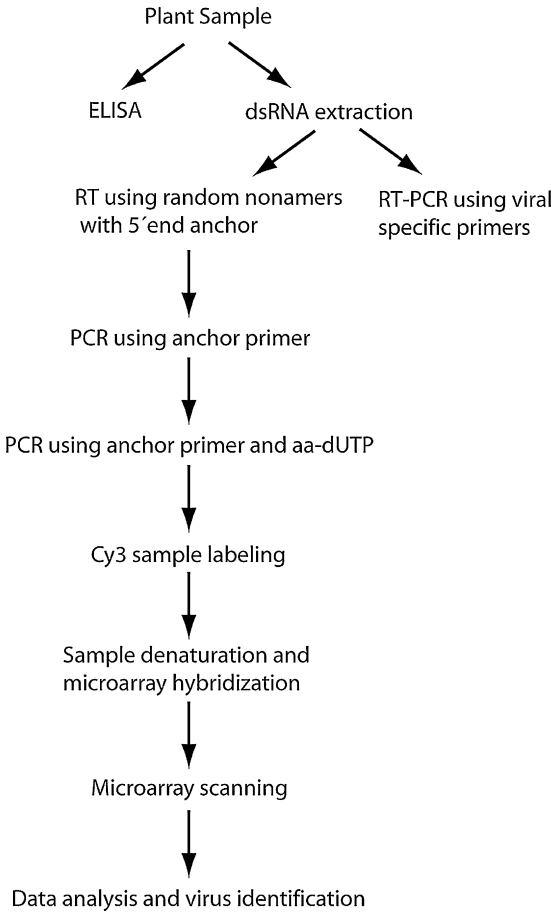

Fig. 1. Schematic representation of the strategy used in the microarray detection assay of grapevine viruses. dsRNA obtained from infected grapevines is reverse transcribed with a random 5′end anchor primer and randomly amplified using an anchor primer. Later, aminoallyl-dUTP is incorporated in an additional PCR with the anchor primer followed by Cy3 labeling. Microarrays are hybridized for 12 h at 65 °C, washed and imaged prior to viral identification by visual inspection of clustered data. In parallel and as part of the validation process, samples are analyzed by alternative techniques such as pathogen-specific RT-PCR and ELISA to determine the viral species present.

RNA lowers the sensitivity and specificity of the detection procedure. Indeed, after assaying several extraction protocols for total grapevine RNA, followed by random primed PCR or direct labeling during RT, no reproducible results were achieved (data not shown). Alternatively, the use of dsRNA as template for microarray detection has the advantage of using samples highly enriched in viral RNA (Valverde et al., 1990). To maximize the sensitivity, dsRNA samples were amplified in a relatively unbiased fashion by using a random primed PCR protocol (Bohlander et al., 1992; Wang et al., 2002). This approach increased the probability to detect all viruses present in a sample, overcoming problems related to low viral titers or to the availability of limited amounts of plant tissue (Fig. 1).

3.3. Testing of the microarray by hybridization to genomic libraries of GLRaV-3 and GFLV

To determine the specificity of the microarray to detect grapevine viruses, initial validation was accomplished by using a mix of plasmids containing DNA fragments that spanned over the whole genome of local viral isolates Cl-766 of GLRaV-3 (Engel et al., 2008b) and Ch-80 of GFLV (Engel et al., 2006). These libraries were labeled with Cy3 and hybridized to the microarray. The GLRaV-3-labeled library (sample L2 in Fig. 2A) coupled specifically to most of the spots designed against GLRaV-3 present in the array.

