Tropical Plant Pathology, vol. 35, 1, 043-047 (2010)

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SHORT COMMUNICATION / *COMUNICAÇÃO*

Development of a species-specific detection method for three Brazilian tomato begomoviruses

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ABSTRACT

The begomoviruses (fam. *Geminiviridae*) have an ssDNA genome, are transmitted by whiteflies, and cause significant losses in tomato fields in Brazil. Nowadays, begomovirus species identification is carried out through the analysis of their complete genome sequence. Due to the high costs and difficulty in applying this technique to large-scale analysis, we aimed to develop a species-specific detection method based on PCR. Three species were targeted for amplification: Tomato severe rugose virus *(ToSRV), Tomato rugose mosaic virus* (ToRMV) and Tomato yellow vein streak virus (ToYVSV). Thirteen primer combinations were designed, and four were finally selected and tested against a group of 82 samples, including cloned DNA and viral DNA from infected plants previously identified by direct sequencing of PCR products. Three primer combinations were selected that could distinguish the three species, and confirmed by sequencing of amplified products. These combinations were validated by evaluation of field-collected tomato samples infected by begomovirus and this demonstrated that PCR can be a useful tool to distinguish the viruses and detect mixed infections caused by three begomovirus species.
Keywords: *Solanum lycopersicum,* PCR detection, geminivirus, ToSRV, ToYVSV.

RESUMO

Desenvolvimento de método de detecção específico à espécie para três begomovirus no Brasil

Os begomovírus (fam. *Geminiviridae*) possuem um genoma de ssDNA, são transmitidos por mosca-branca, e causam danos importantes em campos de produção de tomate no Brasil. Atualmente, a identificação de espécies de begomovírus é realizada pela análise da seqüência completa do genoma. Devido ao alto custo e dificuldade em se aplicar essa técnica para análises em larga escala, o objetivo desse trabalho foi desenvolver um método de detecção espécie-específico baseado em PCR. Três espécies foram avaliadas para amplificação: *Tomato severe rugose virus* (ToSRV), *Tomato rugose mosaic virus* (ToRMV) e Tomato yellow vein streak virus (ToYVSV). Foram testadas treze combinações de primers, e quatro foram selecionadas e avaliadas em um grupo de 82 amostras, incluindo DNA clonado e DNA viral total extraído de plantas infectadas com begomovírus previamente identificados por seqüenciamento direto de produto de PCR. Três combinações de primers capazes de distinguir as três espécies foram selecionadas, e confirmadas por seqüenciamento dos produtos amplificados. Estas combinações foram validadas com amostras de tomate infectadas por begomovírus em campo, demonstrando que a PCR pode ser uma ferramenta útil para separar as espécies virais e detectar infecções mistas causadas pelas três espécies de begomovírus.
Palavras-chave: *Solanum lycopersicum,* detecção por PCR, geminivírus, ToSRV, ToYVSV.

The genus *Begomovirus* encompasses geminivirus species with one or two genomic components, denominated DNA-A and DNA-B, and they are transmitted by whiteflies (Homoptera: Aleyrodidae) to dicotyledonous plants. The DNA-A component codes the coat protein "cp" (AV1), replication-associated protein "rep" (AC1), transactivation protein "trap" (AC2) and replication enhancer protein "ren" (AC3), and has an intergenic region between *cp* and *rep* genes. Begomovirus occurrence has become frequent on the tomato crop in Brazil since the 90's, always associated with the increasing population of whiteflies (Ribeiro et al., 2003). There is an extensive list of begomovirus species reported in tomatoes in Brazil, five of which are accepted as definitive species: *Tomato golden mosaic virus* (TGMV), *Tomato severe rugose virus* (ToSRV), *Tomato rugose mosaic virus* (ToRMV), *Tomato chlorotic mottle virus* (ToCMoV) and *Tomato yellow spot virus* (ToYSV), and at least twelve as tentative species (Castillo-Urquiza et al., 2008; Fauquet et al., 2008; sequence databases).

