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Molecular dissection of a dahlia isolate of potato spindle tuber viroid inciting a mild symptoms in tomato

Daiki Tsushima[a,b], Taro Tsushima[a], Teruo Sano[a,*]

[a] Department of Bio-resources, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036-8561, Japan
[b] Union Graduate school of Agricultural Sciences, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan

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ABSTRACT

The dahlia isolate of potato spindle tuber viroid (PSTVd) accumulates slowly and induces mild disease symptoms in tomato (*Solanum lycopersicum*, cv. Rutgers) plants in contrast to the intermediate isolate (PSTVd-I). The dahlia isolate (PSTVd-D) differs from PSTVd-I in eight locations: 42 and 43 in the terminal left (TL); 64/65, 311, and 312/313 in the pathogenicity (P); 118 and 126 in the variable (V); and 201 in the terminal right (TR) domains. To investigate the molecular determinants in the PSTVd-D genome responsible for the attenuation of symptom severity and lower replication/accumulation in tomato plants, a series of mutants between PSTVd-D and PSTVd-I were constructed by focusing first on the mutations in the TL and P domains in the left-hand half of the molecule. Then, more detailed analysis was performed on the three mutations at positions 118, 126, and 201 in the V and TR domains. One of these mutations is located around the boundary of the right border of the RY-motif, a predicted recognition site of Virp1, a viroid-binding protein. Of 14 mutants (seven based on PSTVd-D and the other seven based on PSTVd-I) examined, 11 propagated stably and three lost infectivity. Mutations in the TL and P domains (42U, 43C, 310U/C, and U or UU insertion to 311/312 in PSTVd mild types) majorly influenced the expression of mild-like symptoms. In contrast, when each of the mutations at 118, 126, and 201 in the V and TR domains were exchanged independently, they minimally influenced systemic accumulation and symptom expression. Mutants based on PSTVd-D with PSTVd-I-type mutations at nucleotide positions 118, 126, and/or 201 showed mild symptoms similar to PSTVd-D, but their systemic accumulation was a little faster than PSTVd-D. In contrast, mutants based on PSTVd-I with PSTVd-D-type mutations at 118, 126, and/or 201 nucleotide positions showed severe symptoms similar to PSTVd-I, and the systemic accumulation was similar to or a little slower than PSTVd-I. The nucleotide at position 201 could be changed to U, G, or A, but C was not acceptable for replication. Because introduction of C at the position 201 can change the loop structure at the right boundary of the RY-motif's consensus sequence, the loop structure may influence recognition by Virp1.

1. Introduction

Viroids are circular, single-stranded RNA molecules, and the smallest known plant pathogens (typically 246–401 nucleotides) (Diener, 1987). Viroids rely on the host transcription machinery to replicate (Daròs and Flores, 2004; Flores et al., 2005) and cause various degrees of symptoms from mild to severe. Viroids are now classified in two families, eight genera, and 32 species (Di Serio et al., 2014). Keese and Symons (1985) have proposed a domain model in that the rod-like secondary structures of viroids in the family *Pospiviroidae* can be divided into five structural domains: terminal left (TL), pathogenicity (P), central (C), variable (V), and terminal right (TR). The members of *Pospiviroidae* share a genus-specific central conserved region (CCR), replicate by asymmetrical rolling circle mechanism, and localize in the nuclei of the infected cells, whereas those in the family *Avsunviroidae* can be folded into a branched rod-like secondary structure lacking a domain structure, including CCR; they replicate by a symmetrical rolling circle mechanism and localize in chloroplasts (Branch and Robertson, 1984; Flores et al., 2005; Sänger, 1987).

Potato spindle tuber viroid (PSTVd) is a species in the family *Pospiviroidae* and has a wide host range in *Solanaceae*, *Asteraceae*, *Gesneriaceae*, and *Lauraceae* plants, most of which are symptomless carriers. Potato (*Solanum tuberosum*) and tomato (*S. lycopersicum*) are symptomatic hosts, and when infected they show stunting, epinasty, leaf distortion, veinal necrosis, apical proliferation, floral variegation, tuber elongation and cracking (potatoes), or reduction of fruit size (tomatoes). Severity of the symptoms (mild to

* Corresponding author.
E-mail address: sano@cc.hirosaki-u.ac.jp (T. Sano).

