Use of Fluorescent Microsphere-Based Assay for Detection of Three Cucurbit-Infecting Viruses

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Abstract

In this study, we describe multiplex polymerase chain reaction (PCR) coupled with the LiquiChip assay for the identification of *Zucchini yellow mosaic virus*, *Cucumber green mottle mosaic virus*, and *Cucumber mosaic virus* by coamplification with plant mRNA as an internal control. Multiplex reverse-transcription (RT)-PCR products were subjected to allele-specific primer extension, then hybridized to carboxylated

microspheres with unique fluorescent identifiers followed by detection using the LiquiChip 200 workstation. This assay is highly specific for distinguishing individual viruses from a mixed viral population and is 10 times more sensitive than multiplex RT-PCR. In addition, the establishment of this method enabled the detection of cucurbit viruses in field samples.

Melon (*Cucumis melo* L.) and watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai) are two high-value cucurbit crops that occupy cultivation areas of more than 5,000 and 12,000 ha, respectively, in Taiwan. Plant viruses are the major pathogens that limit the production of cucurbits. In December 2006, more than 500 ha of melon (*Cucumis melo* L.) cultivation area was seriously damaged by viral infections, resulting in the loss of U.S.\$8 million in Tainan County. In May 2010, an outbreak of serious viral diseases resulted in infection of 60 to 100% of melon crops in the Annan District of Tainan City (Dean 2009). Thus, more accurate tools are required for diagnosing cucurbit viruses in Taiwan. *Cucumber mosaic virus* (CMV; family *Bromoviridae*), *Cucumber green mottle mosaic virus* (CGMMV; family *Virgaviridae*), and *Zucchini yellow mosaic virus* (ZYMV; family *Potyviridae*) are all single-stranded, positive-sense RNA plant viruses. These three viruses are major disease-causing agents found on cucurbitaceous plants not only in Taiwan but also across all continents (Alonso-Prados et al. 1997). They have a great economic impact because they infect crops with high commercial values, including cucumber (*C. sativus* L.), watermelon (*Citrullus lanatus*), bottle gourds (*Lagenaria siceraria* (Mol.)), and zucchini squash (*Cucurbita pepo* L.), and the virus-infected crops often show severe symptoms and produce unmarketable fruit (Gallitelli 2000). According to FAO statistics, the total melon yield in the United States alone was estimated to be 986,066 tons in 2013 (<http://www.fao.org/faostat/en/#data/QC>), which demonstrates the impact and agricultural importance of cucurbit viruses.

The common symptoms of these three viruses include chlorotic lesions, stunting of plant growth, a yellowed mosaic patterning of leaves and fruit, and general fruit deformation. However, necrosis of melon fruit is a specific symptom resulting from ZYMV infection (Desbiez and Lecoq 1997). Though symptoms of CMV infection can vary greatly from host to host, infection of cucumber and other cucurbits with CMV generally causes downward bending of leaves, as well

as severely mottled leaves with alternating light and dark green patches (Gallitelli 2000).

CMV and ZYMV are both transmitted by aphids in a nonpersistent manner but the two viruses can also be mechanically transmitted in fields (Dodds et al. 1984). However, CGMMV is transmitted via direct contact with infected plant parts, contaminated soil, or pollen (Choi 2001; Hollings et al. 1975; Liu et al. 2014; Wu et al. 2011). Furthermore, all three cucurbit viruses are seedborne or seed-transmitted in some plant varieties (Dodds et al. 1984; Liu et al. 2009; Mandal et al. 2008; Nematollahi et al. 2014). Thus, early detection of these viruses is critical for controlling the spread of diseases throughout the world.

