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Simultaneous detection of the seven main tomato-infecting RNA viruses by two multiplex reverse transcription polymerase chain reactions

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Abstract

Cucumber mosaic virus, *Tomato spotted wilt virus*, *Tomato mosaic virus*, *Tomato chlorosis virus*, *Pepino mosaic virus*, *Torrado tomato virus* and *Tomato infectious chlorosis virus* cause serious damage and significant economic losses in tomato crops worldwide. The early detection of these pathogens is essential for preventing the viruses from spreading and improving their control. In this study, a procedure based on two multiplex RT-PCRs was developed for the sensitive and reliable detection of these seven viruses. Serial dilutions of positive controls were analysed by this methodology, and the results were compared with those obtained by ELISA and singleplex versions of RT-PCR. The multiplex and singleplex RT-PCR assays were able to detect specific targets at the same dilution and were 100 times more sensitive than ELISA. The multiplex versions were able to detect composite samples containing different concentrations of specific targets at ratios from 1:1 to 1:1000. In addition, 45 symptomatic tomato samples collected in different tomato-growing areas of Sicily (Italy) were analysed by multiplex RT-PCR, singleplex RT-PCR and commercially available ELISA tests. Similar results were obtained using the RT-PCR techniques, with a higher sensitivity than ELISA, and revealed a common occurrence of mixed infections and confirmed the presence of these seven virus species in Italy.

Keywords: Multiplex RT-PCR; Tomato viruses; Detection; Diagnosis

Tomato (*Solanum lycopersicon*) is the most important vegetable crop, with a world production of 153 million metric tons and a value of over 558 thousand million dollars in 2009 (FAO, 2009). In the last decade, emerging viral diseases of tomato have been reported worldwide and have caused considerable economical losses (Hanssen et al., 2010). In the Mediterranean Basin and other subtropical areas, the following seven RNA viruses have been described as the most important viral pathogens of tomato: i) *Cucumber mosaic virus* (CMV), the type member of the genus *Cucumovirus* of the family *Bromoviridae* and the virus having the widest host plant species range in addition to transmission by aphids (Palukatis and Garcia-Arenal, 2003); ii) *Tomato spotted wilt virus* (TSWV), the type member of the *Tospovirus* genus of the family *Bunyaviridae* that is transmitted by thrips (Adkins, 2000); iii) *Tomato mosaic virus* (ToMV), a member of the genus *Tobamovirus* of the family *Virgaviridae* that infects many solanaceous species, is able to survive for several years in dried plant debris and can be transmitted by seeds or mechanical contact (Broadbent, 1976); iv) *Tomato chlorosis virus* (ToCV) and v) *Tomato infectious chlorosis virus* (TICV), members of the genus *Crinivirus* of the family *Closteroviridae* having limited host range (Martelli et al., 2002) - ToCV is transmitted by *Trialeurodes vaporariorum*, *T. abutilonea* and *Bemisia tabaci* biotypes A and B (Wisler et al., 1998), whereas TICV is only transmitted by *T. vaporiarum* (Duffus et al., 1996); vi) *Pepino mosaic virus* (PepMV), genus *Potexvirus*, family *Alphaflexiviridae*, has a restricted host range and is transmitted by contact (Jones et al., 1980; Mumford and Metcalfe, 2001); and vii) *Torrado tomato virus* (ToTV), the type member of the genus *Torradovirus* of the family *Secoviridae* (Verbeek et al., 2007) that has a restricted host range, including solanaceous species, and is transmitted by whiteflies *B. tabaci* and *T. vaporariorum* (Amari et al., 2008).

