SIMULTANEOUS AND RAPID DIFFERENTIATION OF MEMBERS OF THE *TOMATO YELLOW LEAF CURL VIRUS* COMPLEX BY MULTIPLEX PCR

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SUMMARY

A multiplex PCR method was developed to identify simultaneously either single or multiple infections with members of the *Tomato yellow leaf curl virus* (TYLCV) complex. Several sets of species-specific primers, designed from sequenced genomes of TYLCV from the Mediterranean region (Sicily, Israel and Spain), were tested using plants infected with Egyptian and Tunisian isolates. The results show that this multiplex PCR is rapid, simple and a useful alternative to conventional tools such as PCR-RFLP or PCR-sequencing for typing virus isolates. Furthermore, this approach revealed evidence of recombination among Tunisian TYLCV genomes.

Key words: intergenic region, replicase gene, multiplex PCR, sequence, TYLCV.

INTRODUCTION

Several DNA-based methods such as molecular hybridization, PCR, RFLP and sequencing are much used in plant pathology and have considerably improved disease diagnosis. Since these methods can be costly and time consuming, a multiplex PCR procedure in which several target sequences can be amplified using several sets of primers, was developed for species and strains of the *Tomato yellow leaf curl virus* (TYLCV) complex. Recent studies have provided multiplex PCR protocols, which enhance specificities and sensitivities of detection of infectious agents mainly in the field of clinical and epidemiological virology (Bakaletz *et al.*, 1998; Echevarria *et al.*, 1998; Markoulatos *et al.*, 1999; Luo and Mitchell, 2002). Multiplex PCR has also been successfully applied in the detection of plant pathogens (Jacobi *et al.*, 1998; Nie and Singh, 2002; Shamloul *et al.*, 2002; Ito *et al.*, 2002).

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In Tunisia, tomato yellow leaf curl disease has become a major cause of losses in tomato crops located mainly in open fields and greenhouses in the south and the Sahel (central eastern area) regions of the country. TYLCV was suspected to be the causal agent in the early 1980's (Cherif and Russo, 1983). Its presence was first confirmed in 2002 using molecular procedures (Fekih-Hassan *et al.*, 2003). Detection and differentiation of the species and recombinant strains of the TYLCV complex (Accotto *et al.*, 2000) were always based on PCR coupled either with RFLP or sequencing methods. Moreover, separate parts of the genome such as the conserved coat protein (CP) gene, the replication-associated protein (Rep) gene or the variable intergenic region (IR) need to be characterized for each isolate. A single procedure that is able to simultaneously detect and type TYLCV species is preferable. Multiplex PCR has been successfully applied using one antisense common primer combined with a set of strain-specific sense primers to distinguish among RNA viruses (Nie and Singh, 2002). In this study, we have used this approach for the first time to distinguish among TYLCV species. This assay, involving one step of PCR, has been optimized as a fast, reliable and effective method for detecting and typing Tunisian isolates of the TYLCV complex. The results have allowed further characterization of the genetic diversity of these virus isolates.

MATERIALS AND METHODS

TYLCV isolates. Begomoviral isolates were collected during the 2001 and 2002 growing seasons from plants with typical yellow leaf curl symptoms from several areas in Tunisia. These isolates were previously characterized by PCR amplification and sequencing of both the coat protein gene and the intergenic region (Gorsane *et al.*, 2004). Sequence analysis suggested that these Tunisian isolates cluster with a Sicilian isolate of Tomato yellow leaf curl Sardinia virus (TYLCSV-Sic) (Z28390). A tomato sample infected by an Egyptian isolate was used as a control (Nakhla *et al.*, 1993).