The increasing importance of molecular techniques for virological studies is clearly seen in the begomovirus-tomato pathosystem. Nowadays, detection of begomovirus infection is done by either hybridization or PCR. Identification, however, relies on the analysis of the total genomic sequence (of DNA-A component) or, preliminarily, by comparison of the partial sequence of DNA-A including the common region (CR), and of the regions coding the *cp* and *rep* genes. For the genus *Begomovirus*, the threshold

value of 89% nucleotide identity of the complete DNA-A genome is one of the most important criteria for species demarcation (Fauquet et al., 2008). Earlier studies showed that the begomovirus *cp* gene is highly conserved, while the amino-terminal region of the *cp* (60 to 70 amino acids) is more variable and is thought to represent the genome as a whole (Padidam et al., 1995). PCR analysis using the universal primers suggested by Rojas et al. (1993), particularly the primer pair pARc715 and pALv1978, enables a comparison of this terminal *cp* 5' end, the intergenic region and the terminal *rep* 5' end. This analysis has been used for diversity study on Brazilian begomovirus (Faria & Maxwell, 1999; Ribeiro et al., 2003; Fernandes et al., 2008). Thus far, it has only been possible after sequencing analysis of the amplified DNA fragment. Due to the lack of an easy species-discriminating tool, this study aimed to develop a detection method based on PCR for the two most prevalent viruses in the central part of Brazil (*Tomato severe rugose virus*, Tomato yellow vein streak virus), and additionally for *Tomato rugose mosaic virus*, a virus sharing high identity with *Tomato severe rugose virus* although not widely distributed.

Nucleotide sequence of PCR products after reaction using primers pARc715 and pALv1978 (Rojas et al., 1993) of many tomato samples were analyzed (Fernandes et al., 2008). Nine ToSRV-like sequences were randomly selected and aligned with six ToYVSV-like sequences using ClustalW (Thompson et al.,1994), one ToCMoV sequence (NC003664), one ToYVSV sequence (AY751742), one ToSRV (AY029750) sequence and one ToRMV sequence (AF291705). Conserved regions for each virus and divergent for different viruses were selected for designing eleven species-specific primers for *Tomato severe rugose virus* and Tomato yellow vein streak virus with the program Oligo Analyzer (http://biotools.idtdna.com/analyzer). The primers were ToYVSV1f (AAG GCS TTG GAT AGA TTT TC), ToYVSV6f (GAT GAG ACC CGA TAG CTC), ToYVSV2r (AGC ATC CCG CTT GGG TAT A), ToYVSV3r (AAG TAT GTC GAC CAC TTA), ToYVSV4r (TCA CTC ACA GTG GGA CCA CA), ToYVSV5r (ATT CCC CAA TTC ATT TCG CC), ToYVSV6r (GAG CTA TCG GGT CTC ATC), ToSRV1f (AAG GCG ACG TCT TTG GAA GG), ToSRV7f (GTG CTT CTT CTT TCG ATA AT), ToSRV2r (CTC AGC GGC CTT GTT ATA TTT) and ToSRV5r (CTA CCA TTA CTA CCA ATT C). As *Tomato severe rugose virus* DNA-A (AY029750) shares 88% nucleotide identity with *Tomato rugose mosaic virus* (AF291705), and 81% in the *cp* gene nucleotide sequence, thus being possible that non-specific amplifications could occur, we designed a reverse primer with high specificity to the ToRMV sequence, ToRMV1r (AGGCCCGATGCTAAATGGG). This primer was tested in combination with ToSRV1f primer.