The most important strategy to control these viral diseases in tomato is based on preventing virus introduction and establishment in nurseries and fields and the eradication of infected

plants in the early stages of outbreaks. This approach requires surveillance using specific, sensitive and rapid diagnostic methods. Virus detection in tomato plants is traditionally performed by serological tests (DAS-ELISA) using polyclonal antibodies, but the low viral titre often found in nursery plants or during the early stages of virus infection can produce false negative results (Jacobi et al., 1998). In addition, there are often no commercially available ELISA tests for the main virus species that infect tomato, as is the case for ToCV, TICV and ToTV. Consequently, there is a current need for the implementation and design of reliable diagnostic methods having high sensitivity and specificity for testing plant material. In this study, a procedure based on two multiplex RT-PCR assays was developed and successfully tested in plant material for the detection of CMV, TSWV, ToMV, ToCV, PepMV, ToTV and TICV.

To design appropriate primers that are compatible with a multiplex reaction, nucleotide sequences of different isolates of each virus species were retrieved from the GenBank database. The sequences were aligned using the ClustalW programme implemented within the software Geneious Pro 5.4.6 (Biomatters, New Zealand). To allow the discrimination of the different virus species by electrophoresis, oligo sequences having similar melting temperatures and flanking genomic regions of different sizes were selected using Primer Express 2.0 (Applied Biosystems, USA) and Vector NTI 9.0 (Invitrogen, USA). The nucleotide sequences, genomic position of the primers and size of the amplicons are provided in Table 1.

Greenhouse-grown tomato plants naturally and artificially infected by well-characterised viral species were used as the positive controls. These controls were employed to prepare tenfold serial dilutions using healthy plant extract as diluent and also to obtain specific PCR products that were cloned and used for the optimisation of the multiplex reactions. The plant extracts were prepared by grinding leaves 1:20 (w/v) in PBS buffer, pH 7.2, supplemented with 0.2% DIECA, and 2% (w/v) polyvinyl-pyrrolidone (PVP-10) in individual plastic bags

with a net (Bioerba, Switzerland) to avoid contaminations among the samples. Total RNA was purified from approximately 100 mg of plant leaves using the Ultraclean Plant RNA Isolation Kit (MoBio, USA) according to the manufacturer's instructions. One-step singleplex RT-PCRs were performed for each virus using a Mastercycler gradient thermal cycler (Eppendorf, Germany), which allowed the inclusion of a gradient temperature from 50 to 60ºC to test the optimal annealing temperatures. Optimal RT-PCR reactions were performed in a 25 µl volume consisting of 10 mM Tris-HCl (pH 8.9), 50 mM KCl, 0.3% Triton X-100 (w/v), 1 µM each primer (TICV1/TICV2 for TICV detection; ToTV1/ToTV2 for ToTV detection; TSWV1/TSWV2 for TSWV detection; CMV1/CMV2 for CMV detection; and ToMV1/ToMV2 for ToMV detection) or 1 µM PepMV3, 0.5 µM PepMV1, 0.5 µM PepMV2 for PepMV detection, 250 µM dNTPs, 0.25 units AMV-RT (Promega), 0.5 units GoTaq DNA polymerase (Promega), and 5 µl of RNA template. The one-step RT-PCR was performed at 45ºC for 30 min for the cDNA synthesis, followed by 95ºC for 5 min for denaturation and 40 cycles of amplification (95 ºC for 30 s, 50 ºC for 30 s and 72 ºC for 30 s), with a final step of 72 ºC for 5 min. The expected amplicons were obtained from the virus-infected plants, whereas no amplification products were obtained from the healthy plants. These specific RT-PCR products were inserted into the pGem-T vector (Promega) and cloned into *E. coli* JM-109. The transformants were selected by ampicillin resistance, and the presence of the fragments was verified by PCR using specific primers. The subsequent plasmid purification was performed using the UltraClean Standard Mini Plasmid Prep Kit (MoBio, USA), and the specific nucleotide sequences of the viral targets were confirmed by sequencing in both directions using the ABI PRISM DNA 377 sequencer (Perkin-Elmer, Boston, MA, USA). The plasmid concentration was estimated using the NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). In an initial attempt, a single multiplex assay for the simultaneous detection of the seven viruses was evaluated. For this purpose, 1 ng of each purified viral target-specific plasmid was used to prepare a mixed

