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Influence of the terminal left domain on horizontal and vertical transmissions of tomato planta macho viroid and potato spindle tuber viroid through pollen

Hironobu Yanagisawa[a,b], Teruo Sano[c], Shu Hase[d], Yosuke Matsushita[e,*]

[a] Central Region Agricultural Research Center, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-8666, Japan
[b] The United Graduate School of Agricultural Sciences, Iwate University, Morioka, Iwate 020-8550, Japan
[c] Plant Pathology Laboratory, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki, Aomori 036-8560, Japan
[d] Plant Pathology Laboratory, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata 997-0037, Japan
[e] Institute of Vegetable and Floriculture Science, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-8519, Japan

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ABSTRACT

Viroids can be transmitted vertically and/or horizontally by pollen. Tomato planta macho viroid (TPMVd) has a high rate of horizontal transmission by pollen, whereas potato spindle tuber viroid (PSTVd) does not. To specify the domain(s) involved in horizontal transmission, four viroid chimeras were created by exchanging the terminal left (TL) and/or pathogenicity (P) domains between PSTVd and TPMVd. PSTVd-based chimeras containing TPMVd-TL and P, or TPMVd-TL alone, displayed a high rate of horizontal transmission. TPMVd-based chimeras containing PSTVd-TL and P lost infectivity, and those containing PSTVd-TL alone displayed a low rate of horizontal transmission. In addition, the vertical transmission rate was also higher in the mutants containing TPMVd-TL than in the others. These findings indicate that the sequences or structures in the TL and P (although the role is limited) domains are important not only for horizontal but also for vertical transmission by pollen.

1. Introduction

Pollen-transmission is a major delivery system to transfer viruses and viroids. When the pathogens infect pollen grains, they may infect mother plants through pollination (horizontal transmission), or they may infect the progeny seedlings after fertilization by infected pollen (vertical transmission) (Barba et al., 2007; Card et al., 2007; Mink, 1993). Viroids are economically important pathogens and classified as high-risk plant quarantine pests. Viroids consist of naked, circular, single-stranded RNA—246–475 nucleotides in length—the smallest among plant pathogens, and members of one of two families (***Pospiviroidae*** and ***Avsunviroidae***) (Di Serio et al., 2017; Hammann and Steger, 2012). The genus ***Pospiviroid*** includes nine species [chrysanthemum stunt viroid (CSVd), citrus exocortis viroid (CEVd), columnea latent viroid, iresine viroid 1, pepper chat fruit viroid (PCFVd), potato spindle tuber viroid (PSTVd), tomato apical stunt viroid (TASVd), tomato chlorotic dwarf viroid, and tomato planta macho viroid (TPMVd)]. PSTVd was found to be vertically transmitted through pollen on potato (***Solanum tuberosum***), tomato (***S. lycopersicum***), and petunia (***Petunia hybrida***) plants (Fernow et al., 1970; Kryczyński et al., 1988; Singh et al., 1992, 2003). In addition, vertical transmission through pollen occurred on CSVd-infected tomato (Kryczyński et al., 1988) and on TPMVd- and PCFVd-infected petunia plants (Yanagisawa and Matsushita, 2017). PSTVd was horizontally transmitted through pollen on potato and tomato plants (Kryczyński et al., 1988; Singh et al., 1992). Moreover, horizontal transmission through pollen occurred on CSVd-infected tomato plants (Kryczyński et al., 1988) and on TPMVd-infected petunia plants (Yanagisawa and Matsushita, 2017).

Viroids are localized in the sperm nuclei and the vegetative nucleus of infected pollen grains. They pass through the style with the elongation of the pollen tube after pollination and eventually reach the ovule of the mother plant, resulting in vertical transmission (Matsushita and Tsuda, 2014; Matsushita and Yanagisawa, 2018). Alternatively, horizontal transmission is established when the viroid leaks out of the germinating pollen tube under elongation and infects the lower style and ovary tissues of the mother plant (Yanagisawa and Matsushita, 2018). Interestingly, horizontal transmission can occur even when the pollen tube fails to reach the ovule, indicating that fertilization is not

* Corresponding author.
E-mail addresses: yana1208@affrc.go.jp (H. Yanagisawa), sano@hirosaki-u.ac.jp (T. Sano), s.hase@tds1.tr.yamagata-u.ac.jp (S. Hase), yousuken@affrc.go.jp (Y. Matsushita).