The PCR reactions consisted of a final volume of 10 μL, containing 1 μL (ca. 20ng) of total DNA extracted according to Doyle & Doyle (1983), 1 μL of 10X *Taq* buffer (Invitrogen), 0.35 μL of 50mM $MgCl_2$, 0.4 μL of 2.5mM dNTPs (Pharmacia), 20ng of each primer, and 0.2U of *Taq* polymerase (Invitrogen). PCR reactions were carried out in plates (Eppendorf) using the Mastercycler (Eppendorf) with an initial heating step of 94ºC for 3 minutes and 30 cycles of: denaturing (94ºC/1 minute), primer annealing (50ºC/1 minute) and extension (72ºC/4 minutes), followed by a final extension of 72ºC for 5 minutes. The results were analyzed through electrophoresis in agarose gel (1.3%). As controls, cloned ToSRV (baccatum isolate), ToYVSV (1799 isolate) and ToRMV (provided by Francisco Murilo Zerbini, UFV) were used for PCR condition optimization. Primer testing was done with samples: ToSRV-856, ToSRV-859, ToSRV-860, ToSRV-877 and ToSRV-894 (partial PCR-based sequence with high identity to ToSRV), ToYVSV-1663, ToYVSV-1669, ToYVSV-1673, ToYVSV-1678 and ToYVSV-1776 isolates (high identity to ToYVSV), one sample infected by begomovirus (control positive) and water (negative control). Example of an agarose gel electrophoresis of amplified products is shown in Figure 1.

Initially, the reactions were done with the annealing temperature of 50ºC for all primer combinations. Among the twelve specific combinations (4 for ToSRV and 8 for ToYVSV), nine combinations were eliminated in the first trial because either they were not specific and amplified DNA from other begomoviruses or they did not amplify the specific DNA (data not shown). Therefore, the combinations ToSRV1f-ToSRV2r (amplicon with 820bp), ToYVSV1f-ToYVSV4r (amplicon with 579bp) and ToYVSV1f-ToYVSV6r (amplicon with 435bp) were used in subsequent reactions. As non-specific amplifications were still present using 50ºC of annealing temperature, further testing with temperatures of 55, 60, 62 and 65ºC was carried out. Primer pairs ToYVSV1f-ToYVSV4r and ToYVSV1f-ToYVSV6r amplified only the specific ToYVSV DNA sample at the annealing temperature of 60ºC and 62ºC, and the pair ToSRV1f-ToSRV2r was specific to ToSRV DNA sample with the annealing temperature of 62ºC. Therefore, the annealing temperature of 62ºC was chosen for all subsequent tests. Specificity of the amplification was confirmed by direct sequencing of PCR products and comparison with ToSRV and ToYVSV nucleotide sequence (data not shown).

In order to validate the primers, fifty begomovirus isolates were selected, which were preliminarily identified by PCR product direct sequencing (Fernandes et al., 2008). They all shared a partial genome sequence higher than 90% to either ToSRV or ToYVSV, thus being preliminarily classified as ToSRV-like (34 isolates) and ToYVSV-like (16 isolates) viruses, respectively. Comparison of amplification of ToYVSV-like samples using ToYVSV-specific primers (ToYVSV1f-ToYVSV4r and ToYVSV1f-ToYVSV6r) revealed that the combination ToYVSV1f-ToYVSV6r detected all ToYVSV-samples, whereas the combination using ToYVSV4r primer failed to detect four samples. The combination ToYVSV1f-ToYVSV6r detected ToYVSV-like sequences also in one ToSRV-like sample, suggesting a possible mixed infection. ToSRV-specific primers were

