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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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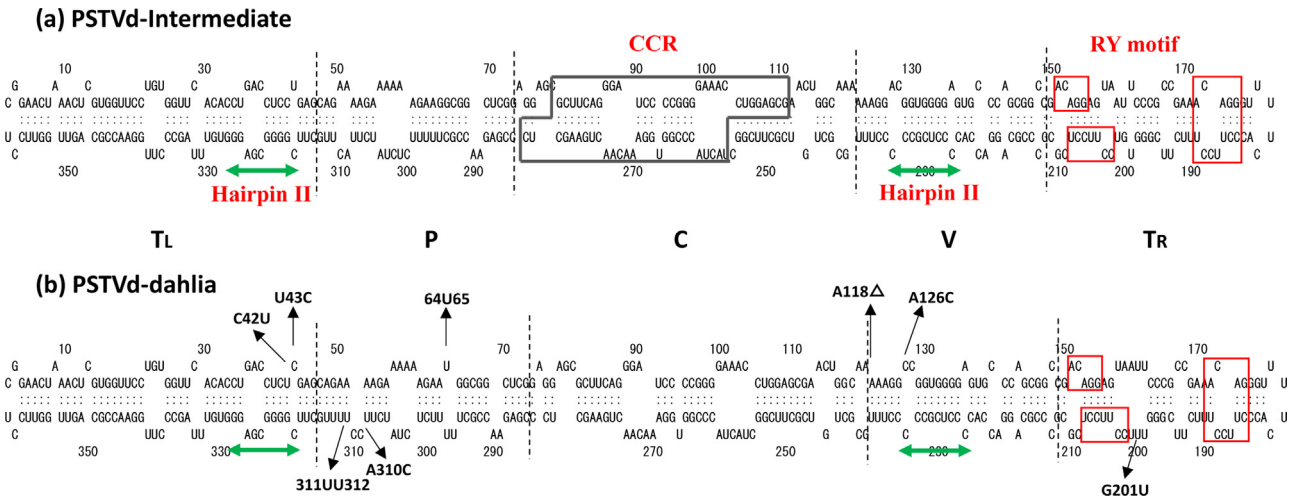

Fig. 1. Comparison of the predicted secondary structures of (a) PSTVd-intermediate and (b) dahlia isolates. Vertical broken lines indicate the border of the five structural domains (from left to right, terminal left, T_L; pathogenicity, *P*; central, *C*; Variable, *V*; terminal right, T_R). Central 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

7.5% polyacrylamide gel electrophoresis (PAGE), self-ligated and inserted into the *Bam* HI site of pBluescript II SK(−) under the control of T7 RNA promoter (Invitrogen, Carlsbad, CA, USA). Plasmids containing dimeric head-to-tail cDNAs of PSTVd-I, PSTVd-D, or several mutants were selected, sequenced, and used for preparing in vitro transcripts. Plasmid DNA (~2 μg) was digested at 37 °C overnight with the *Not* I restriction enzyme (Takara Bio, Otsu, Shiga, Japan) for cleaving downstream of the 3′-end of the cDNA insert. After incubation, the linearized plasmid DNA was extracted by phenol–chloroform, recovered by ethanol precipitation and dissolved in distilled water. Transcription was performed at 37 °C for 2 h with T7 RNA polymerase (Invitrogen, Carlsbad, CA, USA) in a 20 μl reaction mixture according to the manufacturer's instruction. After transcription, the reaction was stopped by adding 2 μl 0.1 M EDTA (pH 7.0), 2.5 μl 4 M LiCl, and 75 μl 99.5% ethanol, and stored for 1 h at −30 °C. The RNA transcripts were recovered by ethanol precipitation, dissolved in 50 μl distilled water, and quantified by UV spectrophotometry.

Inocula were prepared at concentrations of 100 ng RNA/μl in 50 mM sodium phosphate buffer (pH 7.5) containing 1 mg/ml bentonite. Five each of tomato seedlings (*Solanum lycopersicum*, cv. Rutgers) at the first true-leaf stage was dusted with carborundum (600 mesh), then a 10 μl sample of inoculum was placed on the leaf and gently rubbed against the leaf 20 times using a sterile glass rod. Plants were then rinsed immediately with tap water and placed in a growth chamber controlled at 25 °C, with a 16-h day and high light intensity (fluorescent, 40 W × 4).