Current virus detection techniques include enzyme-linked immunosorbent assay (ELISA), immunoblotting, and variations of these serological techniques. Protein-based detection methods are routinely used for quick assessment of large sample loads; however, the detection limit of these methods is highly dependent on the quality of antibodies (Thottappilly et al. 1998). Spectroscopic methods such as transmission electron microscopy are unambiguous and highly accurate approaches for virus detection but are expensive and require experienced operators for sample preparation and observation. Advances in molecular biology have led to nucleic acid-based diagnostic techniques to become more popular (Boonham et al. 2014), particularly regarding the application of polymerase chain reaction (PCR). Specifically, developments in reverse-transcription (RT)-PCR, real-time PCR, immunocapture-PCR, and oligonucleotide array technology have enabled highly sensitive, relatively low-cost, and species-specific detection of DNA and RNA viruses. The LiquiChip assay, based on Luminex xMAP technology (multianalyte profiling beads), is useful in pathogen detection or even disease diagnosis (Dunbar et al. 2003; Liu et al. 2011; Munro et al. 2013; van Brunschot et al. 2014; Xu et al. 2017); when coupled with multiplex PCR, it can meet the need for the detection and quantitation of multiple viral targets (Dunbar 2006; Taylor et al. 2001). In this assay, PCR products are initially purified and subjected to target-specific primer extension (TSPE) or allele-specific primer extension (ASPE). In the newly synthesized strand, dCTP is substituted with biotinylated-dCTP and, subsequently, a short, unique nucleotide sequence is attached at the 5' end. Microspheres hybridize with the TSPE products with covalently linked nucleotide sequences, complementary to the 5' sequence on the TSPE product. Finally, streptavidin-R-phycoerythrin, a reporter dye, complexes with biotin on the TSPE product, and lasers are then used to identify the beads and quantitate the amount of associated reporter fluorescence.

In this study, we demonstrated the application of a multiplex-LiquiChip assay for the simultaneous amplification and identification of CMV, CGMMV, and ZYMV along with an internal control.

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The assay was evaluated by testing its sensitivity and its specificity to the individual target virus followed by comparison with a traditional PCR assay.

Materials and Methods

Virus isolates and cultivation. Isolates of CMV, CGMMV, and ZYMV used in this study were isolated from different field-collected and systemically-infected cucumber leaves (Hseu et al. 1987). All isolates were maintained on zucchini (*Cucurbita pepo* L. var. Zucchini) and squash (cultivar Pepo) plants in the greenhouse at the Taiwan Agricultural Research Institute. Plants showing yellow color or mosaic symptoms were tested for the presence of CMV, CGMMV, and ZYMV by RT-PCR. The primers shown in Table 1 were used to amplify a sequence of the ZYMV, CMV, or CGMMV RNA. The RT-PCR assay was carried out in 20- μ l reaction volumes that contained 2 μ l of Access Quick RT-PCR system (Promega Corp.), 0.5 μ l of each primer (10 μ M), 2 μ l of RNA, and distilled water. The amplification program was performed for 5 min at 94°C; followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min each; and final elongation at 72°C for 10 min. RT-PCR products were detected using 2% agarose gel electrophoresis followed by ethidium bromide staining and observation under UV light. An inoculum of each viral isolate was prepared by grinding infected zucchini leaf tissues in 10 mM potassium phosphate buffer (pH 7.0), and this was mechanically inoculated onto the leaves of host plants (zucchini or melon) using carborundum for subculturing under insect-proof greenhouse conditions.

Total RNA extraction. Total RNA was extracted from 0.1 g of fresh or 0.05 g of freeze-dried infected plant leaves using TriSolution Reagent Plus (GeneMark) as per the manufacturer's instructions. Aliquots of purified RNA were stored at -80°C.

Primer design for PCR and TSPE or ASPE. DNA sequences of the viral coat protein genes and mitochondrial cytochrome oxidase subunit I (COXI) genes of tomato plants were retrieved from the GenBank database. The nucleotide sequences of several ZYMV, CMV, and CGMMV isolates were aligned with the available sequences to identify conserved regions using the ALIGN program (<https://blast.ncbi.nlm.nih.gov>). Primers and probes for the target sequence (Supplementary Figs. S1, S2, and S3) were designed using PrimerPlex 2 (PRIMER Biosoft).