DNA isolation. Total DNA was extracted from leaf

samples by a modified Dellaporta *et al.* (1983) method. Samples of 5 mg leaf tissue were extracted with 1 ml of buffer (50 mM EDTA, 100 mM Tris-HCl, 500 mM NaCl, 10 mM b-mercaptoethanol), vortexed and allowed to stand at 65°C for 10 min. After adding 1/5 volume of potassium acetate (5 M, pH 8), the mixture was incubated on ice for 10 min and clarified by centrifugation at 13,000 rpm for 20 min at 4°C. An equal volume of isopropanol was added to the supernatant fraction, and the mixture was incubated for 10 min at -20°C and centrifuged for 10 min at 13,000 rpm. The pellet was resuspended in sterile water and treated with RNase followed by phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol extraction (24:1). After precipitation with three volumes of ethanol for 30 min at -20°C and centrifugation for 10 min at 13,000 rpm, DNA was washed with 70% ethanol and resuspended in sterile water.

Primers. Species-specific primer sets were designed based on sequences of the Mediterranean complex of TYLCV species retrieved from GenBank database: TYLCSV-Sic (Z28390, Crespi *et al.*, 1995), TYLCV (X15656) and TYLCV-Mal (AF271234). Sequences were aligned using the DNAMAN software package program (Lynnon BioSoft, Quebec City, Canada). Three specific sense primers and one common degenerate complementary-sense primer were designed; their positions are nucleotides from the beginning of the virus genome. One sense primer, PsSic 2267, is located in the Rep gene of the Sicilian strain (position 2267), the second sense primer PsIs 2689 in IR of the Israeli strain (position 2689) and the third, PsSp 2607, in the IR of the Spanish strain. The common reverse degenerate primer PcRvc 397 (position 397) is located in the CP gene.

An additional set of primers including a complementary sense (M15) and a sense degenerate (PRepv1978) primer 5'GCCCACAT(T/C)GTCTT(T/C)CC(A/C/G/T) GT3' (Potter *et al.*, 2003) was used for cloning and sequencing purposes. The complementary strand primer M15: 5'AAAGGATCCCACATATTG3' is situated downstream of the IR and was similar to that used to amplify the IR of Tunisian isolates (Fekih-Hassen *et al.*, 2003).

PCR. For PCR with individual primer pairs, each reaction mixture contained 200 ng of tomato DNA, 10 µM of each primer, 10 mM (each of the four nucleotides), 0.5 U of *Taq* DNA polymerase (Promega, Madison, USA), 1.5 mM $MgCl_2$ in a total volume of 25 µl. PCR conditions were: 3 min of denaturation at 95°C, followed by 35 cycles at 95°C for 50 sec, 55°C for 50 sec and 72°C for 1 min then a final extension step at 72°C for 10 min.

Multiplex PCR. Multiplex PCR reaction contained three sense primers mixed with the PcRvc 397 to produce amplicons with different sizes and gel migration to identify the three target viruses. The 25 µl PCR mixture contained 1 U of *Taq* DNA polymerase (Promega, Madison, USA); all other reagents were the same as described above for single primer pair PCR except the concentration for PcRvc 397 was doubled. Conditions were standardized in a Perkin-Elmer thermal cycler: 5 min of denaturation at 95°C followed by 35 cycles at 95°C for 50 sec, 53°C and 72°C for 1 min followed by a last step at 72°C for 10 min. Hot start was performed to eliminate non-specific reactions.

Amplified products were analysed by electrophoresis in 1.5% agarose gel containing 0.5 µg ml^{-1} ethidium bromide which provided a good resolution for multiplex PCR products.

Cloning and sequencing. The PCR-amplified PRepv1978-M15 product was excised from agarose gel and purified using the GeneClean kit (Qiaex II, gel extraction kit, Qiagen, Valencia, USA). DNA was cloned into pGEM-T easy plasmid vector (Promega, Madison, USA) following the manufacturer's instructions. Three PCR derived clones with an insert of the expected size were selected for sequencing in both directions using dye terminator cycle sequencing. The obtained sequences were compared with those of other begomoviruses retrieved from GenBank database. The sequences of amplification primers were not taken into account in sequence analysis.

