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Differentiation of Cucumber mosaic virus isolates by hybridization to oligonucleotides in a microarray format

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Abstract

A system for microarrays was developed to detect and differentiate Cucumber mosaic virus (CMV) serogroups and subgroups. The coat protein genes of 14 different isolates were amplified using cy3-labelled generic but species–specific primers. These amplicons were hybridized against a set of five different serotype and subgroup specific 24-mer oligonucleotides bound to an aldehyde-coated glass slide via an aminolinker. The results of the hybridization revealed that the method allowed a clear differentiation of the 14 different CMV isolates into the serogroupes 1 and 2, and in addition was able to assign 9 out of 10 different serogroup 1 isolates correctly into subgroups 1a and 1b. This differentiation was not possible by RFLP analysis with the restriction enzyme MspI. The use of amplicons larger than 700 base pairs and their successful differentiation by hybridization to specific oligonucleotides opens avenues to highly parallel, yet sensitive assays for plant viruses.

Keywords: Cucumber mosaic virus; Oligonucleotides; Capture probes; NA-arrays; Detection; Differentiation

1. Introduction

The necessity to apply highly multiplexed methods for the detection and differentiation of plant pathogens grows with the tendency of global production of crops and plants which are im- and exported under increasingly stringent quarantine regulations (Richtlinie 2002/89/EG). Ideally test methods should meet the following requirements: high sensitivity; safety and specificity in addition to speed, reproducibility and affordability.

The best sensitivity is offered by PCR or RT-PCR depending on the type of nucleic acid to be detected. The PCR-methods gain speed, sensitivity and safety when performed under real-time conditions. The application of generic primers that are able to amplify all species of a given genus or family might increase test safety against false negatives. Generic primers have been described, for e.g. Potyviruses (Langeveld et al., 1991), Clostero- and Criniviruses (Dovas and Katis, 2003a, 2003b), Tobamoviruses (Letschert et al., 2002), Tospoviruses (Chu et al., 2001) and Tobraviruses (Weidemann, 1995). It was shown that these generic primers could amplify most members of a genus or even a family. However, in most cases the exact identity of the virus remained to be determined in a separate step.

Differentiation after general amplification can either be done by restriction fragment length polymorphism (RFLP) analyses or by hybridization with specific probes. This allowed the further differentiation into species and even pathotypes (Letschert et al., 2002). Here another advantage of nucleic acid based test methods over serological tests became evident. It has been shown for Tobamoviruses (Letschert et al., 2002) and the Potyvirus PVY_{NTN} (Boonham et al., 2002)

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that mutations leading to new pathotypes can only be detected by nucleic acid-based methodology.

The historical development from dot-hybridization towards the macro- and microarray technology, which nowadays enables highly complex experimental designs, has been reviewed by Jordan (2001). In case of diagnostic applications this allows intensive parallel detection, and would be an ideal supplement to solve the problem of differentiation after generic amplification.

In this article we describe how amplicons from plant viral RNA are used for their differentiation by hybridization to synthetic oligonucleotides (probes) arranged in a two-dimensional array on a glass slide. As model pathogen we have chosen CMV which is known to be highly heterogeneous in its coat protein (Roossinck, 2002). This model system enabled us to compare our results with results obtained by others for the same genus (Anonymous, 1998).

2. Materials and methods

2.1. Virus isolates and serological testing

The CMV isolates (Table 1) were obtained from the ring test organized by COST action 823 (Anonymous, 1998), from the Asian Vegetable Research and Development Centre (AVRDC, Taiwan) and the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). The isolates were purified by local lesion transfer on *Chenopodium quinoa* Willd. or *Vigna unguiculata* L. Walp. before systemic propagation on *Nicotiana glutinosa* L. The purity of isolates and their serological behaviour were confirmed by triple antibody sandwich (TAS) enzyme linked immunosorbent assay (ELISA) using polyclonal antiserum AS 0475,[1] monoclonal antibodies AS 0487–AS 0491 1 as described in Anonymous (1998).