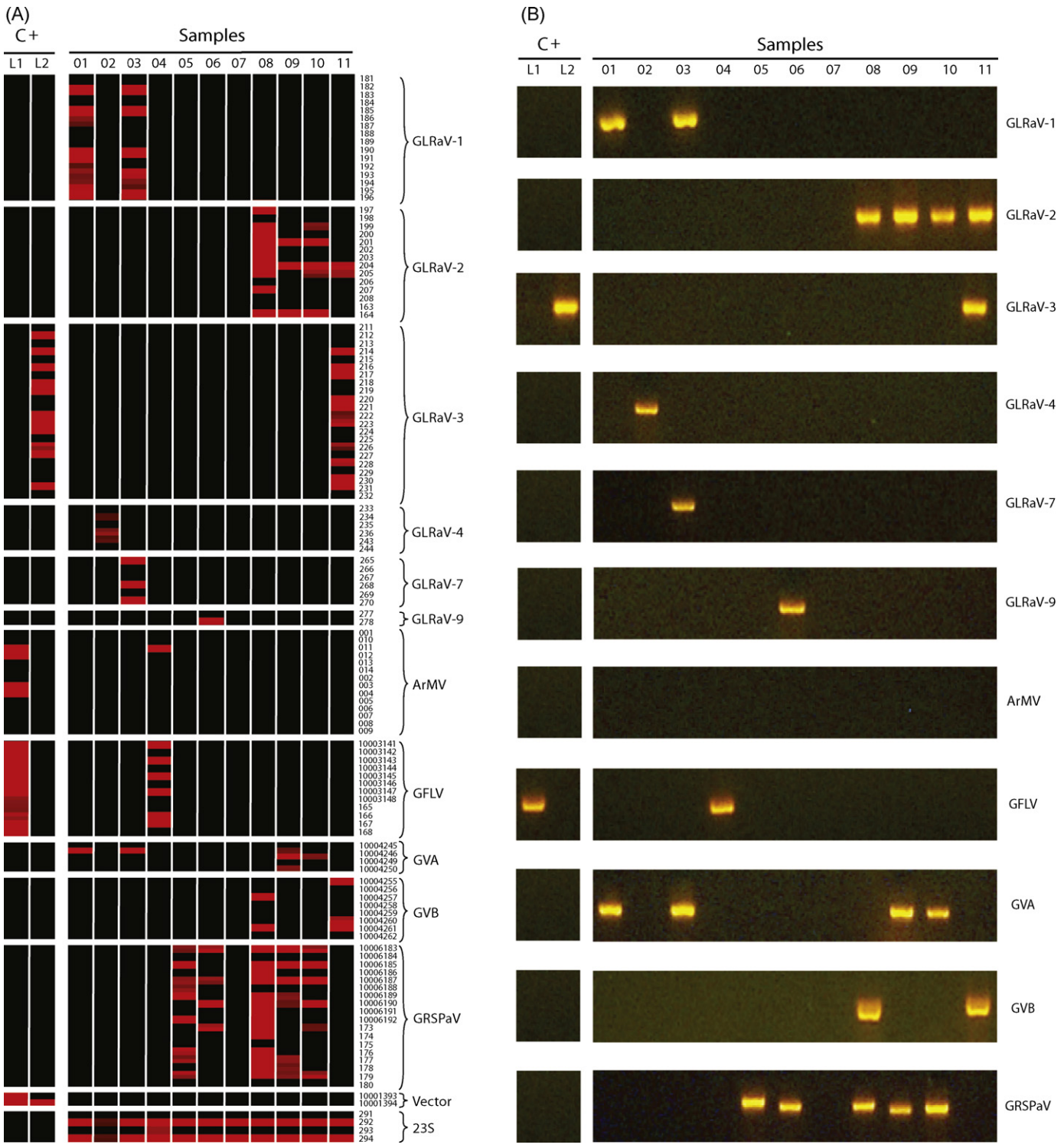

Fig. 2. Multiple virus detection in single and mixed infected grapevines using the oligonucleotide microarray and RT-PCR. (A) Hierarchical clustogram screening of 10 grapevine viruses (*y*-axis) in 13 samples (*x*-axis). Selected oligonucleotide probes considered in the clustogram correspond to horizontal stripes grouped by viral species. The Cy3 hybridization intensity for each spot was plotted as a red linear scale when intensity was above threshold and as a black stripe when Cy3 intensity was below the threshold. "Vector" probes indicate the presence of plasmid in the sample and "23S"correspond to probes hybridizing to the grapevine 23S ribosomal gene. The 13 samples correspond to infected grapevines (samples 1–6, 8–11), uninfected grapevine (sample 7) and GFLV and GLRaV-3 genomic libraries (samples L1 and L2). (B) Virus specific RT-PCR analysis in agarose gels for each of the grapevine samples detailed above(For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