RESULTS

Primer design. Sequences of three divergent members of the TYLCV complex were aligned to identify potential conserved regions suitable as a complementary-sense primer for the three viruses. The choice of the primer set used in multiplex PCR is crucial to decrease the possibility of the formation of non-specific products and to allow amplification of virus-specific fragments different in size and easily distinguishable by gel electrophoresis. Primers should not have significant sequence identity and selection criteria for each primer included either their length (26 to 27 bp) or their % of G+C content.

A degenerate complementary-sense primer was designed from the start of the CP gene. Since this primer is common to the three viruses: TYLCV, TYLCSV-Sic and TYLCSV-Mal, three sense primers were designed to target each of them. The selected primers, their sequences, locations and targets as well as the size of the expected PCR amplicons are presented in Table 1. The specificities of these primers were tested by uniplex and multiplex PCR.

Suitability of primers: application to the Egyptian isolate of TYLCV. To explore the ability of our method to detect and differentiate among Tunisian virus isolates

Table 1. Primer sets designed to amplify DNA from the Mediterranean complex of TYLCV species.

Primer name	Sequence 5' to 3'	TYLCV species	GenBank accession number	Amplicon size (bp)
Sic 2267	TGGAAAGTTCCCCATTCAAGAACATC	Sicily	Z28390	891
Is 2689	ATACTTGGACACCTAATGGCTATTTGG	Israel	X15656	497
Sp 2607	GGAAGCGCTTAGGGGGAGCCATATGG	Spain	AF271234	559
Rvc 397	TGCCTTGGACA(A/G)TGGGG(A/G)CAGCAG	common	_	

associated with the Tomato yellow leaf curl disease, we used an Egyptian isolate of TYLCV (TYLCV-[EG]) as a positive control. With uniplex PCR, each sense primer was individually coupled with PcRvc 397 whereas in multiplex PCR, all primers were mixed together in a single reaction (Fig. 1). Uniplex PCR resulted in an amplified fragment of 497 bp when using PsIs 2689 while amplification failed when using either PsSic 2267 or PsSp 2607. Multiplex PCR reaction using all primers combined resulted in the production of a TYLCV-specific product size (497 bp) indicating the high specificity of the designed primers and the absence of any interaction or interference between them.

Application to Tunisian viral isolates. DNA was extracted from a collection of 11 samples corresponding to freeze-dried tomato (8), pepper (2) and bean (1) leaves harvested from the Sahel (central eastern area) and the south of Tunisia. All samples belong to local Tunisian varieties and were previously selected on the basis of symptom observation and DNA hybridization (Fekih-Hassen *et al.*, 2003). The genetic diversity of these Tunisian isolates was further characterized by PCR-sequencing of both the CP gene and the virus IR (Gorsane *et al.*, 2004). In uniplex PCR, the use of the PsSic 2267 primer gave the expected 891 bp amplicon. Besides this product, we detected a fragment of 559 bp when using PsSp 2607. Multiplex PCR, combining all primers, yielded both fragments. However, the amount of the 559 bp fragment was less than the amount of the 891 bp fragment suggesting a competition between primers to anneal the target DNA (Fig. 2). The use of PsIs 2689 failed to amplify any fragment in uniplex PCR or in multiplex PCR for all Tunisian isolates. PCR assays were negative with healthy or asymptomatic samples (data not shown) for which TYLCV diagnosis was not confirmed by other techniques such as molecular hybridization or PCR amplification.