Table 1
Cucumber mosaic virus isolates used during this work

Virus isolate[a]	Serogroup[b]	Subgroup[c]	Accession No.
rt67 NL	II		AJ810253
rt68 NL	II		AJ810254
PV0420	II		AJ810255
PV0418	II		AJ810256
rt88 ES	I	A	AJ810257
rt52 USA	I	A	AJ810258
KS44 TH	I	B	AJ810259
AN In	I	B	AJ810260
CN03 CN	I	B	AJ810261
rt144 BG	I	B	AJ810262
rt54 CN	I	B	AJ810263
tr15 TH	I	B	AJ810264
rt6 GR	I	B	AJ810265
P3613 TW	I	B	AJ810266

[a] Codes starting with rt are the same as those used in Anonymous (1998); codes in the form PV#### are the DSMZ numbers. The same codes are used in Fig. 1.
[b] Sorting in serogroups according to Anonymous (1998)
[c] Sorting in subgroups according to Roossinck (2002).

2.2. RNA extraction, cDNA synthesis and polymerase chain reaction of the coat protein gene

Total RNA was extracted from leaf tissue with a silica-based procedure described by Rott and Jelkmann (2001). For primer annealing prior to cDNA synthesis 10 μg of total RNA, 1 μl (10 μM) of primer CMV-CPrev (5′-CTG GAT GGA CAA CCC GTT C-3′) in a total volume of 10 μl was heated for 5 min at 95 °C and instantly cooled down on ice. The cDNA was synthesized with 10 U Moloney murine leukemia virus reverse transcriptase (M-MLV-RT, Promega, USA) according to the manufacturers suggestions.

For PCR a 25 μl reaction volume contained 1 μl cDNA, 2.5 μl 10 × PCR-Buffer (Promega), 1.5 μl $MgCl_2$ (25 mM), 1 μl of primer CMV-CPfor (5′-ATG GAC AAA TCT GRA TCW MCC-3′) and primer CMV-CPrev, each 10 μM, 2 μl dNTPs (2 mM), and 1 U Taq-Polymerase (Promega, USA). The amplification was started in a Personal Cycler (Biometra, Germany) with a 5 min denaturation step at 94 °C, followed by 30 cycles of: 94 °C for 30 s, 59 °C for 45 s, and 72 °C for 60 s, and a final extension step at 72 °C for 5 min. To obtain labelled PCR-products we use the same primers, however, labelled with cy3 at their 5′-ends.

2.3. Restriction enzyme digestion and agarose gel electrophoresis

Unpurified PCR product was incubated overnight at 37 °C with 1–2 U of the restriction enzyme MspI.

Untreated PCR products were analysed on a 1% agarose gel in TAE buffer (0.04 M Tris–acetate, 0.001 M EDTA, pH 8.0) whereas the restriction fragments were separated on a 2% agarose gel using the same buffers. The gels contained ethidium bromide and were evaluated with a UV transilluminator (260 nm).

2.4. Cloning, sequencing and sequence analysis

For sequencing the PCR products were purified with the E.Z.N.A. Cycle Pure Kit (PeqLab, Germany) and subsequently ligated into a T-cloning vector (pBlueScript II, Stratagene, USA) according to Marchuk et al. (1991) for transformation into *E. coli*. Positive clones were screened and inserts were sequenced from both directions (MWG Biotech, Germany).

Sequences were analysed using DNA Strider (Marck, 1988), Fasta (Pearson, 1990) and ClustalX (Thompson et al., 1997).

[1] These numbers are DSMZ-collection numbers for antibodies obtained from the plant virus collection of the DSMZ.