The Basic Local Alignment Search Tool (BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to predict the specificity of these primers to their viral target. TSPE primers were designed (Table 1) using PrimerPlex 2, and the design criteria were based on the guidelines for the LiquiChip 200 workstation (Qiagen, Hilden, Germany). The TSPE primers were synthesized with an amino-modified 5' end where a 12-nucleotide linker was attached. The linker had previously been determined to be functionally optimal

for microsphere-based hybridization (Cowan et al. 2004). The LiquiChip assay consists of three main steps that occur in the same micro-well. The general scheme of the entire molecular probing process is as follows (Fig. 1): amplification of viral cDNA by PCR, labeling of the PCR products with virus-specific tags and site-specific biotins by TSPE of tagged primers, capture of the tagged TSPE products by color-addressed microspheres via hybridization of each tag to its precise complementary oligonucleotide conjugated to the surfaces of the microspheres, binding of fluorescent streptavidin-phycoerythrin to the biotins of TSPE products, and reading of fluorescent signals on each microsphere using the LiquiChip 200 instrument (Qiagen, Valencia, CA, USA).

Multiplex RT-PCR. Two-step RT-PCR was used. cDNA was synthesized using the Moloney murine leukemia virus RT (Promega Corp.) in a 20- μ l reaction mixture containing 0.5 μ M each reverse primer, 0.5 μ M deoxynucleoside triphosphates (dNTP), 1 μ l of RT buffer, 0.01 M dithiothreitol, and 5 μ l of nucleic acid at 42°C for 60 min for subsequent multiplex PCR. The multiplex RT-PCR assay was carried out in 25- μ l reaction volumes that contained 2 μ l of dNTP mixture (10 mM dATP, dCTP, dGTP, and dTTP each), 2.5 μ l of 10 $\times$ PCR buffer, 0.25 μ l of Taq polymerase (5 U/ μ l) (Invitrogen), 0.5 μ l of each primer (10 μ M), 2 μ l of cDNA, and distilled water. The optimized concentrations of each primer pair were

Fig. 1. Schematic diagram of LiquiChip assay workflow. The LiquiChip assay consists of three steps: **A**, the target viral cDNA was amplified by polymerase chain reaction; **B**, polymerase extends the primer by incorporating biotin labeled dCTP; and **C**, the color-addressed microsphere coupled with anti-tag sequence hybridized to tag sequences on the target-specific primer extension product. Streptavidin binds to biotin and the fluorescent signal will be detected by the LiquiChip 200 workstation.

Table 1. Primers and MagPlex-TAG beads used in this study

Virus target ^a	Target region ^b	Sequence (5'–3') ^c	Position ^d	Amplicon size (bp)	Bead address ^e
ZYMV	F	AAAGACAAAGAGGATGACAAAGG	8,584–8,606	721	26
	R	CGCTAGTGGTGGCAACAT	9,287–9,304	...	...
	T	<u>TACATTCAACACTCTTAAAT</u> CAAAATTGTGCCGCTCTTTTCGAA	8,701–8,720	...	...
CMV	F	GATGCTAACTTTAGAGTCTGTGTC	1,342–1,364	548	33
	R	GGAATACGCTGGTGCTCAA	1,871–1,889	...	...
	T	<u>ACTACTTATTCTCAAACCTCTAAT</u> AGCAACAGCTTTCGCGACTTA	1,365–1,384	...	...
CGMMV	F	TTATTGCGTTTAGTGCTTCTTATG	5,794–5,817	285	36
	R	CAGTCGTAGGATTGCTAGGAT	6,058–6,078	...	...
	T	<u>ATTAAACAACCTCTTAAC</u> TACACAAGTAAAGTCTGACGGAAC	5,817–5,835	...	...
Cyoxid	F	CCCACCTCTACTAAGGCTGAG	148,358–148,378	124	42
	R	ATTGGTCTGTTCCGATTCTGA	148,461–148,481	...	...
	T	<u>CACTACACATTTATCATAACAAAT</u> AGTCTCTTGCTCCTATTAAGC	148,378–148,398	...	...