Application to mixed isolates. The feasibility of dif-

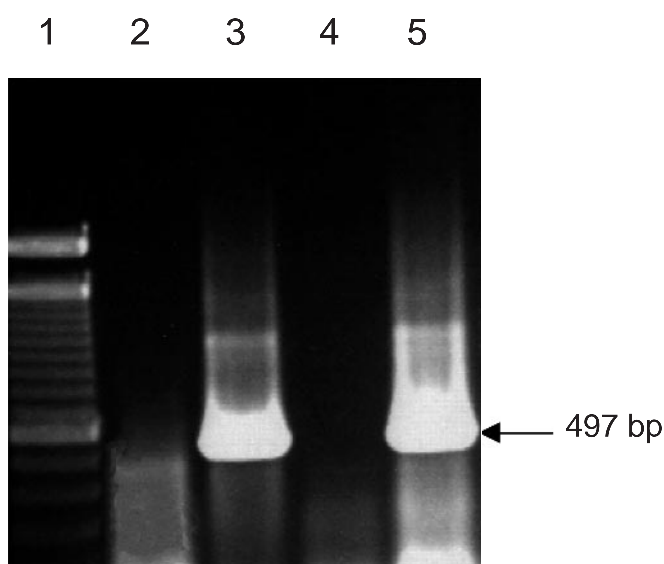

Fig. 1. Uniplex and multiplex PCR using the genomic DNA from a TYLCV-[EG] isolate. Lane 1, 100 bp ladder (Gibco-BRL); Lanes 2 to 4, uniplex PCR performed respectively with sense primers PsSic 2267, PsIs 2689, PsSp 2607 and combined individually with the common reverse PcRvc 397 primer; Lane 5, multiplex PCR combining all primers.

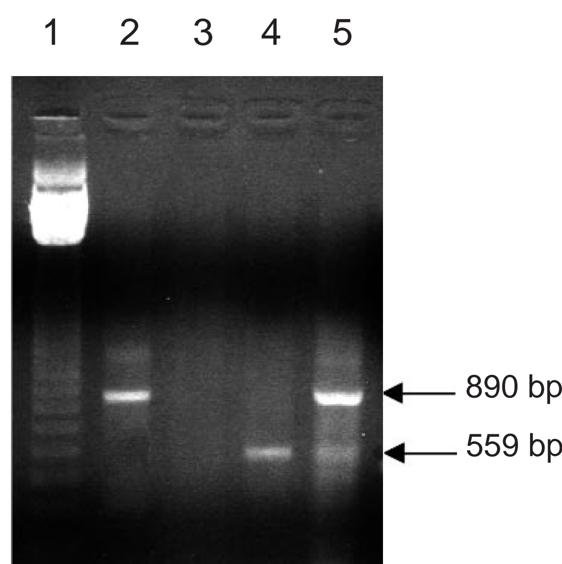

Fig. 2. Uniplex and multiplex PCR using the genomic DNA from a set of Tunisian TYLCV isolates. Lane 1, 100 bp ladder (Gibco-BRL); Lanes 2 to 4, uniplex PCR performed respectively with sense primers PsSic 2267, PsIs 2689, PsSp 2607 and combined individually with the common reverse PcRvc 397 primer; Lane 5, multiplex PCR combining all primers.

ferentiation among strains of TYLCV was demonstrated by the results described above. Possible competition and interference of primers could occur and affect identification when plants with mixed infections are assayed. Therefore, to test the efficiency of this procedure, we used a virus DNA pool prepared by mixing the TYLCV-[EG] DNA with a Tunisian sample that tested positive for TYLCV-Sic and TYLCV-Mal. With uniplex PCR, the three primer sets for TYLCSV-Sic, TYLCSV-Spa and TYLCV amplified PCR fragments of 891 bp, 497 bp and 559 bp respectively. Faint, low molecular weight PCR bands were obtained with the PsSic 2267/PcRvc 397 primers but these would not lead to false diagnosis. Multiplex PCR yielded the three viral amplicons simultaneously, although the viral 891 bp fragment was in low concentration (Fig. 3).

