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A single base pair in the right terminal domain of tomato planta macho viroid is a virulence determinant factor on tomato

Rugang Li[a,b], Chellappan Padmanabhan[a], Kai-Shu Ling[a,*]

[a] U.S. Department of Agriculture – Agricultural Research Service, U.S. Vegetable Laboratory, 2700 Savannah Highway, Charleston, SC, USA
[b] Agdia Inc., 52642 County Road 1, Elkhart, IN 46514, USA

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ABSTRACT

Tomato planta macho viroid (TPMVd), including isolates previously designated as Mexican papita viroid (MPVd), causes serious disease on tomatoes in North America. Two predominant variants, sharing 93.8% sequence identity, incited distinct severe (MPVd-S) or mild (MPVd-M) symptoms on tomato. To identify virulence determinant factor, a series of chimeric infectious clones were generated using synthetic DNA approach to progressively replace each structural domain between the two variants. In bioassays on tomato 'Rutgers', three chimeras containing Terminal Left and Pathogenicity (MPVd-H1), Central (MPVd-H2), or Variable (MPVd-H3) of MPVd-S, incited mild to intermediate symptoms. However, a chimera containing Terminal Right (T_R) of MPVd-S (MPVd-H4) incited severe symptoms. Only one base-pair mutation in the T_R domain between MPVd-M ($_{176}$U:A$_{185}$) and MPVd-S ($_{174}$G:C$_{183}$) was identified. A reciprocal mutant (MPVd-H5) rendered the chimeric viroid mild on tomato. This single base-pair in the T_R domain was determined as the virulence determinant factor for TPMVd.

1. Introduction

Viroids are a group of the smallest pathogens described to date inciting economically important diseases in a number of plants. Unlike viruses, viroids are naked, single-stranded, circular small RNA molecules with 246–401 nucleotides in length (Flores et al., 2011). Since the first discovery of viroids as plant pathogens inducing diseases of potato spindle tuber (Diener and Raymer, 1967; Gross et al., 1978) and citrus exocortis (Semancik and Weathers, 1968), more than 30 viroid species have been recognized (Flores et al., 2011; Di Serio et al., 2014). Because viroids do not encode any protein and also lack any helper virus, the mechanisms of their replication and movement in a host plant, as well as their capacity to alter the host phenotype, have been of interest to the scientific community (Flores et al., 2011; Navarro et al., 2012; Eamens et al., 2014).

The visible macroscopic symptoms in a viroid-infected plant are a result of cellular and developmental disorders (Di Serio et al., 2012). To investigate the functional domains in a viroid that are responsible for inciting the severe disease symptoms, Sano et al. (1992) generated six interspecific chimera between a severe *Tomato apical stunt viroid* (TASVd) isolate and a mild *Citrus exocortis viroid* (CEVd) isolate to study the functions of individual structural domains. Their findings demonstrated that although several structural domains played roles in symptom expression, the stunting symptom incited in the tomato 'Rutgers' by TASVd has been mapped to the Terminal Left (T_L) and the Pathogenicity (P) domains of the viroid (Sano et al., 1992). In *Potato spindle tuber viroid* (PSTVd), the P domain is recognized as a main location of pathogenicity determinants (Dickson et al., 1979; Gross et al., 1981; Herold et al., 1992; Matoušek et al., 2012; Owens and Hammond, 2009; Schnölzer et al., 1985). A recent report showed that expression of an artificial microRNA from the P domain of PSTVd caused abnormal phenotypes in *Nicotiana benthamiana* plants (Eamens et al., 2014). A comparative analysis of sequence variants between mild and severe isolates of CEVd revealed that majority of sequence variations were localized in the P and Variable (V) domains (Visvader and Symons, 1985), and their functions were validated using infectious chimeric cDNA clones (Visvader and Symons, 1986). In other circumstances, the pathogenicity/virulent determinant may also be mapped to sequences outside of the P domain. Qi and Ding (2003) reported that a U257A change within the Loop E of the conserved Central (C) domain was able to convert an intermediate PSTVd strain to a lethal strain that caused severe growth stunting.