2.2. Extraction of low-molecular-weight RNA

Three to six weeks after inoculation (wpi), when control plants showed symptoms, samples of leaves or stems (ca. 1 g) were homogenized in 5 ml of 2× CTAB buffer (10 ml 1 M Tris–HCl [pH 9.5], 46.6 ml 3 M NaCl, 10 ml 0.2 M disodium EDTA [pH 7.0], 2 g CTAB powder, 0.5 ml 2-mercaptoethanol, and 28.4 ml distilled water; final volume 100 ml). Total nucleic acids were extracted once with an equal volume of phenol–chloroform (1:1), precipitated with ethanol, and dissolved in 100–400 μl distilled water. LMW-RNA soluble in 2 M LiCl was obtained by fractionation with 2 M LiCl, and dissolved in 50–100 μl distilled water (Sano et al., 2004; Adkar-Purushothama et al., 2015a). Final RNA extracts were adjusted in water at concentrations ca. 500–1000 ng/μl and store at −30 °C.

2.3. Sequence analysis of PSTVd progeny

LMW-RNAs (ca. 100 ng/μl) extracted from tomato plants infected with PSTVd-I, -D or the mutants, were used for reverse transcription (RT) for 60 min at 37 °C with random hexamer primers (final conc. 0.5 μM) and M-MuLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a total of 25 μl reaction mixture. An aliquot (2 μl) of the RT solution was added to a PCR reaction mixture (final volume of 25 μl) containing 20 pmol each of PSTVd-specific primer set: i.e., PS88M (5′-CCCTGAAGCGCTCCTCCGAG-3′) and PS89P (5′-ATCCCCGGGGAAACCTGGAGCGAAC-3′) (Levy et al., 1994; Weidemann and Buchta, 1998). Double-stranded cDNAs amplified by RT-PCR were ligated directly into pGEM-T vector (Promega, Madison, WI, USA) and used for transformation of *Escherichia coli* DH5α competent cells (Takara Bio, Shiga, Japan). DNA sequencing was performed with an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) or by the Solgent Co. (Seoul, Korea).

2.4. Infectivity assay by RNA gel-blot analysis

LMW-RNAs (ca. 100 ng) were denatured in the presence of formaldehyde and formamide by heating at 68 °C for 15 min, and fractionated by electrophoresis in 1.5% (w/v) agarose gels containing formaldehyde at a final concentration of 0.66 M in 1× MOPS buffer (Tsushima et al., 2015). RNAs were then transferred to a nylon membrane (Biodyne Plus: Pall Corp., Port Washington, NY, USA) and hybridized with a DIG-labeled PSTVd-specific probe (Adkar-Purushothama et al., 2015a) to analyze the infectivity and titer of PSTVd mutants. The hybridized signals were visualized using a Chemidoc-XRS (Bio-Rad) imaging system (ca. 1 h exposure) and quantified using the Quantity One (version 4.6.2) software package.

3. Results

3.1. Infectivity and pathogenicity of 14 PSTVd-I and -D mutants

A total of 14 PSTVd mutants were created by reciprocally exchanging between the intermediate and dahlia isolates the left-hand half of the viroid molecule containing the TL and P domains and also the nucleotides at positions 118, 126 and 201 (numbering referred to PSTVd-I). Namely, these mutations single, double and triple were introduced in the full-length cDNA of PSTVd-I and -D with BamHI termini (from 87G at the 5′-terminal to 86C at the 3′-terminal ends), self-ligated to make tandem dimers, and then ligated into the BamHI site of pBluescript II SK(−). The name of the mutants and their mutated positions are summarized in Table 1. Briefly, PSTVd-ID is a PSTVd-I-based mutant with D-type mutations at positions 118, 126, and/or 201, and PSTVd-DI is a PSTVd-D-based mutant with I-type mutations at positions 118, 126, and/or 201. Similarly, PSTVd-I-series are I-based mutants, and PSTVd-D-series are D-based mutants. The positions and changes of mutations are indicated for example as A118Δ, where A at position 118 was deleted. The nucleotides G and U at position 201 are found naturally in PSTVd. In addition, G (or U) 201A and G (or U) 201C were artificially created in this study.