2.5. Sequence alignments and serogroup allocation testing

For the manual design of the probes, the 181 sequences present on GenBank at 17/02/04 were aligned using the software Vector NTI Version 9.0 (Invitrogen). Serogroup allocation testing was performed with cluster analysis and multidimensional scaling. Unrooted neighbour-joining (NJ) trees were constructed with complete CP-ORF alignments with the software MEGA Version 2.1 (Kumar et al., 2001) using the Kimura 2-parameter distance (Kimura, 1980), including transitions and transversions unweighted and handling gaps by pairwise deletion. Multidimensional scaling was performed using the module Proxscal in the statistics software package SPSS Version 12 (SPSS Inc.) with the following parameters: dissimilarities as proximities, three dimensions, interval proximity transformation, simplex initial configuration, minimized iteration criteria for stress convergence and minimum stress, and maximum 100 iterations.

2.6. Design of the capture probes

Five serogroup specific probes were designed, each 24 base pairs long and containing a 15 T spacer at the 3′end to improve hybridization behaviour. At the very 3′end an aminolinker was attached to bind the probes to the aldehyde-coated glass slides (Genetix, UK). Probes were selected manually based on the sequence alignment to optimize serogroup and subgroup differentiation in a hybridization reaction, i.e., mismatches were positioned towards the probe centre. Two probes targeted serogroup 2 and three targeted serogroup 1, two of which were selectively designed to hybridize only with subgroup 1a or 1b, respectively (Table 2). Because it was not possible to avoid a wobble position in the subgroup specific probes, an inosine was included at the respective position to avoid signal intensity loss due to dilution effects if using degenerate probes (Table 2). All selected probes were tested for thermodynamic properties using the program Vector NTI Advance 9.0 (InforMax Inc., UK) and the best probes were used for the experiments. Because of the stringent constraints on the selection of serogroup specific probes, their specifications were rather tolerant with 37.5–70.8% GC content and a melting temperature (T_m) between 49.7 and 68.1 °C (thermodynamic melting temperature calculation with Vector NTI Advanced 9.0, based on "Nearest Neighbour" theory, with constant parameters of DNA and salt concentration, Table 2).

2.7. Microarray printing and design

Fifty microlitres of the 3′-6-C-aminoterminated probes (Microsynth) were diluted 1:1 in Genetix Microarray Spotting Solution to a final concentration of 25 mM and spotted onto glass slides (Genetix, UK) using a QArrayMini automated spotter (Genetix, UK). Each slide included three replicates of each block (of which only one is shown in Fig. 4), one block including two adjacent replicates of each probe, as well as a cy3-labelled amino-linked probe as standard. The spots are organised such that if positive hybridization in the left half is observed, the isolate belonged to serogroup 1, whereas if positive hybridization was observed in the right half of the array, the isolate belonged to serogroup 2 (Fig. 4). Each spot was stamped three times and spotting was conducted in 75% relative humidity. After spotting, the slides were washed following the protocol for aldehyde-coated slides of the manufacturer (Genetix, UK).

2.8. Hybridization of labelled probes to arrays

PCR products for hybridization were labelled by using 5′-cy3-labelled forward and reverse primers in the PCR reaction. Amplified labelled PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, BRD) according to the manufacturers protocol. The cleaned PCR product was denatured for 10 min at 96 °C, cooled on ice for 3–5 min and then kept on ice until hybridization. After priming the slides with 2× SSC, hybridization was conducted on a Lucidea Slidepro (Amersham Biosciences) by injecting 200 μl of hybridization mix (Bodrossy et al., 2003) containing 1 μg of the purified PCR product, 60 μl 20× SSC, 4 μl 50× Denhardt's solution (Sigma), 2 μl 10% SDS and ultrapure ddH_2O cleaned on a Milli-Q Synthesis A10 (Millipore, USA). The DNA was hybridized at 40 °C for 2 h, followed by three washes under low stringency at 42 °C (6 min in 2× SSC and 0.2% SDS, 2 min in 0.2× SSC and 0.2% SDS, and 2 min in 0.075× SSC). After hybridization the slides were analysed in a GenePix Personal 4100 A microarray scanner (Axon Instruments, USA) at a wavelength of 532 nm with a PMT (photomultiplier tube) gain between 800 and 1000 to avoid overexposure.