template sample containing all of the targets. The multiplex PCR was performed as described above for the one-step RT-PCR, but the reverse transcription step was eliminated due to the DNA nature of the composite template. The amplicons were visualised on 2% agarose gels after staining with ethidium bromide, and problems in the simultaneous detection of the amplicons were observed. Specifically, the primers for ToTV and TICV appeared to be incompatible, showing an inhibition in the amplification of the ToTV and TICV targets when their specific primers were included in the same reaction cocktail. In addition, to evaluate the theoretical sensitivity of the multiplex PCR, tenfold serial dilutions of each prepared viral target plasmid were prepared, ranging from 1 to 10^{-4} ng. The results showed sensitivities that were from 10 to 100-fold less than the singleplex versions. To overcome these drawbacks and to discriminate among the seven viruses, the initial multiplex PCR was divided into two multiplex assays: the first multiplex included the simultaneous detection of PepMV, TSWV, ToTV and ToCV viruses, and the other multiplex allowed the detection of CMV, ToMV and TICV (Figure 1). The reaction cocktail for each multiplex assay was the same as that described above but included only the specific primers for the corresponding virus. The theoretical sensitivity using the dilutions of the specific target plasmids was the same as that obtained by the singleplex assays. Subsequently, tenfold serial dilutions of infected tomato plant extracts diluted into healthy plant extracts were used to compare ELISA, singleplex RT-PCR and the two multiplex RT-PCRs for PepMV, TSWV, CMV and ToMV. However, due to the lack of commercially available ToTV, ToCV TICV ELISA tests, the comparisons of these viruses was performed only between the two RT-PCR versions. In all cases, the multiplex and singleplex RT-PCRs were able to detect specific targets up to the same dilution and were at least 100 times more sensitive than ELISA in those cases in which serological tests were available. To determine the reliability of the viral detection in mixed infections and the interference of amplifications due to relative concentration issues, twenty-eight samples were prepared, combining four or three viral

target plasmids of the different viruses by changing the concentration of one target (1 ng to 10^{-4} ng) and maintaining the same concentration (1 ng) for the other targets. The PCR analyses of these samples showed that any target was detectable in a relative ratio of 1:1000 with respect to the other targets present in the sample (Figure 2).

In addition, these two multiplex RT-PCRs were used to analyse 45 tomato samples collected from greenhouses and fields in different provinces of Sicily in 2011, and the results were compared with the results of ELISA (except for ToCV, TICV and ToTV) and singleplex RT-PCRs described in the literature: PepMV (Mumford and Metcalfe 2001), TSWV (Mumford et al., 1994), ToTV (Pospieszny et al., 2007), ToCV (Louro et al., 2000), CMV (Lin et al., 2004), ToMV (Kumar et al., 2011) and TICV (Vaira et al., 2002). The plant extracts were prepared using a similar methodology as that employed for the infected tomato plants used as the controls. The same crude plant extracts were used for the ELISA tests and for the purification of total RNA. The results are shown in Table 2; we obtained the same diagnostic for all of the plants using both PCR techniques. In the case of the ELISA tests (by which only four viruses could be analysed), we obtained the same results as the RT-PCR methods, with the exception of one sample that tested negative for PepMV by ELISA and positive by RT-PCR versions. The most prevalent virus was ToCV, which was detected in 32 plants, followed by TSWV in 25 plants, CMV in 20 plants, PepMV in 17 plants, ToMV in 12 plants, TICV in 6 plants and ToTV in 2 plants. Three tomato samples were not infected by any virus. Single infections were present in 8 plants, whereas 10 plants were infected by two different viruses, 13 plants by three viruses, 8 plants by four viruses and 3 plants by five viruses (Table 2). These data show that mixed infections with several viruses are very frequent. The interactions between viruses co-infecting the same plant could have an important impact in epidemics and could increase the damage in crops if synergisms arise (Syller, 2012). Testing an additional number of plants should confirm these results for the distribution of certain viruses, which was different in each province in Italy. Although ToCV

was spread throughout Sicily, ToTV and TICV were only found in the Ragusa Province. In addition, CMV and TSWV were more prevalent in Agrigento and Trapani. These different prevalences and infections in each region could be explained in part by the tomato genotypes, vectors and climatic conditions and in part by the different agronomical practices, as tomatoes in Ragusa Province are cultivated mainly under greenhouse conditions and in open fields in Agrigento and Trapani.