Three tomato seedlings (cv. Rutgers) were inoculated with in vitro dimeric transcripts of mutants (100 ng/μl, 10 μl/plant), and the infection was assayed using RNA gel-blot analysis at 2, 3, and 4 wpi. As a result, 11 of the 14 mutants were infectious; all inoculated plants became positive by 4 wpi. Plants infected with I-series mutants showed severe stunting similar to but milder than that induced by PSTVd-I, but D-series mutants showed very mild symptoms similar to but more severe than those caused by PSTVd-D. Mutants that lost infectivity included I-G201C, D-U201C, and D-118A/C126A (Table 1).

3.2. Rate of accumulation and severity of symptoms of PSTVd mutants

LMW-RNAs extracted from 11 PSTVd mutants were analyzed for viroid titers in the infected plants at 2, 3, and 4 wpi via RNA gel-blot analysis followed by visualization and quantification of the hybridization signals using Quantity One software package (version 4.6.2) in a Chemidoc-XRS (Bio-Rad) imaging system.

The results showed that systemic accumulation of all five D-series mutants, DI, D-118A, D-C126A, D-U201A, and D-U201G, progressed very slowly, taking 3–4 weeks for the three plants to react positively, similarly to but a little faster than PSTVd-D (Fig. 2A and B). In contrast, all three plants inoculated with the six I-series mutants, ID, I-A118Δ/A126C, I-A118Δ, I-A126C, I-G201A, and I-G201U, became positive by 2–3 wpi (Fig. 2A), and accumulated similarly to or a little slower than PSTVd-I (Fig. 2B). Collectively, these results indicate that systemic accumulation was mainly controlled by molecular determinants in the TL and P domains, because all the infectious mutants having D-type mutations in the TL and P accumulated slowly. In addition, each of the three mutations

Table 1
Summary of infection assay of 14 PSTVd-I and PSTVd-D based mutants.

	Left Half (TL, P and C)	Mutation Positions in Right Half (V and TR)			Infectivity	Genetic Stability in Tomato	Systemic accumulation	Pathogenicity in Tomato
Wild Type		118	126	201				
PSTVd-Intermediate (I)	I	A	A	G	Yes	stable (10/10)*3	fast	severe
PSTVd-Dahlia (D)	D	Δ*1	C	U	Yes	stable (10/10)	slow	mild
Mutant								
I-series								
PSTVd-ID	I	Δ	C	U	Yes	stable (10/10)	fast	severe
I-A118Δ/A126C	I	Δ	C	G	Yes	stable (10/10)	fast	severe
I-A118Δ	I	Δ	A	G	Yes	stable (10/10)	fast	severe
I-A126C	I	A	C	G	Yes	stable (10/10)	fast	severe
I-G201A	I	A	A	A	Yes	stable (9/10)	fast	severe
I-G201C	I	A	A	C	No	NT	none	none
I-G201U	I	A	A	U	Yes	stable (10/10)	fast	severe
D-series								
PSTVd-DI	D	A	A	G	Yes	stable (10/10)	slow	mild
D-118A/C126A	D	A	A	U	No	NT	none	none
D-118A	D	A	C	U	Yes	stable (10/10)	slow	mild
D-C126A	D	Δ	A	U	Yes	stable (10/10)	slow	mild
D-U201A	D	Δ	C	A	Yes	stable (9/10)	slow	mild
D-U201C	D	Δ	C	C	No	NT	none	none
D-U201G	D	Δ	C	G	Yes	stable (10/10)	slow	mild

*1: Δ means deletion.
*2: Shaded sequence indicates PSTVd-I-type sequence.
*3: Numerical numbers in parenthesis means number of stable cDNA clones per number of sequenced.

at positions 118, 126, and 201 in the V and TR domains were exchangeable between PSTVd-I and -D without noticeable effects upon infectivity. The findings that D-series mutants such as DI and D-U201 G accumulated similarly to but a little faster than PSTVd-D (Fig. 2A), and that I-series mutants such as ID or I-A118Δ accumulated similarly to but a little slower than PSTVd-I (Fig. 2A) indicated that the right-hand half of the PSTVd molecule influenced systemic accumulation to some extent. However, the importance is significantly lower than the left-hand half of the PSTVd molecule.