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necessarily required to establish the horizontal transmission of viroids.

The pospiviroid genome consists of five domains (terminal left (TL), pathogenicity (P), central, variable (V), and terminal right) (Steger et al., 2017). Pathogenicity is affected by a single domain or by multiple domains including the TL, P, and V domains (Hammann and Steger, 2012; Owens and Hammond, 2009; Tsushima et al., 2016). Additionally, Owens and Hammond, 2009 reported that the TL and P domains are mutually involved in pathogenicity, and the TL domain affects pathogenicity over the P domain. The P and V domains also greatly influence infectivity (Owens et al., 1991; Hu et al., 1996) and a mutation in the central domain was involved in determining whether PSTVd could infect *Nicotiana tabacum* (Wassenegger et al., 1996). Both the TL and central domains are also strongly involved in replication (Zhong et al., 2008), and the nucleotides involved in systemic trafficking are scattered across all five domains (Zhong et al., 2008). Accordingly, the differences in the nucleotides of each domain greatly contribute to the biological properties of the viroid.

Severe isolates of PSTVd and TPMVd cause severe symptoms such as stunting and leaf yellowing in susceptible tomato cultivars (Galindo et al., 1982; Hammond, 2017). Although an isolate of TPMVd (accession no. GQ131573) was transmitted vertically and horizontally by pollen in petunias, an isolate of PSTVd (accession no. EU862231) was transmitted only vertically (Yanagisawa and Matsushita, 2018). It was therefore suggested that differences in the nucleotides and/or molecular features of the viroid genomes resulted in differences in transmission. Thus, to specify the domains or regions involved in viroid transmission by pollen, we produced four viroid chimeras from TPMVd and PSTVd by replacing the TL and/or P domains, mechanically infected petunias with them, and collected pollen grains from the infected plants. The pollen grains were then placed on the stigmas of healthy petunias and the distribution of viroid chimera in the pistils of the flower bud after pollination and in the fruits developed by fertilization was observed. Finally, to compare the horizontal and vertical transmission rates of each viroid chimera, tissues from the pollen-inoculated petunia flower organs, such as the stigma, style, ovary wall, placenta, calyx, peduncle, and developing seed, were collected separately and checked for viroid infection using RT-qPCR assays.

2. Results

2.1. The infectivity and symptoms of viroid chimeras to petunias and tomatoes

Petunia plants inoculated with *in vitro* transcripts of four TPMVd-PSTVd chimeras, *i.e.,* TP/PS-TLP (TPMVd-based chimera with PSTVd-TL and –P domains), TP/PS-TL (TPMVd-based chimera with PSTVd-TL), PS/TP-TLP (PSTVd-based chimera with TPMVd-TL and –P), and PS/TP-TL (PSTVd-based chimera with TPMVd-TL) were assayed for infection by RT-qPCR analysis 2 months after inoculation. Three of them, PS/TP-TLP, PS/TP-TL, and TP/PS-TL, were infectious. However, transcripts of TP/PS-TLP were not detected from the uppermost leaves of the inoculated petunia seedlings by RT-qPCR, suggesting that TP/PS-TLP could not systemically infect petunia plants (Fig. 1). None of the viroid chimeras infecting the petunia plants exhibited any symptoms. However, when the chimeras were extracted from the infected petunias and re-inoculated into susceptible tomato plants, dwarfing, leaf chlorosis, and yellowing symptoms developed, which were similar to the symptoms induced by the parental viroids (data not shown).

2.2. The nucleotide sequence of viroid chimeras after infection of petunia plants

Five independent cDNA clones were selected from each sample and sequenced to analyze the nucleotide sequence of the viroid chimeras after infection of petunia plants. Two months after inoculation, PS/TP-TLP had changed in three nucleotides (U_{14} → G, insertion of A_{62}, and U_{167}→ C) (Fig. 1). Similarly, TP/PS-TL had changed in two nucleotides (U_{177} → C and G_{229} → C) and PS/TP-TL had changed in four nucleotides (U_6 → A, C_{84} → U, U_{86} → C, and C_{358} → U). The sequences 8 months after inoculation were also examined but they were all the same as those after 2 months, suggesting that once mutations were gained they were maintained and replicated stably afterwards.