The procedure developed in this study based on two multiplex RT-PCR assays allows for the rapid identification of the main RNA viruses that infect tomato and for the analysis of mixed infections, thus saving time, reagents and costs. This methodology could be included in phytosanitary protocols for early detection of these viruses in nurseries or in certification programmes.

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Table 1. Designed primers, nucleotide sequences and sizes of amplicons for each viral species.

Primer	Virus	Nucleotide sequence (5' to 3')	GenBank (Acc. Number)	Genomic Position	Amplicon Size
TICV 1 TICV 2	TICV	TTGGCTGTGAGTCAAGGAGGT CTGATTTGATAGCCGATTTCCC	FJ815441	5414 RNA2 5528 RNA2	136
ToTV 1 ToTV 2	ToTV	TGGTGCTCAACAGTGCAATCA CACACTGCATCCACTTCTTCCA	DQ388879	859 RNA1 1026 RNA 1	189
ToCV 1 ToCV 2	ToCV	CATTCCGGCTAATCCTAATCGA CCCTAGTGGAGTGTACCTTCAATTTC	AY903448	4461 RNA2 4536 RNA2	101
TSWV 1 TSWV 2	TSWV	GCCATGGTCTTCTTCTGATGAA AGTTATTGTCCCCTGACCCTTC	S48091	161 RNA M 595 RNA M	451
CMV 1 CMV 2	CMV	ATTAACCACCCAACCTTTG TGGGAATGCGTTGGTGCTC	D10538	1413 RNA 3 1872 RNA 3	480
PepMV 1 PepMV 2 PepMV 3	PepMV	CATAGTTGTGCACGGAATTGC TTCCGTCTTGATACTGACCA TGCCGTCTTGATATTGGCCA	AF484251 HQ663892 AF484251	4503 5256 5260	778 782
ToMV 1 ToMV 2	ToMV	GATAATTTGATTGAAGATGAAGCC CTGTACACCTTATAAACATCGCC	AF332868	5644 5895	274

Table 2. Analysis of 45 tomato plants collected in Sicily using multiplex RT-PCR, singleplex RT-PCR and ELISA.