PCR amplification and sequencing of M15-PRepv 1978 viral fragment. We have previously sequenced both IR and CP gene of eleven Tunisian TYLCV isolates from Tunisian crops and demonstrated that they are representative of one spreading TYLCSV-Sic strain regardless of their host and area of origin. As expected, all corresponding samples tested positive with the PsSic 2267 primer; and surprisingly, also yielded an amplicon with a correct size when tested with the PsSp 2607 primer in uniplex and multiplex PCR. To investigate if this result was due to the occurrence of Sicilian and Spanish strains in a mixed infection or to a recombination within a viral genome, we performed PCR amplification of a fragment overlapping the IR and the Rep gene using M15 and PRepv1978. The downstream primer targets a Sicilian TYLCV strain and the upstream degenerate primer targets a conserved region in the Rep gene and was predicted to anneal to all DNA of begomoviruses. PCR products were cloned, and sequenced. The sequence contained PCR primer sequences (M15 and PRepv1978), cis-acting elements involved in DNA replication such as TATA box for the Rep gene and a conserved stem-loop motif with the conserved nonanucleotide sequence (Fig. 4). It also contained a sequence which is 78% homologous to the PsSp 2607 primer confirming previous amplification of the viral 559 bp product in uniplex and multiplex PCR when using this primer.

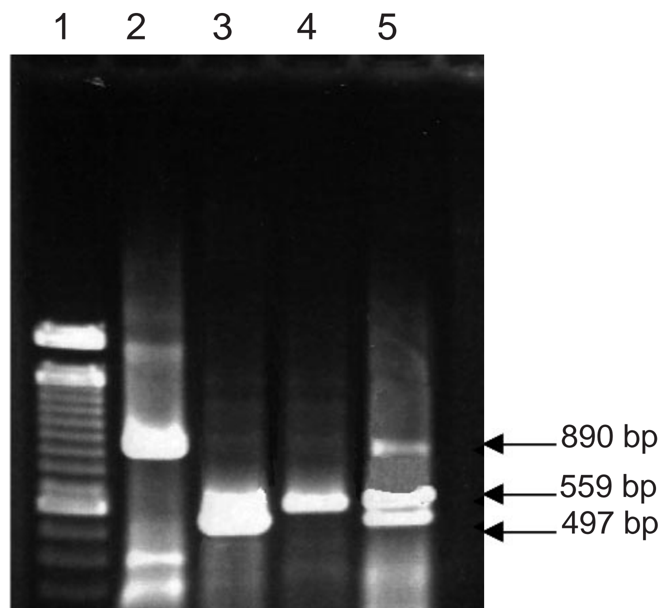

Fig. 3. Uniplex and multiplex PCR using the virus pool mixing the TYLCV-[EG] and the Tunisian isolate DNA. Lane 1, 100 bp ladder (Gibco-BRL); Lanes 2 to 4, uniplex PCR performed respectively with sense primers PsSic 2267, PsIs 2689, PsSp 2607 and combined individually with the common reverse PcRvc 397 primer; Lane 5, multiplex PCR combining all primers.