2.3. Concentration of the infectious viroid chimeras in the pollen grains of the infected petunia plants

First, pollen grains were collected from petunia plants 8 months after inoculation, and the infectivity of pollen grains (50 mg) infected with the several viroid chimeras was compared in an assay with tomato plants followed by RT-qPCR analysis. The reason we used pollen grains 8 months after inoculation was that horizontal and vertical transmission through pollen were stably observed at this time after inoculation in the preliminary tests.

The inoculum was prepared by grinding pollen grains from the infected plants. All three infectious viroid chimeras, as well as the parental viroids, showed infectivity up to 10^{-1} dilution (Table 1), confirming that infectivity in 50 mg of pollen grains was similar among the three viroid chimeras at this time point. When the concentrations of the viroids in 50 mg of pollen grains of the same samples were analyzed by RT-qPCR, it was confirmed that the average Ct values of the pollen samples, including those infected with the parental viroids, were almost equivalent except for PS/TP-TL samples (Table 2).

2.4. Pollen tube elongation of healthy petunia plants at 24 h after pollination by viroid-infected pollen grains

We investigated if the pollen tubes of viroid-infected petunia pollen grains had reached the ovary of healthy petunia plants at 24 h after pollination. The pollen tubes of all pollinated grains 10 months post infection (10 mpi) of each viroid, as well as, the pollen tubes of healthy petunia plants, reached the ovary (Fig. 3). Additionally, a part of these pollen tubes reached the ovule in the ovary (data not shown).

2.5. Distribution of viroid chimeras in the petunia pistils 24 h after pollination by viroid-infected pollen grains

To analyze the distribution of the viroid chimeras in the pistils after pollination by viroid-infested pollen grains, healthy petunia plants were pollinated with pollen grains collected from viroid-infected petunia plants. Twenty-four hours later, the stigma, style, and ovary tissues were collected (Fig. 2A), nucleic acids were extracted, and the viroids were detected using RT-qPCR. When pollinated with pollen grains 8 months post infection (8 mpi) and 10 mpi, PS/TP-TLP was detected in the stigma, style, and ovary of all plants, indicating that the chimera had reached the ovary within 24 h of pollination (Table 3). This chimera also reached the ovary in all individuals pollinated with 6 mpi pollen grains and in 19 individuals pollinated with 4 mpi pollen grains (about 70%). Similarly, PS/TP-TL was detected in the stigma (100% of the individuals), style (100%), and ovary (~70%) of petunia plants pollinated with 8 and 10 mpi pollen grains. The detection rate of this chimera in the ovary was somewhat lower using 6 mpi pollen grains (~60%) and even lower using 4 mpi pollen grains(~20%) (Table 3). The results indicated that the ability of PS/TP-TL to reach the ovary was similar but somewhat lower than that of PS/TP-TLP. In contrast, TP/PS-TL was detected in the stigma of all plants, but was not detected at all in the ovary and in four plants (14.8%) in was detected in the style, even when using 10 mpi pollen grains (Table 3). For 10 mpi pollen grains, TPMVd was detected in the stigma, style, and ovary of all plants, while PSTVd was detected in the stigma of all plants, but only in the style (44.4%) and ovary (7.4%) of same plants. Hence, PSTVd was detected in the style of more plants than TP/PS-TL, but rarely detected in the ovary.