Table 2
Specifications of the serogroup specific probes

Probe name	Specificity	Sequence	%GC	T_m
SG1-2r	1	GCG GAC GGA GCC TCA CCG GTA CTG	70.8	68.1
SG1a2_1r	1a	CAG TCA CIG AAT ATG ATA AGA AGC	37.5	49.7
SG1b2_1r	1b	CAG TCA CIG AGT TCG ATA AGA AGC	45.8	54.1
SG2_3f	2	GTG GGA CGA CCA ATG GCG AGG GTT	62.5	67.3
SG2_4f	2	GAG TCA AAG CAC GCA ACC CTG CAT	54.2	63.1

%GC: percent bases that are either guanine or cytosine; T_m: melting temperature (thermodynamic T_m based on nearest neighbour theory as implemented in Vector NTI Advance 9.0, InforMax, UK). The 15T spacer and the aminolinker at the 3′end of each probe are not indicated.

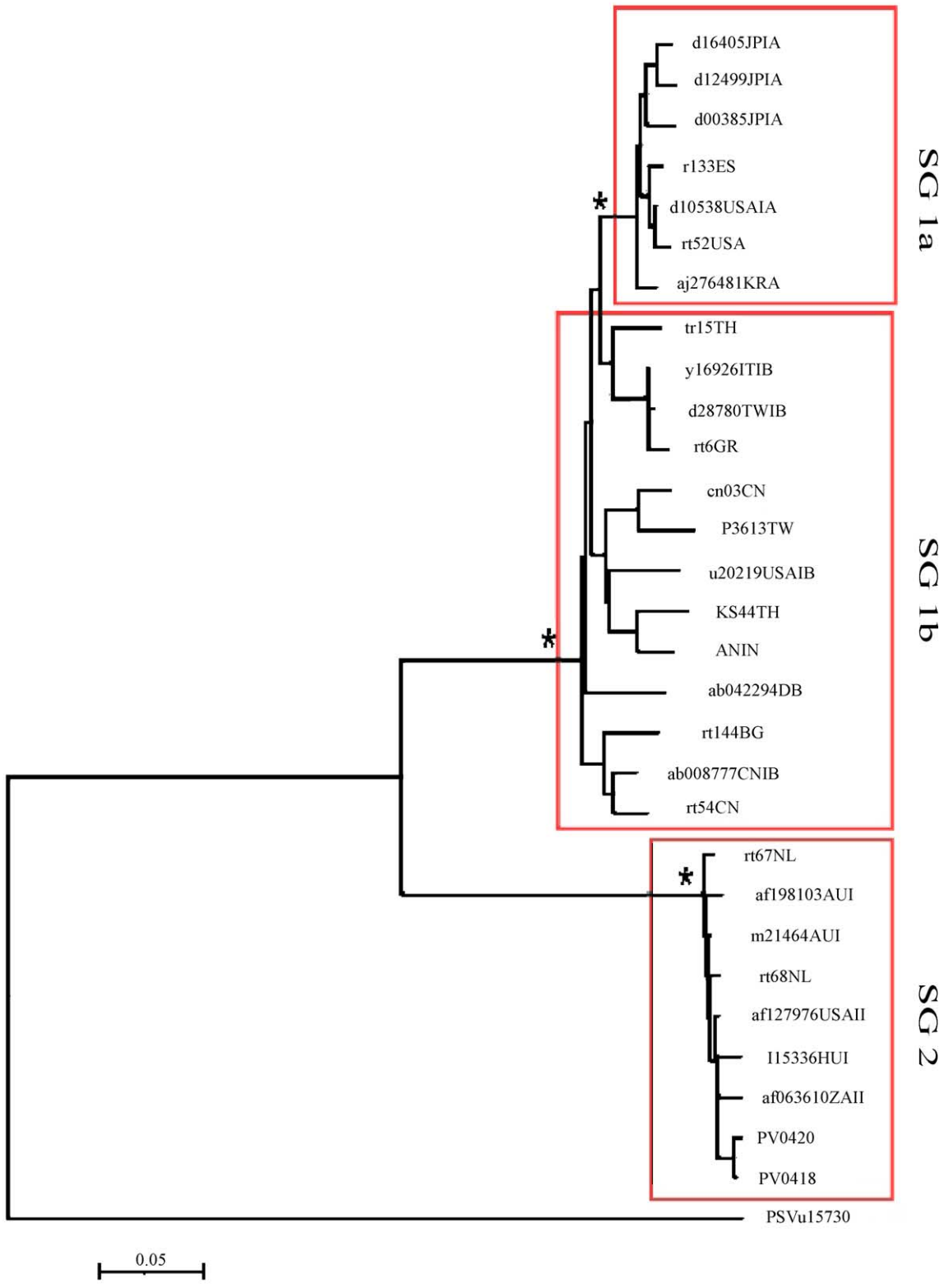

Fig. 1. Phylogenic tree of CMV-isolates based on their coat protein sequences. Phylogenic analysis of 29 CMV coat ORFs with aligned nucleotide sequences. Bootstrap percentage values from 1000 replicates higher than 96% are shown by asterisk. The position of the three CMV serotypes are shown as boxes. Isolate codes are either the same as in Table 1, or accession numbers from databases.

3. Results and discussion

3.1. Comparison of the virus isolates by serology

All 14 virus isolates reacted positively when tested in DAS-ELISA with polyclonal antibodies substantiating their CMV nature. The results of tests with a TAS-ELISA format and serogroup specific monoclonal antibodies substantiated that 4 of the 14 isolates belonged to serogroup 2 and the remainder were serogroup 1. This result confirms the results for the seven isolates (Table 1) that had been fully characterized during the COST-823 ringtest (Anonymous, 1998).

3.2. Comparison by nucleotide sequence of the coat protein genes

Cluster analysis of our 14 new sequences together with the sequences used by Roossinck (2002) resulted in a complete integration of our isolates into the set of sequences used by Roossinck without altering the clustering into sero- and subgroups. Four of our 14 isolates clustered into serogroup 2, which appeared very homogeneous (Figs. 1 and 