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1     GCCCACATCG TCTTCCCTGT CCGGCTGTCA CCCTCAATCA CTATACTCAC CGGTCTCCAA
61    GGCCGCGCAG CGACATCCAT GACGTTCTCG GAAACCCAGT GTTCAAGTTC ATCCGGAACT
121   TGATCGAAAG AAGAAGATAA AAAAGGAGAA ACATATGGTG CCGGAGGCAC CTGGAAAACC
181   ATATCTAAAT TACTACTTAT ATTATGAAAA TGTAAAATGT AATCTCTAGG CGCTAATTCT
241   TTAATTACAT CAAGAGCCTC CGACTTACTT CTTGCCGTTA ATTGCCTTTG CGTAAGCGTC
301   GTTGGCTGTC TGCTGTCCTC CCCTTGCAGA TCGTCCGTCG ATCTGGAAAG TACCCCATTC
361   AAGAACATCT CCGTCCTTGT CGATGTAGGA CTTGACGTCG GAGCTTGATT TAGCTCCCTG
421   AATGTTCGGA TGGAAATGTG CTGATCTGGT TGGGGATACC AGATCGAAGA ATCTGTTATT
481   TTTGCAGTTG AATTTACCCT CGAACTGAAT GAGCATGTGG AGATGAGGTT GCCCATCTTC
541   GTGTAATTCT CTGCAAATCT TAATGTATTT TTTATTTGTT GGTGTTTGTA GTTGAAGTAG
601   TTGTTCTAGG GCTTCTTCTT TGGAGAGAGA ACATTTTGGG AAAGTTAGAA AATAATGTTT
661   TGCATTTATT TGAAAACGCT TAGGCTGAGC CATTTGGTCA ATGGGTACCA ATTGACCTCA
721   GTTTCATTTT ATTCCATGTA TTGGTAGATT GGTAGCTTAT TTATATGTTG GGTACTAAAT
781   GGCATAGATG TAATTATTCA AAGTAATAAA TTTATTTTTT AAATTTTTTT TTGGTAAGGC
841   GGCCATCCGT CTAATATTAC CGGATGGCCG CGCTACCCGA TAAAGAAGTG GGCCCTATGC
901   AGTAATTTAT GTCGACCAAT GAAATTGCAG CCTCAGAGCT TATATAACTG TTTAGCTTTG
961   TTATAAACTT GCTCCCTAAG TTTTAAAAAT ACACAATATGT GGGATCCTTT
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Fig. 4. Nucleotide sequence of the M15-PRepv 1978 fragment. Primers used in PCR amplification are indicated in bold letters. The nonanucleotide sequence is boxed.

This fragment was divided into two parts according to analysis of sequence alignments. The first region encompassing the Rep gene was submitted to the GenBank database. The highest percentage of homology (98%) was with a TYLCSV-Almeria strain (L27708) and a TYLCSV-Sic strain (95% homology) while lower homology (79%) was obtained with the TYLCSV-Mal strain (AF271234). The IR did not follow such pattern of sequence homology since it is 97% identical to the TYLCSV-Sic strain while identity values were below 85% for TYLCV-Mal and TYLCSV-Almeria strains.

DISCUSSION

In recent years, sequence analysis has shown that considerable sequence diversity exists among members of the TYLCV complex. It is very difficult to correlate a given symptom with a particular virus strain since all virus isolates are associated with yellow leaf curl symptoms. Moreover, symptoms seem to be greatly affected by environmental conditions. Molecular tools for identification and clustering isolates into one or other virus species were based essentially on DNA hybridization and PCR amplification coupled to sequencing. Although these methods have been very useful, each one identifies only one species at a time and requires to be coupled with another technique thereby incuring time and expense. Consequently, an alternative to these procedures is required for rapid and reliable screening of isolates related to known members of the TYLCV complex. For this, we used multiplex PCR that seemed particularly promising because of its simplicity and specificity. This method combines three specific sense primers for tomato-infecting begomoviruses, frequently encountered Mediterranean regions, including TYLCV, TYLCSV-Sic and TYLCSV-Mal.

Multiplex PCR was developed using two control target DNA. The first corresponded to a TYLCV-[EG] isolate and the second consisted of eleven Tunisian isolates of TYLCSV-Sic. All primers used gave similar amplification efficiencies for their respective targets in both uniplex and multiplex PCR. The validation of the sensitivity and the specificity of this procedure was done by using DNA pooled for two distinct viral species: TYLCV-[EG] and TYLCSV-Sic. Each one of the sense primers behaved as expected when paired with the complementary sense PcRvc 397 primer and resulted in a specific virus amplification of the target DNA. Furthermore, we have been able to amplify simultaneously multiple specific fragments from both genomes when all primers were combined in multiplex PCR. Although non-specific fragments were sometimes detected, they seem not to interfere with the identification of the begomovirus isolates, because of their size and weak intensity.