Mexican papita viroid (MPVd), considered a genetic variant of *Tomato planta macho viroid* (TPMVd) in the genus *Pospiviroid* and the family *Pospiviroidae* (Di Serio et al., 2014; Verhoeven et al., 2011), was first isolated from wild papita (*Solanum cardiophyllum* Lindl)

* Corresponding author.
E-mail addresses: Rugangli@yahoo.com (R. Li), Chellappan.Padmanabhan@ars.usda.gov (C. Padmanabhan), Kai.Ling@ars.usda.gov (K.-S. Ling).

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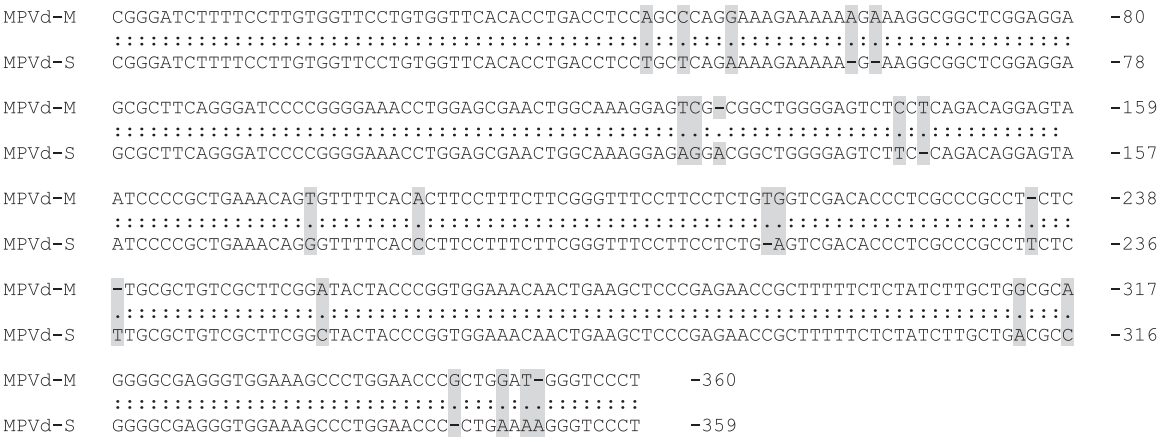

Fig. 1. Sequence comparison between *Tomato planta macho viroid* genotype Mexican papita viroid mild variant (MPVd-M) and severe variant (MPVd-S). Those nucleotide sequences with differences between the mild and severe variants are highlighted.