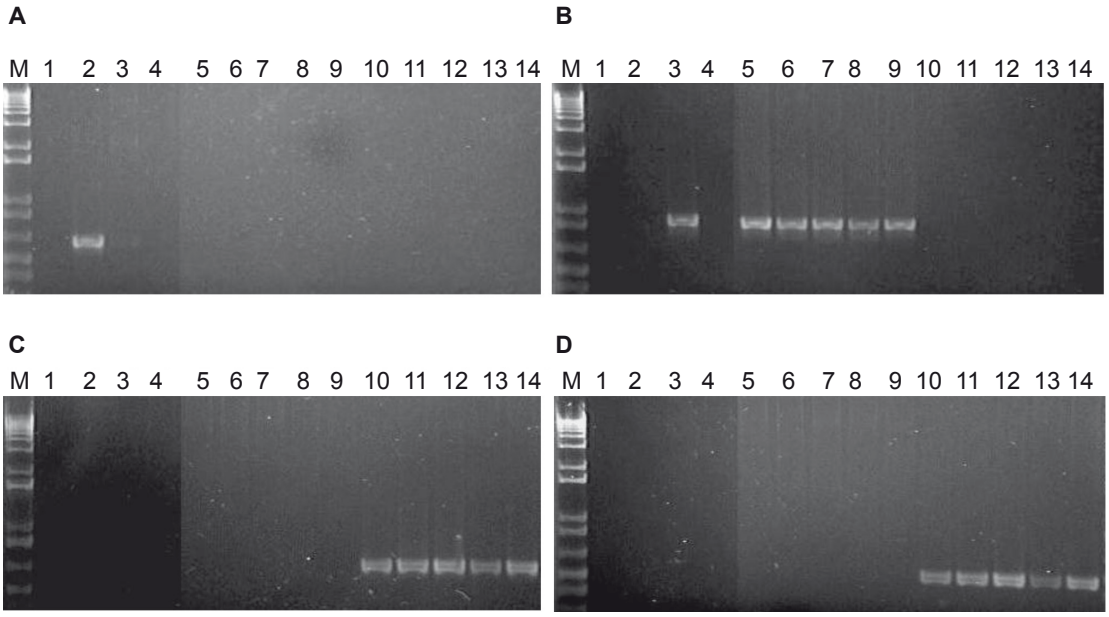

FIGURE 1 - Example of an agarose gel electrophoresis of PCR amplified products using primer pairs ToSRV1f-ToRMV1r with 587bp (A), ToSRV1f-ToSRV2r with 820bp (B), ToYVSV1f-ToYVSV4r with 579bp (C) and ToYVSV1f-ToYVSV6r with 435bp (D). M: 1Kb DNA Plus Ladder, Invitrogen; 1: negative control; 2: ToRMV clone; 3: ToSRV clone; 4: ToCMoV clone; 5: ToSRV-856 sample; 6: ToSRV-859 sample; 7: ToSRV-860 sample; 8: ToSRV-877 sample; 9: ToSRV-894 sample, 10: ToYVSV-1776 sample; 11: ToYVSV-1663 sample; 12: ToYVSV-1669 sample; 13: ToYVSV-1673 sample; 14: ToYVSV-1678 sample.

able to specifically detect all 34 ToSRV-like samples, and amplified the expected DNA fragment in 11 of the ToYVSV-like samples. Again, it indicates the possible occurrence of mixed infections.

All ToSRV-like samples were collected from tomato destined for processing during 2002 to 2003 in Goiás and Minas Gerais states. The sole and apparently mixed ToSRV-like sample infected with ToSRV and ToYVSV was collected in Goiás. The ToYVSV-like samples were collected in 2003 from fresh market tomatoes in Goiás and the Federal District.

Next, unidentified samples were tested using the three primer combinations (Table 1). No amplification was observed in three samples collected at Embrapa Hortaliças and in Goiás from tomato for processing. This suggests that these samples are infected with a begomovirus other than ToSRV and ToYVSV. Fourteen samples (from processing and fresh market tomatoes) were found to be infected with ToSRV and five (from fresh market tomatoes) with ToYVSV. As two of them only amplified with the combination ToYVSV1f-ToYVSV6r, it definitely confirmed that this combination is more suitable for ToYVSV detection. Possible mixed infections with ToSRV and ToYVSV were found in seven samples (from processing and fresh market tomatoes). The amplification specificity was tested by direct sequencing of PCR products. After amplification they were precipitated, re-suspended in 10mM Tris, pH 8.0, and sequenced with the forward primer used for amplification in an automated sequencer (ABI PRISM 377 DNA Sequencer) using BigDye Terminator Cycle Sequencing Kit.