Geographic origin	multiplex RT-PCR							DAS-ELISA (1) and singleplex RT-PCR (2)						
	PepMV	TSWV	ToTV	ToCV	CMV	ToMV	TICV	PepMV (1,2)	TSWV (1,2)	ToTV (2)	ToCV (2)	CMV (1,2)	ToMV (1,2)	TICV (2)
Ragusa Province	+	-	+	-	-	+	+	+,+	-,-	+	-	-,-	+,+	+
	+	-	+	+	-	+	+	+,+	-,-	+	+	-,-	+,+	+
	+	-	-	+	+	-	+	+,+	-,-	-	+	+,+	-,-	+
	+	+	-	+	+	-	+	-,+	+,+	-	+	+.+	-,-	+
	-	+	-	+	-	-	-	-,-	+,+	-	+	-,-	-,-	-
	+	-	-	+	-	-	-	+,+	-,-	-	+	-,-	-,-	-
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	-	-	-	+	-	-	+	-,-	-,-	-	+	-,-	-,-	+
	-	-	-	+	-	-	-	-,-	,-,	-	+	-,-	-,-	-
	+	+	-	+	-	-	-	+,+	+,+	-	+	-,-	-,-	-
	+	-	-	-	-	-	-	+,+	-,-	-	-	-,-	-,-	-
	+	-	-	-	-	-	-	+,+	-,-	-	-	-,-	-,-	-
	-	-	-	+	-	-	+	-,-	-,-	-	+	-,-	-,-	+
Agrigento Province	+	-	-	+	+	+	-	+,+	-,-	-	+	+,+	+,+	-
	+	-	-	+	+	+	-	+,+	-,-	-	+	+,+	+,+	-
	+	+	-	-	+	+	-	+,+	+,+	-	-	+,+	+,+	-
	-	+	-	+	+	-	-	-,-	+,+	-	+	+,+	-,-	-
	-	+	-	+	+	-	-	-,-	+,+	-	+	+,+	-,-	-
	-	-	-	+	-	-	-	-,-	-,-	-	+	-,-	-,-	-
	-	+	-	+	-	+	-	-,-	+,+	-	+	-,-	+,+	-
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	-	-	-	+	-	-	-	-,-	-,-	-	+	-,-	-,-	-
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	-	+	-	+	+	+	-	-,-	+,+	-	+	+	+,+	-
	-	+	-	+	+	-	-	-,-	+,+	-	+	+	-,-	-
	-	+	-	-	+	+	-	-,-	+,+	-	-	+	+,+	-
	-	+	-	-	+	+	-	-,-	+,+	-	-	+	+,+	-
Trapani Province	-	-	-	+	+	-	-	-,-	-,-	-	+	+,+	-,-	-
	-	+	-	+	+	-	-	-,-	+,+	-	+	+,+	-,-	-
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	-	+	-	+	-	-	-	-,-	+,+	-	+	-,-	-,-	-
	-	-	-	-	-	-	-	-,-	-,-	-	-	-,-	-,-	-
	-	+	-	+	+	-	-	-,-	+,+	-	+	+,+	-,-	-
	-	+	-	+	+	-	-	-,-	+,+	-	+	+,+	-,-	-

Figure captions

Figure 1. Panel A: Multiplex PCR for the detection of PepMV, TSWV, ToTV and ToCV; lane M, molecular weight marker 1 kB plus (Invitrogen); lane 1, simultaneous detection of PepMV, TSWV, ToTV and ToCV; lane 2, specific detection of PepMV; lane 3, specific detection of TSWV; lane 4, specific detection of ToTV; lane 5, specific detection of ToCV; lane 6, negative control of a virus-free tomato plant. Panel B: Multiplex PCR for the detection of CMV, ToMV, TICV; lane M, molecular weight marker 1 kB plus (Invitrogen); lane 1, simultaneous detection of CMV, ToMV and TICV; lane 2, specific detection of CMV; lane 3, specific detection of ToMV; lane 4, specific detection of TICV; lane 5, negative control of a virus-free tomato plant.

Figure 2. Panel A: Multiplex PCR for the detection of PepMV, TSWV, ToTV and ToCV; lane M, molecular weight marker 1 kB plus (Invitrogen); lane 1 to lane 4, tenfold serial dilutions of PepMV target (from 1 ng to 10^{-4} ng), maintaining the TSWV, ToTV and ToCV target concentrations at 1 ng; lane 5, negative control of a virus-free tomato plant; lane 6 to lane 9, tenfold serial dilutions of TSWV target (from 1 ng to 10^{-4} ng), maintaining the PepMV, ToTV and ToCV target concentrations at 1 ng; lane 10, negative control of a virus-free tomato plant; lane 11 to lane 14, tenfold serial dilutions of ToTV target (from 1 ng to 10^{-4} ng), maintaining the PepMV, TSWV and ToCV target concentrations at 1 ng; lane 15, negative control of a virus-free tomato plant; lane 16 to lane 19, tenfold serial dilutions of ToCV target (from 1 ng to 10^{-4} ng), maintaining the PepMV, TSWV and ToTV target concentrations at 1 ng; lane 20, negative control of a virus-free tomato plant. Panel B: Multiplex PCR for the detection of CMV, ToMV, TICV; lane M, molecular weight marker 1 kB plus (Invitrogen); lane 1 to lane 4, tenfold serial dilutions of CMV target (from 1 ng to 10^{-4} ng), maintaining the ToMV and TICV target concentration at 1 ng; lane 5, negative control of a virus-free tomato plant; lane 6 to lane