When applied to the Tunisian samples, multiplex PCR assay results correlate with those obtained in uniplex PCR combining each sense primer individually with the common complementary-sense primer. Moreover, these results are consistent with the previous identification of these isolates as members of the TYLCSV-Sic group. We also performed the sequencing of a viral fragment that overlaps the Rep gene and the intergenic region (IR). This part of the TYLCSV Tunisian genome seems to be a hybrid between two strains. The intergenic region, as expected, is remarkably similar to that of the Sicilian strain (97% homology) whereas the Rep gene was 98% identical to a Spanish strain from Almeria. Further analysis demonstrated that the 5' half IR region was 93% identical to TYLCSV-Almeria but the remainder of this sequence was quite different (78% homology). The retention of the 5' part of the IR region with the corresponding Rep sequence draw attention to functional constraint since the N-terminal region of the Rep protein seems to interact specifically with the 5' proximal part of the IR region during replication (Eagle *et al.*, 1994; Orozco *et al.*, 1997). The putative recombination cross-over point was not defined precisely because of the occurrence of identical sets of nucleotides in the involved region. Recombination is not a rare event among begomoviruses and seems to contribute significantly to increased genetic variability of virus genomes leading to the emergence of virulent or well-adapted strains (Harrison and Robinson, 1999; Padidam *et al.*, 1999; Navas-Castillo *et al.*, 2000; Monci *et al.*, 2002; Chatchawankanphanich and Maxwell, 2002).

Such findings revealed another important feature of the multiplex PCR technique, which consists in highlighting potential recombination within virus genomes. Subsequent studies have revealed that recombinant *Potato virus Y* isolates can be detected by this technique (Moravec *et al.*, 2003). Multiplex methodology is thus a valuable tool for one-step identification and typing of viral agents. On the other hand, the identification of such a recombinant strain in Tunisia is of great importance to improve TYLCV diagnosis and to manage effective control strategy based on breeding. This awaits additional knowledge of the genetic viral diversity of the Tunisian isolates of the TYLCV complex by sequence analysis of the complete genome of a large number of virus isolates.

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REFERENCES

Accotto G.P., Navas-Castillo J., Noris E., Moriones E., Lourou D., 2000. Typing of tomato yellow leaf curl virus in Europe. *European Journal of Plant Pathology* **106**: 179-186.

Bakaletz L.O., White G.J., Post J.C., Ehrlich G.D., 1998. Blinded multiplex PCR analyses of middle ear and nasopharyngeal fluid from chinchilla models of single-and mixed-pathogen-induced otitis media. *Clinical and Diagnostic Laboratory Immunology* **5**: 219-224.

Chatchawankanphanich O., Maxwell D.P., 2002. *Tomato leaf curl Karnataka virus* from Bangalore, India appears to be a recombinant Begomovirus. *Phytopathology* **92**: 637-645.

Cherif C., Russo M., 1983. Cytological evidence of the association of a Geminivirus with the Tomato Yellow Leaf Curl Disease in Tunisia. *Phytopathologische Zeitschrift* **108**: 221-225.

Crespi S., Noris E., Vaira A., Accotto G.P., 1995. Molecular characterization of cloned DNA from a tomato yellow leaf curl virus from Sicily. *Phytopathologia Mediterranea* **34**: 93-99.

Dellaporta S.L., Wood J., Hicks J.B., 1983. A plant DNA minipreparation version II. *Plant Molecular Biology Reporter* **1**: 19-21.

Eagle P.A., Orozco B.P., Hanley-Bowdoin L., 1994. A DNA sequence required for geminivirus replication also mediates transcription regulation. *Plant Cell* **6**: 1157-1170.

Echevarria J.E., Erdman D.D., Swierkosz E.M., Holloway B.P., Anderson L.J., 1998. Simultaneous detection and identification of human parainfluenza viruses 1,2 and 3 from clinical samples by multiplex PCR. *Journal of Clinical Microbiology* **36**: 1388-1391.

Fekih-Hassan I., Gorsane F., Djilani F., Fakhfakh H., Nakhla M., Maxwell D., Marrakchi M., 2003. Typing of tomato yellow leaf curl in Tunisia. *EPPO Bulletin* **33**: 347-350.