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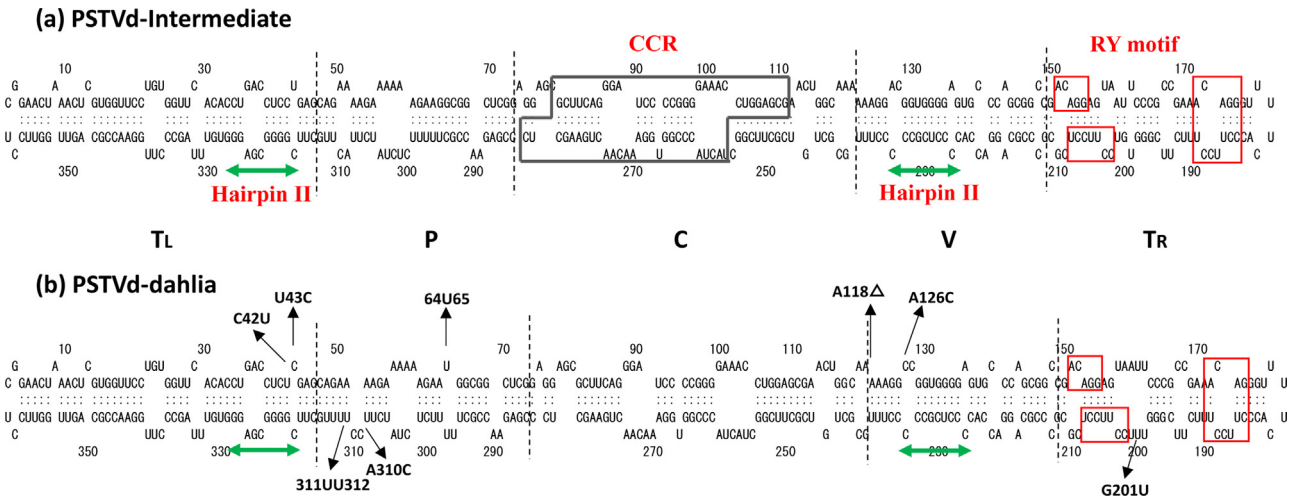

Fig. 1. Comparison of the predicted secondary structures of (a) PSTVd-intermediate and (b) dahlia isolates. Vertical broken lines indicate the border of the five structural domains (from left to right, terminal left, T_L; pathogenicity, *P*; central, *C*; Variable, *V*; terminal right, T_R). Central conserved region (CCR) and RY motif were shown in boxes. Location of the nucleotide sequences which form hairpin II structure are shown in arrows. Five substitutions, 3 insertions and 1 deletion in PSTVd-dahlia are indicated with arrows as compared to PSTVd-Intermediate.