9, tenfold serial dilutions of ToMV target (from 1 ng to 10^{-4} ng), maintaining the CMV and TICV target concentrations at 1 ng; lane 10, negative control of a virus-free tomato plant; lane 11 to lane 14, tenfold serial dilutions of TICV target (from 1 ng to 10^{-4} ng), maintaining the CMV and ToMV target concentrations at 1 ng; lane 15, negative control of a virus-free tomato plant.

Figure 1

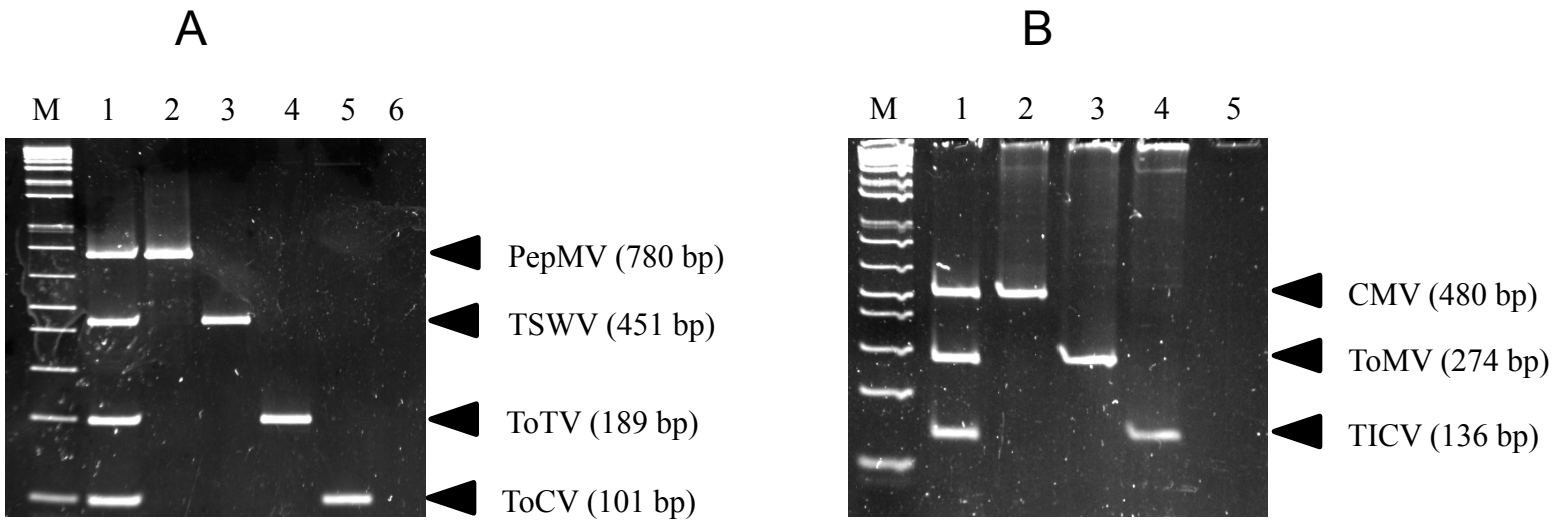