Similar results were obtained when a GFLV library (sample L1 in Fig. 2A) was utilized. In this case all the GFLV spots printed bound to the GFLV library while 4 additional spots against ArMV (Arabis mosaic virus) hybridized also with this library. Samples L1 and 04 (Fig. 2B) were not infected with ArMV according to PCR analysis. ArMV the Nepovirus related most closely to GFLV, gave the expected genus cross-hybridization pattern since GFLV and ArMV share approximately 70% of the genome nucleotide identity. These

Table 1
Oligonucleotides used for the detection of GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-7, GLRaV-9, ArMV, GVA, GVB, GFLV and GRSPaV by RT-PCR.

Virus	Primer name	Primer sequence	Fragment size (bp)	References
GLRaV-1	LR1CPF1 LR1CPR1	CTAGCGTTATATCTCAAAATGA CCCATCACTTCAGCACATAAA	502	This study
GLRaV-2	LR2 12474U LR2 12806D	TTGACAGCAGCCGATTAAGCG CTGACATTATTGGTGCGACGG	333	(Nakaune and Nakano, 2006)
GLRaV-2	LR2 V2dCPf2 LR2 V2CPr1	ACGGTGTGCTATAGTGCGTG GCAGCTAAGTACGAATCTTC	497	(Bertazzon and Angelini, 2004)
GLRaV-3	LR3 LC1F LR3 LC2R	CGCTAGGGCTGTGGAAGTATT GTTGTCCCGGGTACCAGATAT	546	(Turturo et al., 2005)
GLRaV-4	LR4 HSPV-F LR4 HSPC-R	ACATTCTCCACCTTGTGCTTTT CATACAAGCGAGTGCAATTACA	321	(Osman et al., 2007)
GLRaV-7	LR7 F LR7 R	TATATCCCAACGGAGATGGC ATGTTCCTCCACCAAAATCG	502	(Engel et al., 2008a)
GLRaV-9	LR9 F LR9 R	CGGCATAAGAAAAGATGGCAC TCATTCACCACTGCTTGAAC	393	(Alkowni et al., 2004)
ArMV	ArMVfor ArMVrev	TGACAACATGGTATGAAGCACA TATAGGGCCTTTCATCACGAAT	402	(Gambino and Gribaudo, 2006)
GFLV	FL C3310 FL H2999	GATGGTAACGCTCCCGCTGCTCTT TCGGGTGAGACTGCGCAACTTCCTA	312	(MacKenzie et al., 1997)
GVA	GVA 6540U GVA 6880D	TTTGGGTACATCGCGTTGGT TCTAAGCCCGACGCGAAGT	341	(Nakaune and Nakano, 2006)
GVA	GVA H587 GVA C995	GACAAATGGCACACTACG AAGCCTGACCTAGTCATCTTGG	429	(Minafra and Hadidi, 1994)
GVB	GVB C410 GVB H28	GTGCTAAGAACGTCTTCACAGC ATCAGCAAACACGCTTGAACCG	460	(Minafra and Hadidi, 1994)
GRSPaV	RSP UP1 RSP DO2	TGAGATGGTYGCTAATATCG CTATTAGTACGGTATTCCAG	242	(Nakaune and Nakano, 2006)

results indicate that at the genus level, the libraries contained viral sequences belonging to the Ampelovirus and Nepovirus genera, while at the species level, the results demonstrate the presence of GFLV and GLRaV-3, thus confirming the suitability of the probe design and selectivity of the hybridization protocol. Notably, the observed cross-hybridization suggests that unreported members of the Nepovirus family may be detected also in plant samples. Two additional probes included in the microarray were used to confirm the presence of plasmid vectors in samples L1 and L2 (Fig. 2A).