2). The remaining serogroup 1 isolates revealed a more heterogeneous pattern, but followed the grouping of Rossinck into two subgroups, 1a with two of our isolates, and 1b containing the remaining eight isolates. The comparison by nucleotide sequence data indicated that the suggested clustering of serogroup 1 isolates into two subgroups (Roossinck, 2002) seems to be stable, since the addition of 10 new isolates did not alter it.

This was confirmed by a completely different analysis methods, the multidimensional scaling (Fig. 2), which shows that the sero- and subgroups are again well separated and in full agreement to those obtained by cluster analyses. The fact that this analysis was even stable when using the complete set of 181 available different sequences plus our additional 14 ones indicates the value and stability of this grouping.

3.3. Amplification of CP gene and RFLP analysis

The primers CMV-CPrev and CMV-CPfor resulted in fragments of 773 bp (serotype 1) and 788 bp (serotype 2), respectively. The amplicons comprise the complete CP gene and part of the 3′ non-translated region.

We wanted to examine if patterns generated with restriction enzyme digestion of CMV amplicons would substantiate the grouping obtained by the phylogenic analyses of the CP sequences as proposed by Anonymous (1998). In Fig. 3 we show the obtained patterns for isolates that belonged to subgroup 1b. It becomes evident, that RFLP patterns, at least using solely the restriction enzyme MspI, which had been used in the previous ringtest, are not congruent with the clustering. Three isolates belonging to subgroup 1b revealed three different RFLP-patterns when their amplicons were digested with MspI (Fig. 3, lanes 3–5). This is in contradiction to the results from the COST 823 ringtest, where a clear grouping of the tested isolates according to their RFLP pattern was presented (Anonymous, 1998). However, the grouping from their comparison was never backed by sequence data, which Rossinck and we have used.