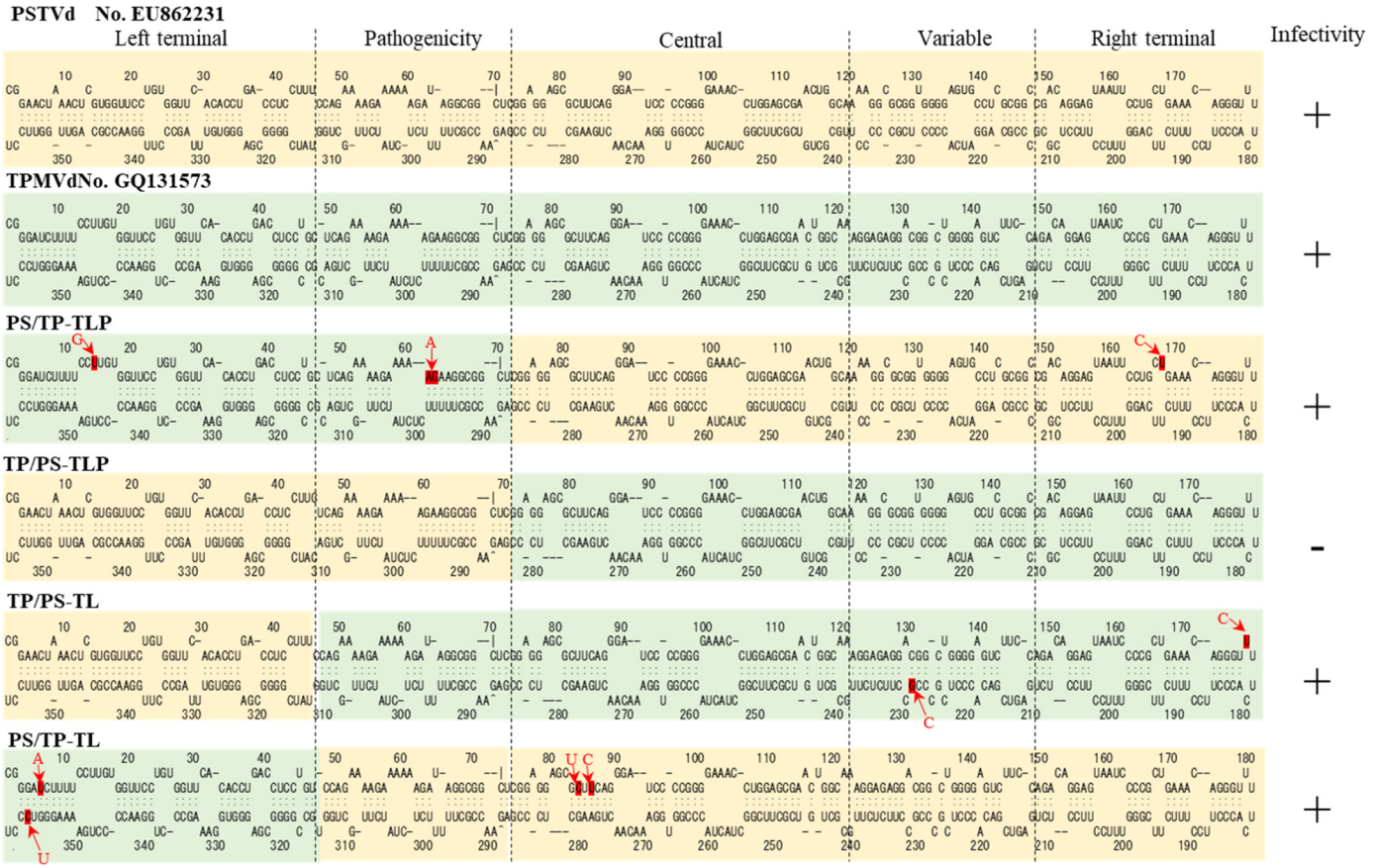

Fig. 1. Secondary structure of each viroid chimera recombined from tomato planta macho viroid (TPMVd, accession no. GQ131573) and potato spindle tuber viroid (PSTVd, accession no. EU862231). Pale yellow and pale green boxes indicate sequences of PSTVd and TPMVd, respectively. The base marked in red is where the mutation occurred. The infectivity column indicates the infectivity of each viroid or chimera in petunia plants.

Table 1
Infectivity of petunia pollen grains infected by each of viroid constructs in tomato plants.

Viroid or chimera name	Dilution of inocula (infected pollen extracts)				
	10^{0}[a]	10^{-1}	10^{-2}	10^{-3}	10^{-4}
TPMVd	3/3[b]	3/3	0/3	0/3	0/3
PSTVd	3/3	3/3	0/3	0/3	0/3
PS/TP-TLP	3/3	3/3	0/3	0/3	0/3
TP/PS-TL	3/3	3/3	0/3	0/3	0/3
PS/TP-TL	3/3	3/3	0/3	0/3	0/3

[a] Inocula obtained from 50 mg petunia pollen grains homogenized in 0.1 M phosphate buffer (pH 7.5).
[b] Number of viroid-infected tomato plants / number of tomato plants inoculated with viroid-infected pollen grains.

2.6. Distribution of the viroid chimeras in the petunia fruits 24 days after pollination with viroid-infected pollen grains

We analyzed the distribution of the viroid chimeras in the petunia fruits developed after pollination. Twenty-four days after pollination, the ovary wall, placenta, developing seed, calyx, and peduncle were collected separately (Fig. 2B), and analyzed using the same methods as above.