3.4. Microarray hybridization and detection of multiple viruses in grapevine samples with single and mixed infections

In order to determine the performance of the microarray on virus-infected plants, 10 field-collected samples were analyzed by the microarray (Fig. 2A and Fig. S1) and by RT-PCR with viral primers (Fig. 2B and Table 1). Results obtained with grapevine samples with single or mixed infection showed consistency between the microarray and the PCR analyses. The microarray was especially useful for samples with viral co-infections, where standard mono-detection techniques are time-consuming. A total of 11 samples (one uninfected and 10 naturally infected) were analyzed and the results are shown in Fig. 2. The hierarchical clustering analysis of the microarray results provided a simple alternative to determine the viruses present in a sample through a fast visual examination. The procedure detected the presence of 10 grapevine viruses belonging to three different families (Closteroviridae, Comoviridae and Flexiviridae). Two of the samples (samples 9 and 10) showed mixed infection of GLRaV-2, GRSPaV and GVA, sample 1 showed mixed infection of GLRaV-1 and GVA and sample 3 was infected with GLRaV-1, GLRaV-7 and GVA. Sample 5 presented a single infection of GRSPaV and sample 6 had a multiple infection of GLRaV-9 and GRSPaV. Sample 11 had infections with GLRaV-2, GLRaV-3 and GVB and sample 8 had infections with GLRaV-2, GRSPaV and GVB. Samples 4 and 2 showed single infections with GFLV and GLRaV-4 respectively. Sample 7 corresponds to an uninfected grapevine control which hybridized only to grapevine 23S rRNA probes. Notably, 50% of the infected grapevines exhibited multiple infections of up to 3 viruses. It is important to mention that 3 of the 10 viruses detected (GLRaV-4, GLRaV-7 and GLRaV-9), had been previously reported for the first time in Chile as a result of microarray testing (Engel et al., 2008a; Escobar et al., 2008). The total number of probes that reacted with a specific virus from different samples showed variations that may be explained by sequence and viral load differences between samples. However, in all cases analyzed, the hybridization signatures identify clearly the viral species present either in single or mixed infected samples. Microarray probes that hybridized with grapevine 23S genes are shown in the clustogram as controls (Fig. 2A).

3.5. RT-PCR and sequence analysis of microarray positive samples

In order to validate the microarray results and evaluate its performance, a parallel analysis of 10 grapevine viruses was done by RT-PCR using viral specific sequences (Table 1 and Fig. 2B). Samples showing infection with GLRaV-1 (**GQ415404**), GLRaV-2 (**GQ415405**), GLRaV-3 (**EU344896**), GLRaV-4 (**EU746618**), GLRaV-7 (**EU334662**), GLRaV-9 (**EU334663**), GVA (**GQ415406**), GVB (**GQ415407**), GFLV (**GQ415403**) and GRSPaV (**GQ415408**) were cloned and sequenced to confirm viral presence. Considering a total of 13 samples and 10 viruses, the results obtained by microarray (Fig. 2A and S1) and RT-PCR (Fig. 2B) techniques were in concordance with 100% of agreement.