severe) depend on PSTVd strains in combination with host plant varieties. Many sequence variants are known in PSTVd (Góra et al., 1994; Gross et al., 1978, 1981; Herold et al., 1992; Lakshman and Tavantzis, 1993; Nie, 2012; Owens et al., 1992, 2009; Puchta et al., 1990), with the variations being mostly concentrated in the P and V domains. Comparative sequence analyses of phenotypically dissimilar, natural PSTVd isolates strongly suggests that the P domain plays a major role in pathogenicity (Góra et al., 1994, 1996; Owens et al., 1995, 1996; Schmitz and Riesner, 1998; Schnölzer et al., 1985), although the other domains of the molecule also contribute to the replication and pathogenicity in *Citrus exocortis viroid*, another member of the same genus (Chaffai et al., 2007; Murcia et al., 2011; Sano et al., 1992; Skoric et al., 2001; Visvader and Symons, 1985, 1986). Infectivity studies of six intraspecific chimeras of PSTVd variants constructed by exchanging the P and V domains between mild, severe, and lethal isolates of PSTVd showed that the P domain is directly responsible for the severity of symptoms in the tomato. However, symptom severity was not correlated with viroid accumulation (Góra et al., 1996). Mutational analysis within the P domain demonstrated that mutations that stabilize the pre-melting region (PM) 1 led to a reduction in PSTVd replication/accumulation (Owens et al., 1995). In addition to the P domain, a single nucleotide substitution within the V domain is sufficient to greatly reduce/abolish infectivity (Owens et al., 1991; Hu et al., 1996), and a C to U substitution at nucleotide 259 in loop E of the CCR converted PSTVd variant KF440-2 from noninfectious to infectious in *Nicotiana tabacum* (Wassenegger et al., 1996). An U to A substitution at nucleotide 257 in the CCR also converted PSTVd-intermediate (PSTVd-I; one of the most characterized variant that incites intermediate symptoms) to a lethal strain that caused severe growth stunting and premature death of infected plants, indicating a new pathogenicity determinant that functions independently from the P domain (Qi and Ding, 2003). Meanwhile, it was suggested that the RY motif in the TR domain contributes to PSTVd infectivity in tomato probably facilitating systemic spreading by recruitment of Virp1, a viroid RNA binding protein (Gozmanova et al., 2003; Hammond 1994; Kalantidis et al., 2007; Maniataki et al., 2003; Martínez de Alba et al., 2003).

A variant of PSTVd detected from dahlia plants (*Dahlia* Cav.) accumulates slowly and induces very mild symptoms in tomato (cv. Rutgers) plants in contrast to PSTVd-I (Tsushima et al., 2011). The dahlia isolate (PSTVd-D) shares 97% sequence identity with PSTVd-I, but PSTVd-D differs at eight positions affecting nine nucleotides: five substitutions at positions 42, 43, 126, 201, and 310; two insertions at 64/65 and 311/312; and one deletion at 118. These changes are located in the TL (42 and 43), P (64/65, 310, and 311/312), V (118 and 126), and TR (201) domains. Interestingly, the nucleotides in the V (126) and TR (201) domains were unique to PSTVd-D and can form distinct loop structures among those reported thus far (Fig. 1). Deep-sequencing analysis of PSTVd-specific small RNAs (PSTVd-sRNA) that accumulate in PSTVd-infected tomato plants revealed that the number of PSTVd-sRNA reads extensively decreased in PSTVd-D-infected tissues compared with those in PSTVd-I, particularly those derived from the V and TR domains containing the nucleotides 118, 126 and 201, in which the nucleotide sequences differed between PSTVd-I (severe) and PSTVd-D (mild) (Tsushima et al., 2015).

To elucidate the importance of mutations in PSTVd-D with respect to attenuation, a series of mutants based on PSTVd-I and -D were created by initially focusing on the mutations lying in the TL and P domains and then on those in the V and TR domains. To define the significance of mutations at 118, 126, and 201 on attenuation as well as those in the TL and P domains, a total of 14 mutants were created by exchanging these mutations and then assaying for infectivity and pathogenicity. In addition, because one of the mutations at position 201 is located in an unique loop structure in the TR domain at the right boundary of the internal RY-motif, this mutation was assayed for effect on infectivity, systemic accumulation, and symptom expression. The results defined major determinants for the attenuation in the TL and P domains. Also, the role of three mutations in the V and TR domains were found to be minor, contributing minimally to systemic accumulation and symptom severity.

2. Materials and methods

2.1. Construction of PSTVd dimeric cDNA clones

PSTVd dimeric cDNA clones with various mutations were constructed according to the protocol described previously (Adkar-Purushothama et al., 2015a; Tsushima et al., 2015). Briefly, a unit-length cDNA fragment of PSTVd-I (accession no. M16826), PSTVd-D (accession no. AB623143) or the mutants with *Bam* HI termini was excised from pUC18-based plasmid vectors, purified by