When 10 mpi pollen grains were used, PS/TP-TLP was detected at a high rate in the ovary wall (~92%), placenta (100%), and developing seeds (100%), and at relatively high rates in the calyx (~42%) and peduncle (~75%) (Table 4). In contrast, TP/PS-TL was only detected in the developing seeds, even after pollination with 10 mpi pollen grains (Table 4). For 10 mpi pollen grains, the detection rate of TPMVd in fruit tissues was high, while PSTVd was only detected in for the developing seeds. The results revealed that the ability to distribute in fruit tissues was high in PS/TP-TLP and PS/TP-TL, similar to TPMVd, and extremely low in TP/PS-TL, as in PSTVd. However, it should be noted that distribution into the developing seeds was an exception as the detection rate was also relatively high (~17–58%) for TP/PS-TL, suggesting that a different mechanism is involved.

The nucleotide sequence of two of the viroid chimeras, PS/TP-TLP and PS/TP-TL, detected in the placenta and calyx were identical to those detected in petunias 2 months after infection, indicating that the mutations which took place after the infection of the petunias were maintained stably in these chimeras after horizontal transmission.

2.7. Vertical transmission of viroid chimeras by pollination with viroid-infected pollen grains

From the results presented above, it became clear that the P and especially the TL domains were strongly involved in the ability of

Table 2
Viroid concentration of petunia pollen grains infected by TPMVd, PSTVd, and three viroid chimeras.

Viroid or chimera name	Test no.	Viroid detection in petunia pollen grains		Internal control of actin 11 mRNA in petunia pollen grains	
		Viroid detection (Ct)	Average of Ct	Internal control (Ct)	Average of Ct
TPMVd	1	24.0[a]	16.1[b]	20.2[a]	20.9[b]
	2	11.0		21.1	
	3	13.4		21.3	
PSTVd	1	20.6	16.1	20.1	20.1
	2	14.2		20.1	
	3	13.7		20.2	
PS/TP-TLP	1	19.1	14.1	19.8	20.1
	2	11.5		20.3	
	3	11.5		20.3	
TP/PS-TL	1	20.2	15.6	19.8	20.3
	2	13.2		20.9	
	3	13.2		20.3	
PS/TP-TL	1	15.1	11.8	20.2	20.5
	2	10.4		20.8	
	3	9.8		20.6	

50 mg of pollen was collected from seedlings infected with each viroid. Pollen grains were collected 8 months after inoculation. RNA was extracted from these pollen grains, and 100 ng RNA was used as a template for the detection of viroids and action 11 of mRNA in petunia pollen grains using RT-qPCR assays.
[a] Mean Ct value of three replications of same template.
[b] Mean Ct value of three tests.

viroids to spread horizontally through pollination by the infected pollen grains. Therefore, we further analyzed the possibility that the TL and/or P domains are involved in vertical transmission of viroids. Using the same methods as above, healthy petunia seedlings were pollinated with pollen grains collected 4, 6, and 8 mpi with the three viroid chimeras, as well as the parental viroids. The infected plants were grown in a growth chamber and matured seeds were harvested.

As in the case of horizontal transmission, PS/TP-TLP showed the highest seed transmission rate of the three chimeras. The average seed transmission rates were 92.6%, 86.3%, and 54.9%, for the pollen grains collected 8, 6, and 4 mpi, respectively, which was comparable to, but slightly lower, than the parental species, TPMVd (Table 5). PS/TP-TL was the second highest, with rates of 54.45%, 4.7%, and 3.2%, for 8, 6, and 4 mpi, respectively. The difference in vertical transmission rate between these two chimeras was greater than it was for horizontal transmission. Interestingly, the seed transmission rate of PS/TP-TL was higher than that of PSTVd when 8 mpi pollen grains were used for pollination, but it was significantly lower when 6 and 4 mpi pollen grains were used. Again, TP/PS-TL was the lowest of all, with 4.7%, 1.2%, and 1.7% for 8, 6, 4 mpi, respectively (Table 5).

Finally, we examined the nucleotide sequences of three infectious viroid chimeras in the seed-transmitted petunia plants. The sequences were the same as those detected from petunias 2 months, indicating that the mutations which took place after the infection of the petunias were maintained stably in these chimeras after seed transmission.