4. Discussion

Approximately 1200 viral species have been described to infect plants (Boonham et al., 2007; Fauquet and Fargette, 2005) and

about 60 have been reported solely to infect grapevines (Martelli and Boudon-Padieu, 2006). Simultaneous multi detection systems like the one presented here are highly desirable, especially to analyze plant diseases caused by multiple viruses. In fact, grapevine plants are prone to contain several co-infecting viruses, demonstrating the value of having a simultaneous detection system. The microarray developed in this study contains probes designed against species-specific regions, to discriminate between closely related genus members, and against highly conserved regions at the family level, to enable detection of highly divergent viruses or even previously unidentified viruses. The possibility to detect unknown viruses has been validated widely with this approach, especially for novel human or animal viruses (Chiu et al., 2007; Mihindukulasuriya et al., 2008; Wang et al., 2003a) and it has an advantage over conventional detection techniques where the user needs to have a preconceived idea of what to screen for. Thus, in this work a total of 570 unique probes were designed and printed in duplicates to target grapevine viruses (Table S1 and Fig. S1). 70-mer oligonucleotides combine specificity and versatility since they are able to bind divergent viral templates without the need to have a perfect sequence match, while at the same time they discriminate between related viral species (Bozdech et al., 2003; Chou et al., 2004; Wang et al., 2003b).

One key step that needed to be validated was the correct probe design, particularly because unlike fungal or bacterial pathogens, viral genomes do not have universal sequence domains such as ribosomal genes, which could serve as probe targets. The initial validation process consisted in the hybridization of whole genome viral libraries against the microarray to demonstrate the specificity of the printed probes. This was confirmed by using Cy3 labeled GLRaV-3 and GFLV libraries that gave the expected strong Cy3 signal intensities with the respective viral species probes and with some probes of closely related virus such as ArMV in the case of GFLV (Fig. 2A samples 4 and L1). The cross-hybridization demonstrates the usefulness of having highly conserved viral sequences in the microarray as a means to detect new viral members not represented explicitly in the array.

A second step in the validation process was to demonstrate that nucleic acid from uninfected grapevine samples would not cross-hybridize with viral probes. As can be seen in Fig. 2A, uninfected sample 7 gave the expected result hybridizing only with grapevine 23S rRNA control probes.

It has been reported widely that microarrays may not be sensitive enough to detect target sequences present at low concentration without amplification steps (Boonham et al., 2007; Lenz et al., 2008). To overcome sensitivity issues due to the fact that plant viruses are distributed randomly with low titers in plant tissues, samples were amplified using a random primed PCR protocol prior to the hybridization step. In addition, to maximize the virus/host RNA ratio, dsRNA enriched samples were used as templates instead of total RNA. Previous results indicate that detection of plant viruses from grapevine total RNA or dsRNA without an amplification process raises the possibility of obtaining false negative results and diminishes the efficiency and sensitivity of the detection method (data not shown). In conclusion, randomly amplified dsRNA-enriched samples overcome these limitations by lowering the host RNA background and increasing the amount of viral DNA available for hybridization, hence maximizing the chances of detecting viruses independently of the viral titer present in the sample. This is particularly important during the summer season when the titer of some grapevine viruses is lower. Notably, this approach allows processing of small amounts of plant tissue in order to obtain reproducible results since less than 1 g of plant tissue was enough to proceed with the RT-PCR and hybridization steps.

Consistent results were obtained when data of grapevine samples analyzed with RT-PCR were compared with those obtained with the microarray. The efficacy of the microarray with grapevine samples collected in different seasons and from different geographical regions of Chile was tested. Importantly, 3 of the 10 viruses reported (GLRaV-4, GLRaV-7, GLRaV-9) were previously detected for the first time in Chile due to the microarray hybridization signatures obtained, confirming their presence in local samples (Engel et al., 2008a; Escobar et al., 2008). It is important to mention that occasional cross-hybridization was obtained between some GLRaV-4 and GLRaV-9 probes, suggesting that these viruses could be different strains of the same species rather than two different members. The taxonomic status of GLRaV-4, GLRaV-5, GLRaV-6 and GLRaV-9 is currently under study and might suffer modifications in the future (Martelli, 2009).