Expression of symptoms and its severity paralleled systemic accumulation. Five infectious D-series mutants having D-type mutations in the TL and P domains showed very mild stunting with mild leaf curling, which is comparable to those induced by PSTVd-D (Fig. 2C). In contrast, six I-series mutants having the I-type mutations in the TL and P domains exhibited severe stunting and leaf curling, which is similar to or a littler milder than those induced by PSTVd-I (Fig. 2C). These results confirmed that the molecular determinant of PSTVd-D regulating the attenuated symptom expression fall in the left-hand half (more specifically in the TL and/or P domain) of the PSTVd molecule, corroborating previous reports of the severity of other PSTVd isolates (Góra et al., 1996; Owens et al., 1996). In addition, the findings that PSTVd-D incited the mildest symptoms indicated that the right-hand half of the viroid contributes minimally to the attenuation of symptom expression.

3.3. Genetic stability of PSTVd mutants

Because there were obvious differences in the accumulation level and severity of the symptom observed in 11 infectious mutants of I-series and D-series, the genetic stability of the progeny was examined to confirm that the differences could be attributed to the mutations. LMW-RNA extracts from three infected plants at 4 wpi were combined and used for RT-PCR amplification, cloning, and sequencing of full-length cDNA of PSTVd progeny. Sequencing of ten cDNA clones per mutant randomly selected showed that the 11 infectious mutants replicated stably. Although a couple of non-specific mutations were randomly incorporated at positions other than 118, 126, and 201, these mutations were present in only a minor fraction of the population (Fig. S1). For example, only 1 of 10 cDNA clones had a singleton mutation in I-A118Δ/A126C, I-A118Δ, and I-A126C, and 2 of 10 cDNA clones had a singleton mutation in I-G201A and D-U201A. Consequently, the differences in the accumulation and pathogenicity observed in the 11 PSTVd mutants can be associated with the introduced mutants.

At position 201, mutants with A as well as the wild-type G and U were infectious and replicated stably; however, mutants with C lost the infectivity. Analysis of the secondary structures by mfold prediction revealed that mutants with A at the position 201 can form a similar stem-loop structure to that found in the wild type (i.e., G and U); however, the stem-loop structure is disrupted and changed in mutants with C at this position (Fig. 3 and 4). These structural changes seem to have impacted negatively infectivity. The mutation effects at positions 118 and 126 were more complicated. I-G201U was infectious and replicated stably; however, D-118A/C126A lost the infectivity (Table 1). Because these mutants share the same right-hand half (i.e., A118Δ, A126C, and G201U) but have a different left-hand half of the viroid molecule, it is possible that the less infectious left-hand half molecule derived from PSTVd-D contributed to the inactivity of D-118A/C126A.

4. Discussion

Molecular analysis of the pathogenesis determinants of PSTVd has been previously performed using several combinations of PSTVd variants (Schnölzer et al., 1985; Owens et al., 1995, 1996;