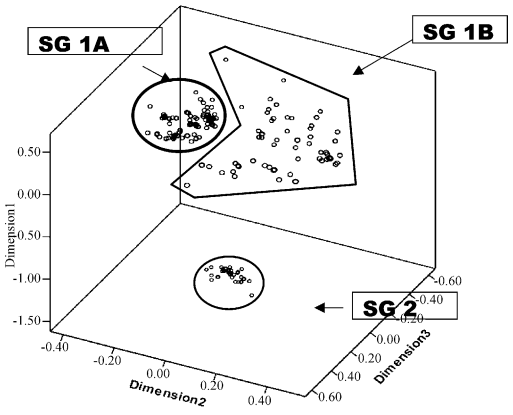

Fig. 2. Serogroup allocation analysis by multidimensional scaling. Serogroup allocation analysis by three-dimensional scaling using the proxscal module of SPSS Version 12. The three serogroups (SG) are outlined. The degree of genetic variation of each serogroup is visualized by the covered area that indicates limited genetic variation in serogroups 1A and 2 and a large genetic variation in serogroup 1B.

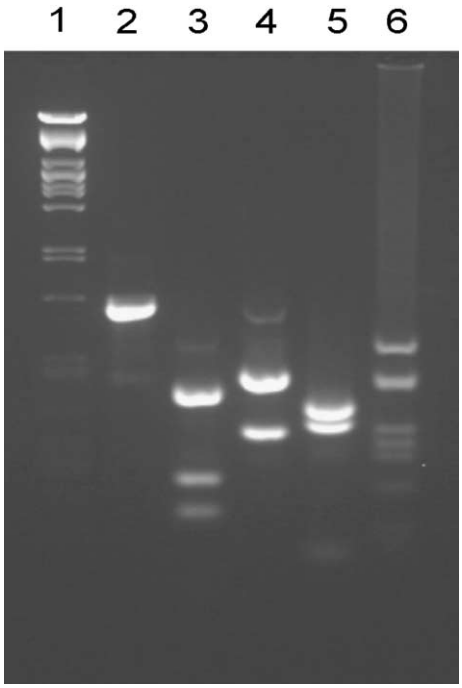

Fig. 3. RFLP Analysis of three different CMV-isolates from subgroup 1B Amplicons from the three CMV isolates belonging to subgroup 1b were digested with MspI and the resulting fragments separated on a 2% TAE agarose gels. Lane 1: lambda DNA pstI cut; lane 2: undigested; lane 3: CMV KS44 TH; lane 4: CMV AN In; lane 5: CMV tr15 TH; lane 6: puc9 Hae III cut.