As more viral sequences become available, additional probes can be designed to span over more viral genes, raising the possibility of detecting a virus as well as all the divergent isolates or quasispecies. Having probes available against different genomic regions is another advantage over PCR methodology, where primers often target only one specific region of the viral genomes. Since microarrays are very easy to upgrade, a new version with additional probes against recently completed or newly described viral genomes is currently being developed in our laboratory. The microarray based format is flexible, robust, easy to upgrade, and has the potential to increase detection throughput while reducing simultaneously the cost per unit (Martin et al., 2000).

To our knowledge, this is the first report of an oligonucleotide microarray able to detect simultaneously all the known (and possibly new) grapevine viruses. Since glass planar microarrays can easily fit 30,000 probes, it is realistic to think of a large generic plant virus microarray. In this case, more than 20 different probes could be printed against each of the 1200 plant viruses currently described. Considering that Chile is among the main fruit exporters worldwide, a generic plant virus microarray for high throughput detection and certification purposes is highly desirable.

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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.2009.11.009.

References

Abdullahi, I., Rott, M., 2009. Microarray immunoassay for the detection of grapevine and tree fruit viruses. J. Virol. Methods 160, 90–100.
Agindotan, B., Perry, K.L., 2008. Macroarray detection of eleven potato-infecting viruses and potato spindle tuber viroid. Plant Dis. 92, 730–740.
Alkowni, R., Rowhani, A., Daubert, S., Golino, D., 2004. Partial characterization of a new ampelovirus associated with grapevine leafroll disease. J. Plant Pathol. 86, 123–133.
Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Bertazzon, N., Angelini, E., 2004. Advances in the detection of grapevine leafroll-associated virus 2 variants. J. Plant Pathol. 86, 283–290.
Bohlander, S.K., Espinosa III, R., Le Beau, M.M., Rowley, J.D., Diaz, M.O., 1992. A method for the rapid sequence-independent amplification of microdissected chromosomal material. Genomics 13, 1322–1324.
Boonham, N., Tomlinson, J., Mumford, R., 2007. Microarrays for rapid identification of plant viruses. Annu. Rev. Phytopathol. 45, 307–328.
Boonham, N., Walsh, K., Smith, P., Madagan, 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.
Bowtell, D., Sambrook, J., 2003. DNA Microarrays: A Molecular Cloning Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Bozdech, Z., Zhu, J., Joachimiak, M.P., Cohen, F.E., Pulliam, B., DeRisi, J.L., 2003. Expression profiling of the schizont and trophozoite stages of *Plasmodium falciparum* with a long-oligonucleotide microarray. Genome Biol. 4, R9.
Bystricka, D., Lenz, O., Mraz, I., Piherova, L., Kmoch, S., Sip, M., 2005. Oligonucleotide-based microarray: a new improvement in microarray detection of plant viruses. J. Virol. Methods 128, 176–182.
Conejero-Goldberg, C., Wang, E., Yi, C., Goldberg, T.E., Jones-Brando, L., Marincola, F.M., Webster, M.J., Torrey, E.F., 2005. Infectious pathogen detection arrays: viral detection in cell lines and post-mortem brain tissue. Biotechniques 39, 741–751.
Chang, S., Puryear, J., Cairney, J., 1993. A simple and efficient method for isolating RNA from pines trees. Plant Mol. Biol. Rep. 11, 113–116.