Gorsane F., Fekih-Hassan I., Nakhla M.K., Fakhfakh H., Maxwell D.P., Marrakchi M., 2004. Molecular evidence of tomato yellow leaf curl virus-Sicily spreading on tomato, pepper and bean in Tunisia. *Phytopathologia Mediterranea* **43**: (in press).

Harrison B.D., Robinson D.J., 1999. Natural genomic and antigenic variation in Whitefly-transmitted Geminiviruses (Begomoviruses). *Annual Review of Phytopathology* **37**: 369-398.

Ito T., Ieki H., Ozaki K., 2002. Simultaneous detection of six citrus viroids and *Apple stem grooving virus* from citrus plants by multiplex reverse transcription polymerase chain reaction. *Journal of Virological Methods* **106**: 235-239.

Jacobi V., Bachand G.D., Hamelin R.C., Castello J.D., 1998. Development of a multiplex immunocapture RT-PCR assay for detection and differentiation of tomato and tobacco mosaic tobamoviruses. *Journal of Virological Methods* **74**: 167-178.

Luo G., Mitchell T.G., 2002. Rapid identification of pathogenic fungi directly from cultures by using Multiplex PCR. *Journal of Clinical Microbiology* **40**: 2860-2865.

Markoulatos P., Samara V., Siafakas N., Plakokefalos E., Spyrou N., Moncany M.L., 1999. Development of a quadriplex polymerase chain reaction for human cytomegalovirus detection. *Journal of Clinical Laboratory Analysis* **13**: 99-105.

Monci F., Sanchez-Campos S., Navas-Castillo X., Moriones E., 2002. A natural recombinant between the Geminivirus *Tomato yellow leaf curl Sardinia virus* and *Tomato yellow leaf curl virus* exhibits a novel pathogenic phenotype and is becoming prevalent in Spanish populations. *Virology* **303**: 317-326.

Moravec T., Cerovska N., Boonham N., 2003. The detection of recombinant, tuber necrosing isolates of *Potato Virus Y* (PVY^{NTN}) using a three-primer PCR based in the coat protein gene. *Journal of Virological Methods* **109**: 66-69.

Nakhla M.K., Mazyad H.M., Maxwell D.P. 1993. Molecular characterization of four tomato yellow leaf curl isolates from Egypt and development of diagnostic methods. *Phytopathologia Mediterranea* **32**: 163-173.

Navas-Castillo J., Sanchez-Campos S., Noris E., Louro D., Accotto G.P., Moriones E., 2000. Natural recombination between Tomato yellow leaf curl virus-Is and Tomato leaf curl virus. *Journal of General Virology* **81**: 2797-2801.

Nie X., Singh R.P., 2002. A new approach for the simultaneous differentiation of biological and geographical strains of *Potato virus Y* by uniplex and multiplex RT-PCR. *Journal of Virological Methods* **104**: 41-54.

Orozco B.M., Miller A.B., Settlage S.B., Hanley-Bowdoin L., 1997. Functional domains of a geminivirus replication protein. *Journal of Biological Chemistry* **272**: 9840-9846.

Padidam M., Sawyer S., Fauquet C.M., 1999. Possible emergence of new geminiviruses by frequent recombination. *Virology* **265**: 218-225.

Potter J.L., Nakhla M.K., Mejia L., Maxwell D.P., 2003. PCR and DNA hybridization methods for specific detection of bean-infecting begomovirus in the Americas and Caribbean. *Plant Disease* **83**: 29-32.

Shamloul A.M., Faggioli F., Keith J.M., Hadidi A., 2002. A novel multiplex RT-PCR probe capture hybridization (RT-PCR-ELISA) for simultaneous detection of six viroids in four genera: *Apscaviroid, Hostuviroid, Pelamoviroid and Pospiviroid. Journal of Virological Methods* **105**: 115-121.

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