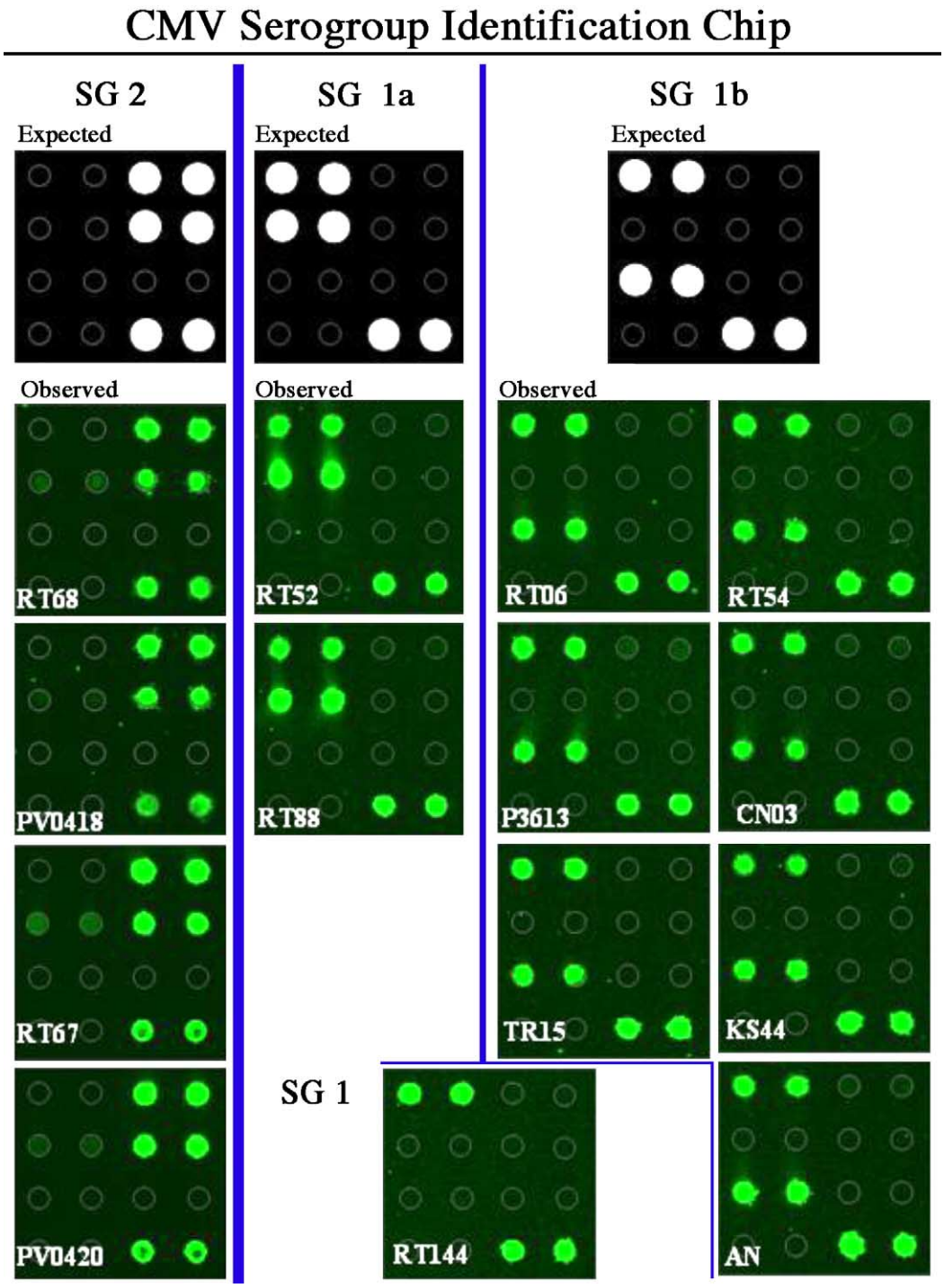

Fig. 4. Hybridization results of 14 different CMV Isolates on a two-dimensional array. Cy3-labelled DNA amplicons were hybridized to oligonucleotide capture probes bound onto glass slides. Fluorescent patterns were recorded with a GenePix Personal 4100 A microarray scanner (Axon Instruments, USA) at a wavelength of 532 nm.

Table 3
Sequence comparison between the serogroup 1b specific probe

Virus isolate	Serogroup	Serogroup 1b specific probe (5′ to 3′) CAG TCA CIG AGT TCG ATA AGA AGC
KS44 TH	1b	CAG TCA CTG AGT TCG ATA AGA AGC
AN In	1b	CAG TCA CTG AGT TCG ATA AGA AGC
CN03 CN	1b	CAG TCA CAG AGT TCG ATA AGA AGC
RT144 BG	1b	CAG T**T**A CAG A**A**T TCG A**C**A AGA A**A**C
RT54 CN	1b	CAG TCA CGG A**A**T TCG ATA AGA AGC
Tr15 TH	1b	CAG TCA CGG AGT TCG ATA AGA AGC
RT6 GR	1b	CAG TCA CGG AGT TCG ATA AGA AGC
P3613 TW	1b	CAG TCA CAG AGT TCG ACA AGA AGC
RT88 ES	1a	CAG TCA CGG A**A**T **AT**G ATA AGA AGC
RT52 USA	1a	CAG TCA CGG A**A**T **AT**G ATA AGA AGC
RT67 NL	2	CAG TCA CGG A**C**T **AT**G ATA AGA AGC
RT68 NL	2	CAG TCA CGG A**C**T **AT**G ATA AGA AGC
PV-0420	2	CAG TCA CGG A**C**T **AT**G ATA AGA AGC
PV-0418	2	CAG TCA CGG A**C**T **AT**G ATA AGA AGC

Capture probe sequences are shown without the 15T spacer and the aminolinker at the 3′end. The corresponding DNA section is shown as reverse strand for easy comparison of the 14 isolates. Mismatches are marked in bold. Codes are the same as in Table 1. RT144 BG did not hybridize to the subgroup 1b specific probe.