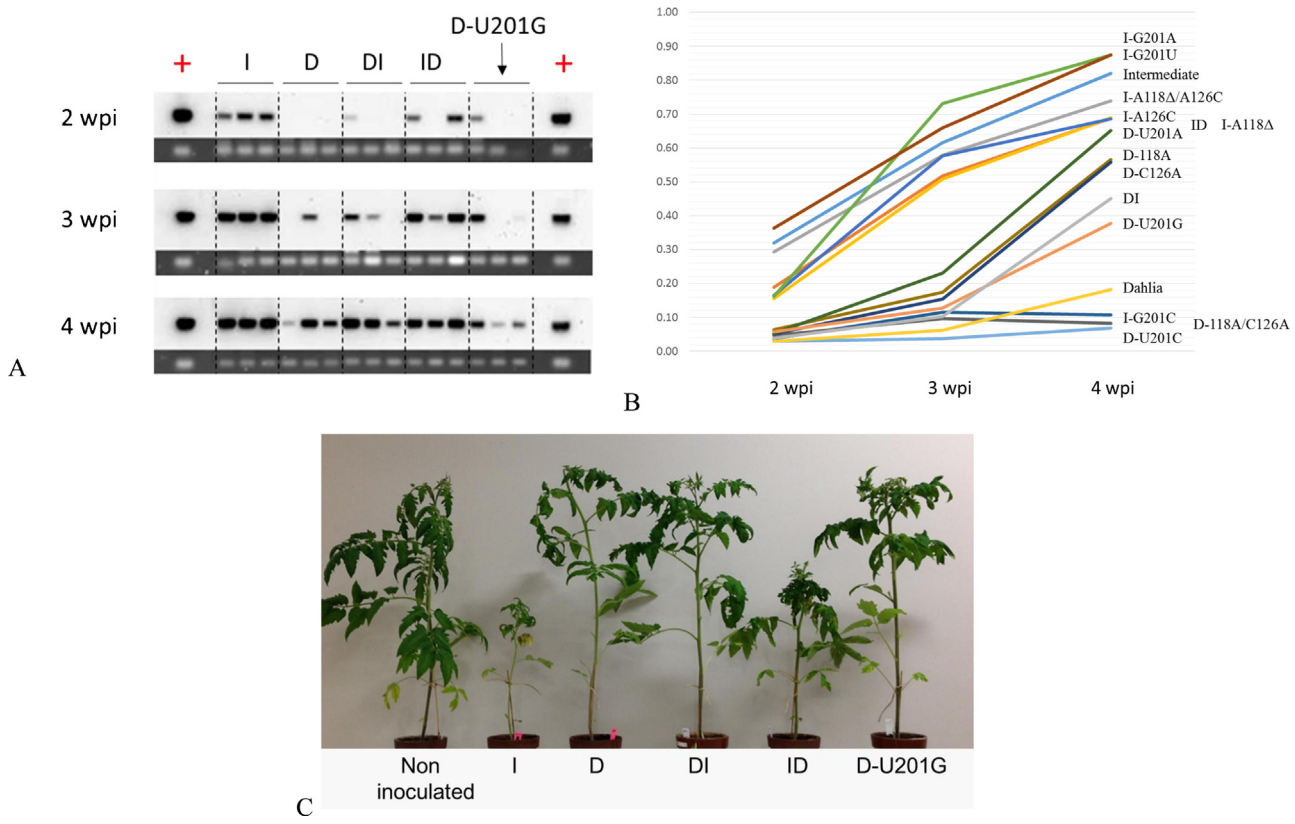

Fig. 2. Comparison of the rate of accumulation and systemic symptoms of PSTVd mutants. (A) Accumulation of the five stable mutants (from left to right, PSTVd-I (I), PSTVd-D (D), PSTVd-DI (DI), PSTVd-ID (ID), D-U201G) in three replicates of infected plants at 2, 3 and 4 wpi were analyzed by RNA gel-blot analysis followed by visualization using a Chemidoc-XRS (Bio-Rad) imaging system. + marks denoted with red color indicate positive control of PSTVd. LMW-RNAs (l00 ng) were fractionated by electrophoresis in 1.5% (w/v) agarose gels and used for loading controls (below the hybridization signals). (B) The relative accumulation was quantified based on the hybridization signals using Quantity One software package and the averages of three replicates were shown in a line graph (B). (C) Representative symptoms of tomato (cv. Rutgers) plants infected with PSTVd-I, -D, and their mutants (from left to right, non-inoculated tomato, PSTVd-I (I), PSTVd-D (D), PSTVd-DI (DI), PSTVd-ID (ID), D-U201G). Photos were taken 4 wpi. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Góra et al., 1996; Itaya et al., 2001; Matoušek et al., 2012). Using natural variants, artificial mutants, or chimeric recombinants created in vitro, it has been shown that the P domain plays a major role in the severity of symptom expression in tomato. PSTVd-D isolated from dahlia plants (Tsushima et al., 2011) used in this study induced mild symptoms in this host, whereas PSTVd-I induced more severe symptoms. PSTVd-D and PSTVd-I differs in nine nucleotides located in the TL, P, V, and TR domains. Comparative infection assays performed in our study using a series of 14 mutants based on PSTVd-D and PSTVd-I identified determinants for the attenuation of PSTVd-D in the TL and P domains. This result corroborates previous findings using PSTVd variants such as M and RG1 or QFA, C3, and AS1. Because PSTVd-D is different from PSTVd-I in the TL (nucleotides 42 and 43) and P (nucleotides 64/65, 310, and 311/312) domains (Fig. 1), the roles of these nucleotides on the pathogenicity is extremely interesting. For example, since the proposal of the hypothesis on the role of RNA silencing in the pathogenicity of viroids (Wang et al., 2004), at least four supporting data were reported in the