^a Zucchini yellow mosaic virus (ZYMV), Cucumber mosaic virus (CMV), Cucumber green mottle mosaic virus (CGMMV), and the internal control (Cyoxid).

^b F, R, and T indicate forward primer, reverse primer, and TAG allele-specific primer extension primer, respectively.

^c Underlined and bold indicates the MagPlex-TAG sequences provided by Luminex Corp.

^d Position of primers for ZYMV, CMV, CGMMV, and Cyoxid are based on accession numbers NC_003224, KC019301, NC_001801, and FJ374974, respectively.

^e The MagPlex-TAG bead product number assigned by Luminex Corp.

evaluated using mixed viral cDNA. The concentration ratios of specific primers were found to be 0.05 to 0.4 μM for ZYMV primers, 0.1 to 0.4 μM for CMV primers, 0.04 to 0.4 μM for CGMMV primers, and 0.04 to 0.4 μM for internal control primers. The amplification efficiency of viral genes was stable in spite of changing primer concentrations. The reaction procedure involved pretreatment at 95°C for 5 min followed by 3 min at 95°C; 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30; and one final extension step at 72°C for 7 min before arresting the reaction at 4°C. The product of multiplex RT-PCR was analyzed using agarose gel electrophoresis with UV illumination. The optimal conditions were determined and selected for further multiplex RT-PCR detection.

TSPE or ASPE. RT-PCR products were treated with 2 U of alkaline phosphatase (GE Healthcare) and 4 U of exonuclease I (GE Healthcare) in a final reaction volume of 10 μl of at 37°C for 30 min, then heat inactivated at 80°C for 20 min. TSPE reaction was carried out in 20 μl of reaction volume, which contained 5.0 μl of treated RT-PCR product, 1 $\times$ TSPE reaction buffer, 1.0 mM MgCl_2 , 0.20 μM each TAG-ASPE primer, 0.03 U of Tsp DNA polymerase, 100 μM each dNTP except for dCTP, and 4.0 μM biotin-14-dCTP (Invitrogen). Thermocycling conditions for the TSPE reactions were 96°C for 2 min followed by 30 cycles of 94°C for 30 s, 58°C for 1 min, and 74°C for 1 min.

Bead hybridization and analysis. The hybridization reaction consisted of one TSPE product and a mixture of each of the five Luminex xTAG beads sets (Table 1). The hybridization was performed in 96-well flat-bottom plates with a reaction volume of 50 μl , containing 25 μl of xTAG beads mixture (2 $\times$ Tm hybridization buffer: 0.4 M NaCl, 0.2 M Tris, 0.16% Triton X-100 [pH 8.0], and 100 beads of each bead set per microliter), 5 μl of TSPE reaction, and 20 μl of distilled water. The hybridization was conducted at 37°C for 30 min. A no-template control from the PCR assay was used as a background control. The xTAG beads were separated by a magnetic separator (Luminex Corporation) and washed twice with 75 μl of 1 $\times$ Tm hybridization buffer. The xTAG beads were resuspended in 75 μl of 1 $\times$ Tm hybridization buffer containing streptavidin-R-phycoerythrin (Invitrogen) at 4 $\mu\text{g ml}^{-1}$ followed by incubation at 37°C for 15 min. The end product was analyzed at 37°C on the LiquiChip 200 workstation according to the system manual, and the results were expressed as mean fluorescence intensity (MFI). Any signal that was greater than three times the highest background MFI signal for a given bead set was considered as positive.

Sensitivity of RT-PCR and LiquiChip assay. CMV-, ZYMV-, and CGMMV-infected Zucchini (squash) cDNA were mixed in

equal amounts. A 10-fold serial dilution of mixed cDNA was prepared from total RNA (10^{-8} to 10^{-17} g) with distilled water. Multiplex RT-PCR and LiquiChip assays were performed using this dilution series. The sensitivity was determined by the highest dilution endpoint using a detectable product. For the LiquiChip assay, a positive result was defined by an average MFI reading three times higher than the background reading. MFI values from the samples were analyzed in triplicate.