3. Discussion

Viroids are small, self-replicating, non-protein-coding RNAs, and single mutations can induce marked changes in their functions in the hosts, such as replication, trafficking, pathogenicity, and host range (Kovalskaya and Hammond, 2014; Škarić, 2017; Zhong et al., 2008). This implies that certain genome sequences and/or structures can change the horizontal and vertical transmission of viroids through pollen. In vertical (or seed) transmission, for example, PSTVd and TCDVd share a very similar overall nucleotide sequence homolog,

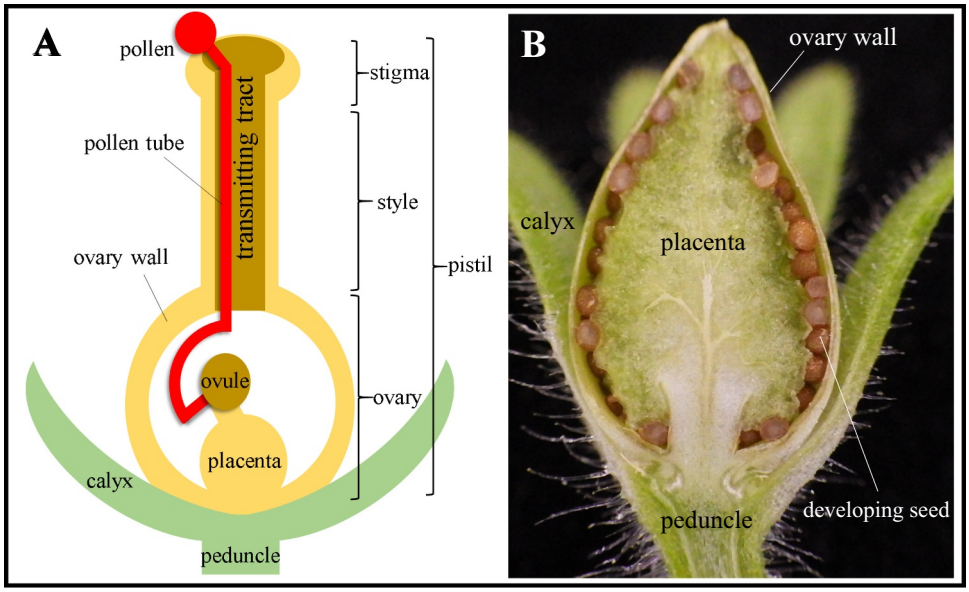

Fig. 2. (A) Pollen tube pathway in petunia plant 24 h after pollination. (B) The cross-sectional view of a petunia fruit 24 days after pollination.

Table 3
Infection rates of each viroid chimera in various parts of the pistils of petunia plants at 24 hours after pollination using pollen grains collected at different periods after inoculation.

Viroid or chimera name	Part of pistil	Elapsed time after inoculation[a]							
		4 months		6 months		8 months		10 months	
		Viroid detection	Internal control	Viroid detection	Internal control	Viroid detection	Internal control	Viroid detection	Internal control
TPMVd	Stigma	nt	nt	nt	nt	nt	nt	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)
	Style	nt	nt	nt	nt	nt	nt	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)
	Ovary	nt	nt	nt	nt	nt	nt	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)
PSTVd	Stigma	nt	nt	nt	nt	nt	nt	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)
	Style	nt	nt	nt	nt	nt	nt	12/27 (4/9, 6/9, 2/9)	27/27 (9/9, 9/9, 9/9)
	Ovary	nt	nt	nt	nt	nt	nt	2/27 (1/9, 1/9, 0/9)	27/27 (9/9, 9/9, 9/9)
PS/TP-TLP	Stigma	27/27[b] (9/9[c], 9/9, 9/9)	27/27[c] (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)
	Style	23/27 (7/9, 9/9, 7/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)
	Ovary	19/27 (6/9, 7/9, 6/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)
TP/PS-TL	Stigma	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)
	Style	2/27 (0/9, 0/9, 2/9)	27/27 (9/9, 9/9, 9/9)	0/27 (0/9, 0/9, 0/9)	27/27 (9/9, 9/9, 9/9)	1/27 (0/9, 1/9, 0/9)	27/27 (9/9, 9/9, 9/9)	4/27 (2/9, 1/9, 1/9)	27/27 (9/9, 9/9, 9/9)
	Ovary	0/27 (0/9, 0/9, 0/9)	27/27 (9/9, 9/9, 9/9)	0/27 (0/9, 0/9, 0/9)	27/27 (9/9, 9/9, 9/9)	0/27 (0/9, 0/9, 0/9)	27/27 (9/9, 9/9, 9/9)	0/27 (0/9, 0/9, 0/9)	27/27 (9/9, 9/9, 9/9)
PS/TP-TL	Stigma	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9,9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)
	Style	18/27 (5/9, 6/9, 7/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9,9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)	27/27 (9/9, 9/9, 9/9)
	Ovary	5/27 (2/9, 2/9, 1/9)	27/27 (9/9, 9/9, 9/9)	16/27 (4/9, 6/9, 6/9)	27/27 (9/9, 9/9, 9/9)	19/27 (7/9, 7/9, 5/9)	27/27 (9/9, 9/9, 9/9)	19/27 (6/9, 6/9, 7/9)	27/27 (9/9, 9/9, 9/9)