Chiu, C.Y., Alizadeh, A.A., Rouskin, S., Merker, J.D., Yeh, E., Yagi, S., Schnurr, D., Patterson, B.K., Ganem, D., DeRisi, J.L., 2007. Diagnosis of a critical respiratory illness caused by human metapneumovirus by use of a pan-virus microarray. J. Clin. Microbiol. 45, 2340–2343.
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 Acids Res. 32, e99.
Dawson, E.D., Moore, C.L., Dankbar, D.M., Mehlmann, M., Townsend, M.B., Smagala, J.A., Smith, C.B., Cox, N.J., Kuchta, R.D., Rowlen, K.L., 2007. Identification of A/H5N1 influenza viruses using a single gene diagnostic microarray. Anal. Chem. 79, 378–384.
Deyong, Z., Willingmann, P., Heinze, C., Adam, G., Pfunder, 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.
Dovas, C.I., Katis, N.I., 2003. A spot multiplex nested RT-PCR for the simultaneous and generic detection of viruses involved in the aetiology of grapevine leafroll and rugose wood of grapevine. J. Virol. Methods 109, 217–226.
Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D., 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U.S.A. 95, 14863–14868.
Elnifro, E.M., Ashshi, A.M., Cooper, R.J., Klapper, P.E., 2000. Multiplex PCR: optimization and application in diagnostic virology. Clin. Microbiol. Rev. 13, 559–570.
Engel, E., Arredondo, V., Girardi, C., Gonzalez, A., Escobar, P., Fiore, N., Arce, P., Valenzuela, P.D.T., 2006. Grapevine viruses in chile: genomics and detection based on immunocapture and microarray technologies. In: 5th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG), pp. 118–119.
Engel, E.A., Escobar, P., Montt, C., Gomez-Talquenca, S., Valenzuela, P.D.T., 2008a. First Report on the Occurrence of *Grapevine leafroll-associated virus 7* and *9* in chilean grapevines. Plant Dis. 92, 1252.
Engel, E.A., Girardi, C., Escobar, P.F., Arredondo, V., Dominguez, C., Perez-Acle, T., Valenzuela, P.D., 2008b. Genome analysis and detection of a chilean isolate of *Grapevine leafroll associated virus-3*. Virus Genes 37, 110–118.
Escobar, P.F., Fiore, N., Valenzuela, P.D.T., Engel, E.A., 2008. First detection of *Grapevine leafroll-associated virus 4* in chilean grapevines. Plant Dis. 92, 1474.
Fauquet, C.M., Fargette, D., 2005. International committee on taxonomy of viruses and the 3,142 unassigned species. Virol. J. 2, 64.
Gambino, G., Gribaudo, I., 2006. Simultaneous detection of nine grapevine viruses by multiplex reverse transcription-polymerase chain reaction with coamplification of a plant RNA as internal control. Phytopathology 96, 1223–1229.
Hughes, T.R., Mao, M., Jones, A.R., Burchard, J., Marton, M.J., Shannon, K.W., Lefkowitz, S.M., Ziman, M., Schelter, J.M., Meyer, M.R., Kobayashi, S., Davis, C., Dai, H., He, Y.D., Stephaniants, S.B., Cavet, G., Walker, W.L., West, A., Coffey, E., Shoemaker, D.D., Stoughton, R., Blanchard, A.P., Friend, S.H., Linsley, P.S., 2001. Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. Nature 19, 342–347.
Kistler, A.L., Gancz, A., Clubb, S., Skewes-Cox, P., Fischer, K., Sorber, K., Chiu, C.Y., Lublin, A., Mechani, S., Farnoushi, Y., Greninger, A., Wen, C.C., Karlene, S.B., Ganem, D., DeRisi, J.L., 2008. Recovery of divergent avian bornaviruses from cases of proventricular dilatation disease: identification of a candidate etiologic agent. Virol. J. 5, 88.
Lee, G.P., Min, B.E., Kim, C.S., Choi, S.H., Harn, C.H., Kim, S.U., Ryu, K.H., 2003. Plant virus cDNA chip hybridization for detection and differentiation of four cucurbit-infecting Tobamoviruses. J. Virol. Methods 110, 19–24.