3.4. Comparison by array hybridization

The capture probes were manually designed based on an alignment of 195 different cp sequences which were either from GenBank or from our own sequencing work. The probes targeted regions optimal for the differentiation between serogroups 1 and 2 or between subgroups 1a and 1b. As shown in Fig. 4, the serogroups 1 and 2 specific probes worked perfectly and detected the respective virus isolates without any false positive result. The faint cross hybridization signals of the probe for subgroup 1a (SG1a2_1r) in hybridizations with serogroup 2 isolates (Fig. 4, RT67, RT68, PV0420) originates from the fact that it differs from serogroup 2 sequences by only a single base pair in the centre of the probe sequence (see discussion below). The differentiation into the two subgroups of serogroup 1 was possible with one exception. The two subgroup 1a isolates hybridized correctly, whereas among the eight subgroup 1b isolates one, RT144 BG, did not react with its subgroup specific capture probe. The failure was not due to experimental design, but could be explained by sequence data revealing that this isolate disposes of four mismatches to the probe sequence in the respective DNA sequence (Table 3). Nevertheless, the hybridization pattern of the isolate RT144 BG clearly allows correct allocation to serogroup 1.

Sequence comparison and our array hybridization results also revealed that the success of single-base mismatch hybridisation was dependent on position and type of mismatch, and probably also on pH and sequence context, well in accordance with previously described hybridization characteristics (Allawi and SantaLucia, 1998, and references therein; Brown et al., 2003). For example, hybridization was successful with one mismatch between the isolate DNA and the probe if the mismatch was located near the 3′end of the probe, as in the hybridization between the serogroup 1b specific probe and P3613 TW (Table 3). If a mismatch occurred in the centre region of the probe, hybridization success was strongly dependent on the mismatch type. Hybridization between the serogroup 1b specific probe and the isolate RT54 CN was nearly optimal although there was a G·T mismatch close to the probe centre, which may be explained by the fact that this mismatch is among the most stable mismatches observed in DNA (Allawi and SantaLucia, 1998, and references therein). In contrast, the very faint cross-hybridization (Fig. 4) between the probe for subgroup 1a (SG1a2_1r) in hybridizations with serogroup 2 isolates indicates almost complete destabilisation of duplex formation with a centre-located A·G mismatch. Although this mismatch is generally believed to be rather stable, there are large differences in the destabilising effect between the four different possible structures and there is a large effect of pH and sequence context in this mismatch as described by Brown et al. (2003). These effects may explain the strong hybridization inhibition observed in our experiments.

Our results on differential hybridization against five specifically selected oligonucleotides clearly demonstrates the great potential of oligonucleotide-based microarray technology for virus isolate diagnostics. This is the first time, that a diagnostic chip for plant viruses was established with oligonucleotides, where so far, cDNAs were used as capture probes (Boonham et al., 2003; Lee et al., 2003). These are of course specific for the homologous virus, however, because of their size, are limited in their discriminating ability to sequence homologies <80% (Boonham et al., 2003). In our experiments it was possible to show for the first time, that short oligonucleotides are suitable as capture probes for the discriminative hybridization of isolates differing by less than 8% in an amplified PCR product larger than 700 base pairs. The proven ability to hybridize successfully generic PCR products in parallel against oligonucleotides is one step towards a possibly general plant virus chip, where non-specific random oligonucleotides could serve as capture probes for genus-specific amplification products. This procedure might be faster than sequencing the amplicons when it comes to

new strains or even species. At least it could enable the fast detection of new viruses of a given genus.

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