last four years. Among them, three specific PSTVd-sRNA species which can target and silence host genes expression via RNAi pathway through homologous sequence-depending manner were identified in combinations of PSTVd-tomato (Adkar-Purushothama et al., 2015b), PSTVd-*Nicotiana* species (Eamens et al., 2014), and tomato planta macho viroid (TPMVd)-tomato (Avina-Padilla et al., 2015). Although different viroid mutants or species were used in these analysis, the three PSTVd or TPMVd-sRNA sequences of the interest correspond to the nucleotide regions of 39–59, 46–56, and 293–313 in the P domains of PSTVd-I. Since the nucleotide differences found between PSTVd-I and PSTVd-D at positions 42, 43, 64/65, 310, and 311/312 are all included in the three PSTVd or TPMVd-sRNAs, the significance of these individual nucleotides or their structures awaits further detailed analyses.

Comparisons of the molecular structures and mutations found in PSTVd isolates characterized thus far showed that nucleotides (in reference to PSTVd-I) 46G, 47C, and 317U are present in intermediate-severe and mild isolates, while 310U/C and insertion of U or UU in 311/312 were specific of mild isolates (Fig. 3). A BLAST database search revealed that the sequence from 310 to 318 in PSTVd-D, ^{310}(U/C) CUUUGCUU^{318} (the underlined sequences show those characteristic to mild and/or intermediate-severe), is conserved in some isolates reported as mild (accession numbers X76844, M88681, M88677) and in those detected from Russian potato isolates (Owens et al., 2009) inciting mild-type symptoms in tomato (accession numbers JQ889841, JQ889850–JQ889853, EU879915–EU879923, etc.).

Because most of the studies related to PSTVd pathogenicity have focused on the functions of the P domain thus far, our study is unique in analyzing mutants in the V and the TR domains. PSTVd-D is different from PSTVd-I in the V (nucleotides 118 and 126) and TR (nucleotide 201) domains, and the significance of the three nucleotides in the attenuation of PSTVd-D was verified in our study. Deletion of 118A was not specific to PSTVd-D but observed in other mild and severe isolates (Fig. 3). The same deletion was frequently found in Russian potato PSTVd mild to severe isolates and shown to be neutral in pathogenicity in tomato (Owens et al., 2009). Two