Leinberger, D.M., Schumacher, U., Autenrieth, I.B., Bachmann, T.T., 2005. Development of a DNA microarray for detection and identification of fungal pathogens involved in invasive mycoses. J. Clin. Microbiol. 43, 4943–4953.
Lenz, O., Petrzik, K., Spak, J., 2008. Investigating the sensitivity of a fluorescence-based microarray for the detection of fruit-tree viruses. J. Virol. Methods 148, 96–105.
MacKenzie, D.J., McLean, M.A., Mukerji, S., Green, M., 1997. Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. Plant Dis. 81, 222–226.
Martelli, G.P., 2009. Grapevine virology highlights 2006–2009. In: 15th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG), pp. 15–23.
Martelli, G.P., Boudon-Padieu, E., 2006. Directory of Infectious Diseases of Grapevines and Viruses and Virus-like Diseases of the Grapevine: Bibliographic Report 1998–2004. Options Méditerranéennes B55.
Martin, R., James, D., Levesque, C.A., 2000. Impacts of molecular diagnostic technologies on plant disease management. Annu. Rev. Phytopathol. 38, 207–239.
Mihindukulasuriya, K.A., Wu, G., St Leger, J., Nordhausen, R.W., Wang, D., 2008. Identification of a novel coronavirus from a beluga whale by using a panviral microarray. J. Virol. 82, 5084–5088.
Minafra, A., Hadidi, A., 1994. Sensitive detection of grapevine virus A, B, or leafroll-associated III from viruliferous mealybugs and infected tissue by cDNA amplification. J. Virol. Methods 47, 175–187.
Nakaune, R., Nakano, M., 2006. Efficient methods for sample processing and cDNA synthesis by RT-PCR for the detection of grapevine viruses and viroids. J. Virol. Methods 134, 244–249.
Osman, F., Leutenegger, C., Golino, D., Rowhani, A., 2007. Real-time RT-PCR (TaqMan) assays for the detection of grapevine leafroll associated viruses 1–5 and 9. J. Virol. Methods 141, 22–29.
Osman, F., Leutenegger, C., Golino, D., Rowhani, A., 2008. Comparison of low-density arrays, RT-PCR and real-time TaqMan RT-PCR in detection of grapevine viruses. J. Virol. Methods 149, 292–299.
Pasquini, G., Barba, M., Hadidi, A., Faggioli, F., Negri, R., Sobol, I., Tiberini, A., Caglayan, K., Mazyad, H., Anfoka, G., Ghanim, M., Zeidan, M., Czosnek, H., 2008. Oligonucleotide microarray-based detection and genotyping of Plum pox virus. J. Virol. Methods 147, 118–126.
Saldanha, A.J., 2004. Java Treeview—extensible visualization of microarray data. Bioinformatics 20, 3246–3248.
Sergeev, N., Distler, M., Courtney, S., Al-Khaldi, S.F., Volokhov, D., Chizhikov, V., Rasooly, A., 2004. Multipathogen oligonucleotide microarray for environmental and bio-defense applications. Biosens. Bioelectron. 20, 684–698.
Strange, R.N., Scott, P.R., 2005. Plant disease: a threat to global food security. Annu. Rev. Phytopathol. 43, 83–116.
Turturo, C., Saldarelli, P., Yafeng, D., Digiaro, M., Minafra, A., Savino, V., Martelli, G.P., 2005. Genetic variability and population structure of grapevine leafroll-associated virus 3 isolates. J. Gen. Virol. 86, 217–224.
Valverde, R.A., Nameth, S.T., Jordan, R.L., 1990. Analysis of double-stranded RNA for plant virus diagnosis. Plant Dis. 74, 255–258.
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., Urisman, A., Liu, Y.T., Springer, M., Ksiazek, T.G., Erdman, D.D., Mardis, E.R., Hickenbotham, M., Magrini, V., Eldred, J., Latreille, J.P., Wilson, R.K., Ganem, D., DeRisi, J.L., 2003a. Viral discovery and sequence recovery using DNA microarrays. PLoS Biol. 1, E2.
Wang, H.-Y., Malek, R., Kwitek, A., Greene, A., Luu, T., Behbahani, B., Frank, B., Quackenbush, J., Lee, N., 2003b. Assessing unmodified 70-mer oligonucleotide probe performance on glass-slide microarrays. Genome Biol. 4, R5.