plants in 1996 from Mexico (Martínez-Soriano et al., 1996). Beginning in 2009, several disease outbreaks caused by TPMVd genotype MPVd have been reported in greenhouse tomatoes grown in Canada and Mexico (Ling and Bledsoe, 2009; Ling and Zhang, 2009; Verhoeven et al., 2011). This sudden emergence of TPMVd genotype MPVd in several greenhouse tomato facilities has caused great concerns to tomato growers and seed producers. However, little biological information was available about TPMVd genotypes, despite the sequence information on complete genome in several isolates with TPMVd genotype MPVd (Ling and Bledsoe, 2009; Ling and Zhang, 2009; Verhoeven et al., 2011). Numerous questions as to their molecular and biological properties remain unanswered. The lack of a local lesion host makes it difficult to isolate MPVd from a field sample that was often in a mixed infection with another endemic virus (*i.e.*, *Pepino mosaic virus*). Thus, we created infectious cDNA clones from two Mexican variants with TPMVd genotype MPVd which share only 93.8% sequence identity. To our surprise, symptoms incited on the inoculated tomato plants 'Rutgers' using infectious cDNA clones derived from these two TPMVd-MPVd variants were distinctive. MPVd-Mex8 (herein after referred to as MPVd-S) induced severe plant stunting, severe vein necrosis on leaves, and with tiny or no fruits formed in the infected plants. On the other hand, tomato plants 'Rutgers' infected by MPVd-MX (herein after referred to as MPVd-M) showed more subtle mild symptoms of leaf chlorosis, normal plant growth and fruit production despite some general reduction in the fruit size. The objective of the present study was to determine which functional domain of TPMVd is responsible for inciting the severe disease symptoms on tomato. Five chimeric viroid molecules were chemically synthesized *in vitro*. In a series of mutants, each individual structural domain of MPVd-M was progressively replaced with its corresponding domain from MPVd-S. Interestingly, a chimera with Terminal Right (T_R) domain of MPVd-S incited severe stunting and vein necrosis symptoms that were similar to that of an MPVd-S infection. Three chimera with replacement of the T_L, P or V domain of the MPV-S genome incited only mild disease symptoms similar to that of an MPVd-M infection. These results provided direct evidence to support the notion that the T_R domain of MPVd-S was involved in regulating severe disease symptom expression on tomato 'Rutgers' plants. A closer examination on the sequence differences in the T_R domain between MPVd-S and MPVd-M revealed only one base-pair change from MPVd-M ($_{176}$U:A$_{185}$) to MPVd-S ($_{174}$G:C$_{183}$). To validate that this pair of nucleotides in the T_R domain was the virulence determinant factor, an additional chimera (MPVd-H5) was developed by replacing the T_R domain of MPVd-S with that of MPVd-M. Bioassay showed that MPVd-H5 generated a mild disease symptom on the infected tomato plants similar to that of MPVd-M. This mild symptom response confirmed that the single base pair change in the T_R domain of MPVd-S was indeed responsible for severe symptom expression.

2. Results

2.1. Identification and genetic characterization of tomato-infecting TPMVd genotype MPVd variants from Mexico

To examine the genetic diversity of field-collected isolates of TPMVd genotype MPVd with only mild disease symptoms on tomatoes, 48 full-length sequences were obtained, of which 37 (77%) had identical sequences in their 360 nt genomes (GenBank accession no. KF683198) and 11 others (23%) had either one or two nucleotide substitutions or deletions. Multiple sequence alignments using the Clustal W method (DNASTAR) revealed that the predominant sequence variant (designated as MPVd-M) has only one nucleotide substitution (C255A) when compared to the TPMVd isolate MPVd-VF2 (GenBank accession no. FJ824844) previously identified in Canada (Ling and Bledsoe, 2009). This MPVd-M variant was also closely related to the sequences from 10 papita-infecting isolates of MPVd (Martínez-Soriano et al., 1996), with sequence identities of 98.9–99.7%. However, the MPVd-M variant exhibited greater genetic divergence to the type member of TPMVd (GenBank accession no. K00817, Kiefer et al., 1983), with 92.4% nucleotide sequence identity. Moreover, the MPVd-M variant shared only 93.8% nucleotide sequence identity to another MPVd variant (MPVd-Mex8, GenBank accession no. GQ131573, herein after referred to as MPVd-S) (Ling and Zhang, 2009). MPVd-S was identified on tomatoes from the same greenhouse facility in Mexico two years prior as that of MPVd-M, but incited severe disease symptoms on tomato, which shared only 92.7% nucleotide sequence identity to the type member of TPMVd. Thus, MPVd-M and MPVd-S were two distinct variants in the TPMVd genotype MPVd. A pairwise alignment analysis revealed a total of 23 nucleotide changes between the two MPVd variants, with 14 substitutions and nine insertions/deletions (Fig. 1). Comparative evaluation of their secondary structures, generated through folding of primary sequences on the WEB-based Mfold server (http://mfold.rna.albany.edu/?q=mfold) (Zuker, 2003), showed presence of 31 loops in the MPVd-M variant, but only 28 loops in the MPVd-S variant (Fig. 2). Both MPVd variants assumed the rod-like conformation with extensive base-pairing characteristics similar to that of other pospiviroids. A closer examination between these two variants in the TPMVd genotype MPVd showed that majority of sequence variations were localized in the T_L, P and V domains, with some other changes in the T_R domain. Thus, it was necessary to carry out a functional analysis through mutagenesis to identify the virulence determinant factor.