Results

Optimization of multiplex RT-PCR. For RT-PCR, the optimal concentrations of each primer pair were determined as 0.05 μM ZYMV-specific primer, 0.1 μM CMV-specific primer, 0.04 μM CGMMV-specific primer, and 0.1 μM each plant Cyoxid internal control primer, which generated the clear part of each band; that is, 124 bp (Cyoxid), 285 bp (CGMMV), 548 bp (CMV), and 721 bp (ZYMV) respectively. The amplification efficiency of viral genes was stable in spite of changing primer concentrations (Fig. 2), and analysis of RT-PCR products by gel electrophoresis showed that each primer pair was specific to its respective target without cross-reactivity between primers and templates (Fig. 3A, lanes 2 to 4). The CMV, CGMMV, and ZYMV viral cDNA yielded amplification products from all three viruses with product sizes as expected, albeit with a slight loss in PCR efficiency indicated by the reduction in band intensities shown in Figure 3A, lane 5. The internal control was consistently detected in all the reactions, except the no-template control, as expected.

Specificity of the LiquiChip assay for CMV, ZYMV, and CGMMV. The specificity of the LiquiChip assay was assessed by performing separate TSPE reactions using all four TAG-TSPE primers on RT-PCR products amplified from each virus, along with the internal control. The synthesized TSPE products were then hybridized to beads and analyzed using the LiquiChip 200 workstation. A high MFI reading was obtained from the bead that corresponded to its designated template when using a single template (Fig. 3B). No cross-interaction was observed between TAG-TSPE primers and noncognate RT-PCR amplicons, because no signals were obtained from other beads; this suggested that the TSPE primers were highly specific for their respective amplicons. Similar to the multiplex RT-PCR, all three viruses could be detected simultaneously with little loss to MFI when a mixture of the templates was used in the TSPE reaction. The consistent coamplification and detection of internal controls confirms the integrity of RNA used in the assay.

Sensitivity of LiquiChip assay for the detection of cucurbit viruses. The detection limit of the LiquiChip system was tested using

Fig. 2. Optimization of multiplex polymerase chain reaction (PCR) with different concentration ratios of primer sets. The assay was performed with the primer pairs of Zucchini yellow mosaic virus (ZYMV), Cucumber mosaic virus (CMV), Cucumber green mottle mosaic virus (CGMMV), and the internal control (Cyoxid). The primer concentration ratio of each treatment is shown on the top of the figure. The number indicates the ratio of each primer pair final concentration in PCR. The PCR products of ZYMV, CMV, CGMMV, and Cyoxid identified by 2% agarose gel electrophoresis are indicated by the arrows. Lane M: 100-bp DNA ladder.

a 10-fold serial dilution of CMV-, CGMMV-, and ZYMV-infected zucchini RNA as a template. RT-PCR products were analyzed by gel electrophoresis, which detected CMV and ZYMV at 10^{-12} g and CGMMV RNA at 10^{-14} g (Fig. 4A). LiquiChip assay was approximately 10-fold more sensitive than RT-PCR, with a detection limit of CMV and ZYMV at 10^{-13} g and CGMMV at 10^{-15} g (Fig. 4B).

The feasibility of the LiquiChip assay to detect cucurbit viruses on field samples was evaluated by blind assay of several melon plants with symptomatic mosaic or chlorotic leaves. From 40 melon plants

randomly collected from the field in Taichung, Taiwan in 2015, ZYMV accounted for 40% (16 of 40), CMV accounted for 25% (10 of 40), and CGMMV accounted for 5% (2 of 40) of the infections detected in collected samples. Single infections accounted for 30% of the samples. However, two viruses were detected in 20% of the samples, of which 15% (6 of 40) were infected with ZYMV and CMV, whereas 5% (2 of 4) were infected with ZYMV and CGMMV (Table 2). No plants were infected with all three viruses in our survey. The samples that tested negative for CMV, ZYMV, and CGMMV