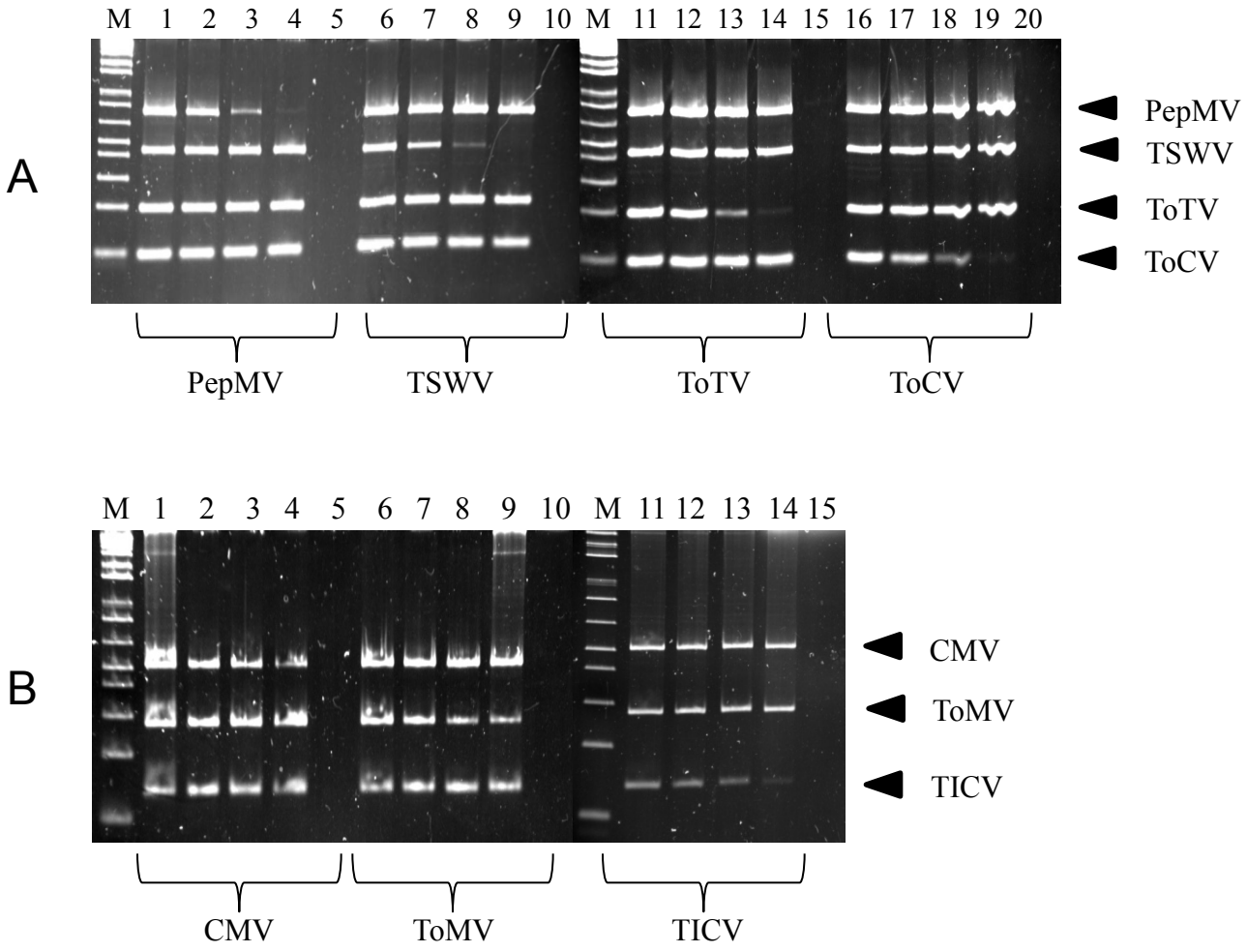

A
M 1 2 3 4 5 6
PepMV (780 bp)
TSWV (451 bp)
ToTV (189 bp)
ToCV (101 bp)
B
M 1 2 3 4 5
CMV (480 bp)
ToMV (274 bp)
TICV (136 bp)

Figure 2


A
M 1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15 16 17 18 19 20
PepMV
TSWV
ToTV
ToCV
PepMV
TSWV
ToTV
ToCV
B
M 1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15
CMV
ToMV
TICV
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