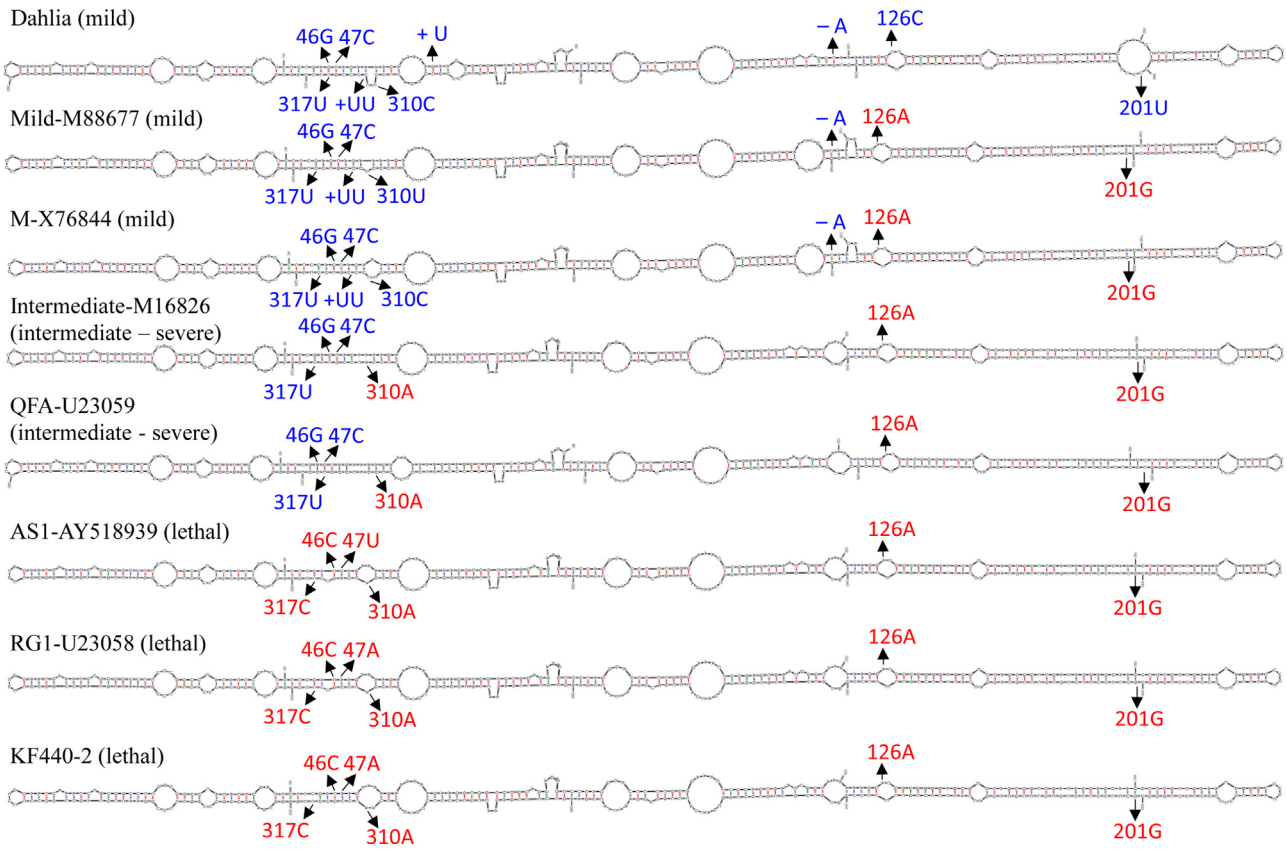

Fig. 3. Mapping in the predicted PSTVd secondary structure of the mutations found in the dahlia and in other characterized isolates of this viroid inducing mild, severe, and lethal symptoms in tomato. Mutations denoted with blue characters indicate the substitutions specific to PSTVd-D, and others with red characters are specific to lethal isolates of PSTVd. Mild-M88677 (Schnölzer et al., 1985), M-X76844 (Góra et al., 1996), QFA-U23059 (Gruner et al., 1995), AS1-AY518939 (Matoušek et al., 2012), RG1-U23058 (Owens et al., 1996), and KF440-2 (Góra et al., 1996). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mutants, I-A118Δ and D-118A, created in our study showed similar stunting, leaf curling, and systemic accumulation compared with PSTVd-I and PSTVd-D, respectively (Fig. 2B). The change in nucleotide 118 caused a certain but not a big influence upon the severity of symptoms and systemic accumulation. A126C and G201U substitutions were unique to PSTVd-D, and a BLAST analysis showed that the variants with A at position 126 were unusual in the subviral RNA database (Rocheleau and Pelchat, 2006), with isolates from tomato (AY372397) and Iranian potato (DQ308556) among those. Indeed, in 13 of the 156 PSTVd sequences deposited, there is C and in the remaining an A at the nucleotide 126. Even though 126C is infrequent, our mutational analysis clearly showed that I-A126C was an infectious and stable mutant, indicating that C was actually viable for position 126, which forms part of the 18th loop in the predicted secondary structure model. Our comparative infectivity assay indicated that A–C or C–A change in the nucleotide 126 did not cause a noticeable difference in the severity of symptoms, namely, I-A126C and PSTVd-I or D-C126A and PSTVd-D showed very similar stunting and leaf curling. However, they were a little different in systemic accumulation compared to PSTVd-I and -D, respectively. Particularly, I-A126C accumulated slower than I and D-C126A accumulated faster than D (Fig. 2B). We concluded that a change at nucleotide 126 did not cause a noticeable difference in symptom severity but influenced systemic accumulation.