Fig. 3. Specificity of multiplex reverse-transcription polymerase chain reaction (RT-PCR) and LiquiChip assay for the detection of *Zucchini yellow mosaic virus* (ZYMV), *Cucumber mosaic virus* (CMV), and *Cucumber green mottle mosaic virus* (CGMMV). **A**, Multiplex RT-PCR products from different virus-infected RNA templates. Lane 1 represents no-template control; lanes 2, 3, and 4 contained product amplified from ZYMV-, CMV-, and CGMMV-infected total melon RNA, respectively; lane 5 represents products from a mixture of three virus-infected total RNA in equal amounts; and lane M represents 100-bp DNA ladder. **B**, Bar chart of the result of LiquiChip assay using different RNA templates. RNA templates used in the assay: NC1 and NC2 represent background fluorescence and no template in the reaction, respectively; Mix represents ZYMV-, CMV-, and CGMMV-infected melon RNA mixed in equal amount. The mean fluorescence intensity (MFI) represents the average reading of three experimental repeats, and error bars represent the standard deviation.

are likely to be either fertilizer deficient or infected with another virus. To sum up, the assay determined that approximately 50% of symptomatic melon plants were infected with CMV, ZYMV, or CGMMV. Both single infections of ZYMV, CMV, and CGMMV and mixed infections of two of these viruses were detected. However, no single infection of CGMMV was found in any of the field samples. These samples also were tested by RT-PCR, and the results were mostly consistent for the two assays, except for two samples, which were shown to be positive for CMV and ZYMV by the LiquiChip assay but negative by RT-PCR.

Discussion

The LiquiChip detection technology, Luminex xMAP, requires a PCR amplification step, and its limit of detection can be determined by fluorescent signals. A LiquiChip assay was used to detect multiple targets derived from viruses and virus-infected plants by reading and analyzing the MFI signals from hybridized bead samples. In addition, amplicon labeling requires only one additional PCR step. (Dunbar 2006; Dunbar et al. 2003; Liu et al. 2011; Munro et al. 2013; van Brunschot et al. 2014; Xu et al. 2017). However, use of this method of plant virus detection should include the essential step of RT from

total RNA, because most plant viruses have RNA genomes. In this study, we developed an accurate and sensitive LiquiChip xTAG-bead hybridization assay for the detection of CMV, CGMMV, and ZYMV. Primers were designed to target genes encoding viral-coat proteins, and were able to amplify successfully in multiplexed RT-PCR. We demonstrated the ability of the LiquiChip assay to simultaneously detect three cucurbit viruses. The TSPE primers have high specificity for their respective target viruses; therefore, the detection of multiple viruses in the same reaction can be carried out without cross-reactivity between noncognate primers and templates. Chang et al. (1987) reported a high incidence of mixed infection of CMV, ZYMV, and several other cucurbit viruses in Taiwan. We replicated the detection of a mixed viral infection in a host plant by multiplexing the reaction using a mixture of viral templates. Consistent amplification of the host plant's genes as an internal control reduced the possibility of false-negative detection. The sensitivity of the LiquiChip assay was shown to be 10-fold higher than that of RT-PCR. Although it is known that multiplexing reduces amplification efficiency, virus detection was possible from template samples as small as 10^{-15} g. Highly sensitive assays are essential for early-stage detection of the infection when hosts are symptomless.

Fig. 4. Sensitivity of multiplex reverse-transcription polymerase chain reaction (RT-PCR) and LiquiChip assay for detection of *Zucchini yellow mosaic virus* (ZYMV), *Cucumber mosaic virus* (CMV), and *Cucumber green mottle mosaic virus* (CGMMV). **A**, Agarose gel of the multiplex RT-PCR products amplified from a 10-fold serially diluted template. Lane M represents the 100-bp ladder; lane 1 represents no-template control; lanes 2 to 10 used 10^{-8} to 10^{-16} g of template, respectively. **B**, The bar chart shows the result of the LiquiChip assay with decreasing amounts of template. NC1 and NC2 indicate the background fluorescence and no-template reaction, respectively. The amounts of template used in each reaction are labeled in grams. The mean fluorescence intensity (MFI) shows the average of three experimental repeats. The standard deviations are indicated by the error bars.