A total of 14 amplification products from primer combination ToSRV1f-ToSRV2r and ToYVSV1f-ToYVSV6r were sequenced using the forward primer. All sequences shared >95% identity with the corresponding specific virus, demonstrating the specific amplification. The primer ToRMV1r was tested in combination with ToSRV1f primer (amplicon with 587bp) for all tested samples (Table 1). No DNA sample from the field was amplified with this primer combination. Amplification of the DNA of *Tomato rugose mosaic virus* clone was obtained and demonstrated its ability to amplify the specific DNA fragment. This result strongly suggests that *Tomato rugose mosaic virus*-like isolates are not widespread in the sampled area of Goiás, Minas Gerais and the Federal District.

Thirteen primer pairs were used to test their ability to distinguish three begomovirus species, but only four were potentially specific to our isolates. These four combinations were finally used to test field samples by PCR. Sequencing of amplified products demonstrated that one pair (ToSRV1f-ToSRV2r) could specifically detect ToSRV-like samples, another pair (ToYVSV1f-ToYVSV6r) ToYVSV-like samples and the pair ToSRV1f-ToRMV1r was possibly suitable for detection of ToRMV-like samples. After testing many field samples, we could conclude that samples with mixed infection were commonly found. They were particularly detected in ToSRV and ToYVSV mixed samples collected from fresh market tomatoes. As observed earlier (Fernandes et al., 2008), ToSRV-like isolates predominate in Goiás from tomatoes for processing and this is the

TABLE 1 - Analysis of begomovirus infection in tomato samples

Samples	ToSRV	ToYVSV		ToRMV
	ToSRV1f - ToSRV2r	ToYVSVf- ToYVSV4r	ToYVSV 1f- ToYVSV 6r	ToSRV1f- ToRMV1r
ToSRV -clone	+	-	-	-
ToYVSV-clone	-	+	+	-
ToRMV - clone	-	-	-	+
Previously identified samples				
33 ToSRV -like	+	-	-	-
1 ToYVSV-like	-	+	+	-
1 ToYVSV-like	-	-	+	-
11 ToYVSV-like	+	+	+	-
1 ToSRV - like and 3 ToYVSV-like	+	-	+	-
Unindentified samples				
14	+	-	-	-
3	+	+	-	-
2	-	+	-	-
4	+	+	+	-
3	-	+	+	-
3	-	-	-	-

only virus isolated in most samplings. On the other hand, ToYVSV-like samples predominated in the Federal District from fresh market tomatoes and it seems that these samples were also mixed infected with ToSRV-like isolates. As they were preliminarily identified as ToYVSV-like viruses based on the PCR-sequencing, it is most likely that ToYVSV DNA-A molecules (the target of PCR amplification and sequencing) was more abundant than ToSRV-like DNA-A. It is not known if ToYVSV replicates more efficiently than ToSRV in the varieties or hybrids used in fresh market tomatoes.

Three samples were negative using the four primer combinations, indicating that another species may be present in these samples. Two samples were collected in the Embrapa experimental field and the third collected at a processing tomato field in Goiás state. Our preliminary studies suggested that at Embrapa isolates of Tomato mottle leaf curl virus predominate, while in Goiás isolates of *Tomato chlorotic mottle virus* were sometimes found (unpublished data) and may explain this negative result. We could finally conclude that these primers were useful to quickly discriminate the begomovirus species present in the sample and to demonstrate the high incidence of mixed infections caused by this virus, specially in fresh market tomato fields.

ACKNOWLEDGMENTS

The ToRMV clone was provided by Dr. Francisco Murilo Zerbini, Universidade Federal de Viçosa. We thank Carolina de Mello Franco and Leonardo Queiroz Correia for technical assistance.

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TPP 9074 - Received 2 June 2009 - Accepted 20 January 2010
Section Editor: F. Murilo Zerbini