nt; not-tested.

[d]Number of samples positive for *Petunia hybrida* putative monosaccharide transporter mRNA (accession no. AF061106; used as an internal control) among the 18 total samples tested.

[a] Pollen grains collected at 4, 6, and 8 months after inoculations of petunia plants were then used to pollinated heathy petunia plants. Healthy petunia seedlings grown for 2 months after seeding were pollinated with infected pollen grains, and then the pistils were collected 24 hours after pollination to the healthy plants.

[b] Number of samples containing viroids / number of samples tested.

[c] The experiments were conducted three times with nine samples each.

Table 4
Infection rates of each of viroid chimera in the fruit parts of petunia plants after pollination using pollen grains collected at different periods after inoculation.

Viroid or chimera name	Part of fruit	Elapsed time after inoculation[a]			
		4 months Viroid detection	6 months Viroid detection	8 months Viroid detection	10 months Viroid detection
	Ovary wall	nt	nt	nt	9/12 (5/6, 4/6)
	Placenta	nt	nt	nt	12/12 (6/6, 6/6)
TPMVd	Developing seed	nt	nt	nt	12/12 (6/6, 6/6)
	Calyx	nt	nt	nt	6/12 (4/6, 2/6)
	Peduncle	nt	nt	nt	5/12 (3/6, 2/6)
	Ovary wall	nt	nt	nt	0/12 (0/6, 0/6)
	Placenta	nt	nt	nt	0/12 (0/6, 0/6)
PSTVd	Developing seed	nt	nt	nt	12/12 (6/6, 6/6)
	Calyx	nt	nt	nt	0/12 (0/6, 0/6)
	Peduncle	nt	nt	nt	0/12 (0/6, 0/6)
PS/TP-TLP	Ovary wall	9/12[b] (4/6[c], 5/6)	8/12 (3/6, 5/6)	12/12 (6/6, 6/6)	11/12 (5/6, 6/6)
	Placenta	11/12 (5/6, 6/6)	9/12 (5/6, 4/6)	12/12 (6/6, 6/6)	12/12 (6/6, 6/6)
	Developing seed	12/12 (6/6, 6/6)	12/12 (6/6, 6/6)	12/12 (6/6, 6/6)	12/12 (6/6, 6/6)
	Calyx	4/12 (1/6, 3/6)	5/12 (3/6, 2/6)	9/12 (5/6, 4/6)	5/12 (3/6, 2/6)
	Peduncle	6/12 (2/6, 4/6)	2/12 (1/6, 1/6)	8/12 (5/6, 3/6)	9/12 (5/6, 4/6)
TP/PS-TL	Ovary wall	0/12 (0/6, 0/6)	0/12 (0/6, 0/6)	0/12 (0/6, 0/6)	0/12 (0/6, 0/6)
	Placenta	0/12 (0/6, 0/6)	0/12 (0/6, 0/6)	0/12 (0/6, 0/6)	0/12 (0/6, 0/6)
	Developing seed	6/12 (2/6, 4/6)	2/12 (0/6, 2/6)	4/12 (3/6, 1/6)	7/12 (5/6, 2/6)
	Calyx	0/12 (0/6, 0/6)	0/12 (0/6, 0/6)	0/12 (0/6, 0/6)	0/12 (0/6, 0/6)
	Peduncle	0/12 (0/6, 0/6)	0/12 (0/6, 0/6)	0/12 (0/6, 0/6)	0/12 (0/6, 0/6)
PS/TP-TL	Ovary wall	0/12 (0/6, 0/6)	12/12 (6/6, 6/6)	12/12 (6/6, 6/6)	9/12 (4/6, 5/6)
	Placenta	0/12 (0/6, 0/6)	12/12 (6/6, 6/6)	12/12 (6/6, 6/6)	12/12 (6/6, 6/6)
	Developing seed	6/12 (3/6, 3/6)	12/12 (6/6, 6/6)	12/12 (6/6, 6/6)	12/12 (6/6, 6/6)
	Calyx	0/12 (0/6, 0/6)	4/12 (3/6, 1/6)	6/12 (2/6, 4/6)	5/12 (2/6, 3/6)
	Peduncle	0/12 (0/6, 0/6)	3/12 (2/6, 1/6)	5/12 (2/6, 3/6)	5/12 (2/6, 3/6)