We defined a positive detection in LiquiChip assay as when the obtained signal was threefold higher than the background noise (negative control). Because the technique is very sensitive, there are far greater chances of obtaining false-positive results. Such an approach (using an arbitrary cutoff) is potentially subjective, and different batches of samples may have their own distinct range of signals (i.e., an amplicon with a low percentage of cytosine yields less fluorescence). This would be worth investigating with a statistical approach to data analysis, which may provide more consistency when comparing results between experiments.

Serological assays are still widely used in many laboratories for the detection of plant viruses, because they are suitable for quick assessment of large batches of samples. However, obtaining high-quality monoclonal antibodies is often time consuming and expensive. Nucleic-acid-based assay bypasses the need for antibody production, and this alone drastically reduces the cost and time required. The downside of conventional nucleic-acid-based assays is that independent reactions are required for the detection of different viruses. An increase in sample size and multiple viral strains implies that the assay can become laborious and impractical. The LiquiChip assay demonstrated here allowed detection of multiple cucurbit viruses in a single reaction. The only limitation of the LiquiChip assay would be that the starting material required RNA purification, unlike ELISA, in which the sample preparation can be as simple as homogenized tissue sap. Through the LiquiChip assay, we were able to obtain some insight as to the population of viruses that are present in the field and the potential mechanism by which the infection spreads. Our field survey for diseased melon showed that ZYMV and CMV were the most prevalent viruses; CGMMV was less common in symptomatic melon plants, which suggests that aphids are likely to be highly responsible for the spread of the diseases (ZYMV and CMV).

Multiplex RT-PCR can be a rapid and cost-effective approach to the simultaneous detection of multiple virus infections in plants; however, agarose gel electrophoresis does not readily resolve PCR products of similar sizes. The advantage of this targeted LiquiChip system is the multiplex detection of the three cucurbit viruses that may significantly reduce detection cost and labor and, therefore, increase processing efficiency. The newly emerging LiquiChip detection assay based on xMAP technology can meet the need for rapid diagnosis and enable the detection and quantitation of multiple DNA targets. When coupled with multiplex PCR, the assay has the capacity to simultaneously identify multiple infectious agents in the same reaction well. Thus, LiquiChip assay can deliver fast, quantitative data to facilitate the diagnosis of mixed infections and better understand etiology (Dunbar 2006). This study, for the first time, has demonstrated a method of detecting three cucurbits viruses simultaneously in a single reaction with a high degree of specificity and sensitivity. We further continued demonstrating the ability of the LiquiChip assay to distinguish symptomatically similar viruses in a large-scale field assay. Thus, we validated the assay as a reliable

diagnostic tool to detect cucurbit viruses in fields. This technology may help in limiting the worldwide spread of plant diseases by serving as a quarantine detection measure.

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Table 2. Number of samples (percentage in parentheses) of melon plants collected from the field testing positive by reverse-transcription polymerase chain reaction (RT-PCR) and LiquiChip

Viruses detected ^a	Number of sample detected (%)	
	RT-PCR	LiquiChip
ZYMV	8 (20)	8 (20)
CMV	4 (10)	4 (10)
CGMMV	0 (0)	0 (0)
ZYMV + CMV	4 (10)	6 (15)
ZYMV + CGMMV	2 (5)	2 (5)
CGMMV + CMV	0	0
ZYMV+ CMV + CGMMV	0	0
ND	22 (55)	20 (50)
Total	40 (100)	40 (100)

^a Zucchini yellow mosaic virus (ZYMV), Cucumber mosaic virus (CMV), and Cucumber green mottle mosaic virus (CGMMV). ND indicates that ZYMV, CMV, and CGMMV were not detected from the sample.