In position 201, only G and U have been found in the natural variants reported thus far: 17 of 156 variants in the subviral RNA database possess U and the remaining G. Because this nucleotide is located at the right boundary of the lower strand of the highly conserved RY-motif, we examined the influence of the four nucleotides upon infectivity and pathogenicity. Interestingly, not only G and U

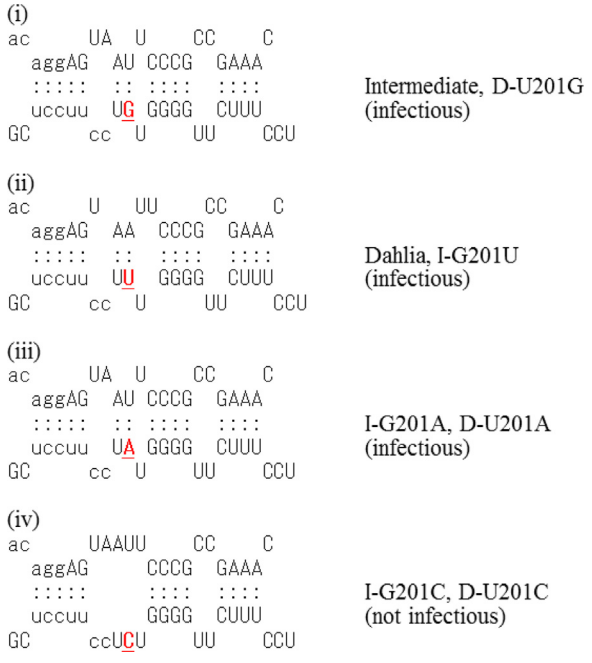

Fig. 4. Comparison of predicted secondary structures of a part of TR domain including the nucleotide 201 (red). Predicted secondary structures were similar in the infectious mutants (i–iii) with G, U or A at the nucleotide 201, which formed stem. On the other hand, C residue at the nucleotide 201 can cause significant structural change in the mutants (iv) which lost the infectivity. Lower letters indicate the sequences that form RY-motif. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

found in the nature but also A was viable in PSTVd-I and -D without causing a noticeable negative effect upon infectivity, genetic stability, and systemic accumulation. Only the mutants with 201C (i.e., I-G201C and D-U201C) lost infectivity. Because the introduction of C at position 201 can change the structure in the right boundary of the RY-motif (Fig. 4), it is possible that change could have a negative effect on the RY-motif ability to recruit Virp1 and result in the loss of infectivity. Comparative infectivity assays revealed that all the three viable nucleotides G, U, and A at position 201 did not affect the severity of symptoms because I-G201 (U or A) and D-U201 (G or A) showed very similar stunting and leaf curling compared to PSTVd-I and -D, respectively. However, they were a little different in the rate of accumulation compared with PSTVd-I and -D, respectively. I-G201U and I-G201A accumulated similarly to I, but D-U201 G and D-U201A accumulated faster than D (Fig. 2B). Thus nucleotide position 201 did not affect the severity of symptoms but influenced the accumulation rates.

The three mutations found in the right-hand half of PSTVd-I and -D were exchanged without causing noticeable differences in the severity of symptoms, but differences in the systemic accumulation rates were observed (in some cases). All three D-series mutants with single I-type mutations accumulated almost similarly to DI with three mutations; thus, the three mutations may have independently contributed to the attenuation of the PSTVd-D isolate.

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