nt; not-tested.
[a] Pollen grains collected at 4, 6 and 8 months after inoculations of petunia plants were then used to pollinate heathy petunia plants. Healthy petunia seedlings grown for 2 months after seeding were pollinated with the infected pollen grains.
[b] Total number of samples containing viroids / total number of samples tested.
[c] The experiments were conducted twice with six samples each.

however, PSTVd showed the ability to invade the ovule whereas TCDVd did not, and thus only PSTVd was transmitted through seeds (Matsushita et al., 2011). In coleus blumei viroid 1, a point-mutation at nucleotide 25 in loop five in the genome switches on the potential to transmit through seeds (Tsushima and Sano, 2018). In horizontal transmission, on the other hand, an isolate of TPMVd, but not PSTVd, was highly transmitted horizontally through pollen (Yanagisawa and Matsushita, 2018). Since PSTVd and TPMVd also share relatively high overall nucleotide sequence homology, the result suggested that certain nucleotide(s) and/or structure(s) can determine the efficiency of horizontal and/or vertical transmission of viroids by pollen.

In this study, we focused on the domains of TPMVd involved in efficient horizontal transmission and found that a TPMV-PSTVd chimera with the TL and P domains of TPMVd was more efficiently transmitted horizontally from style to ovary by pollination with viroid-infected pollen grains. The other chimera with only the TL domain of TPMVd showed the second highest horizontal transmission rate. Moreover, the chimeras with the TL domain of TPMVd were also transmitted vertically by pollen at the highest rate. Therefore, we concluded that the TL domain of TPMVd is the major, and the P domain of TPMVd is a secondary, determinant for both horizontal and vertical transmission of TPMVd by pollination with viroid-infected pollen grains.

Our previous study revealed that it is essential for viroids to move

Table 5
Vertical transmission rates of each viroid chimera through viroid chimera-infected pollen using pollen grains collected at different periods after inoculation.

Viroid or chimera name	Elapsed time after inoculation[a]					
	4 months		6 months		8 months	
	Viroid detection	Transmission rate (%)	Viroid detection	Transmission rate (%)	Viroid detection	Transmission rate (%)
TPMVd	162/185[b] (78/92[c], 84/93)	85.57[d]	188/188 (94/94, 94/94)	100.0	142/144 (48/48, 94/96)	98.6
PSTVd	35/195 (19/100, 16/95)	18.0	43/187 (22/93, 19/94)	23.0	58/187 (19/92, 39/95)	31.0
PS/TP-TLP	106/193 (57/100, 49/93)	54.9	164/190 (80/95, 84/95)	86.3	174/188 (88/94, 86/94)	92.6
TP/PS-TL	4/233 (1/100, 3/133)	1.7	2/188 (0/94, 2/94)	1.0	9/190 (6/95, 3/95)	4.7
PS/TP-TL	6/186 (2/93, 4/93)	3.2	9/190 (3/95, 6/95)	4.7	104/191(49/96, 55/95)	54.45

[a] Pollen grains collected at 4, 6 and 8 months after inoculation of petunia plants were then used to pollinated heathy petunia plants. Healthy petunia seedlings grown for 2 months after seeding were pollinated with infected pollen grains.
[b] Total number of samples containing viroids / total number of samples tested.
[c] The experiments were conducted twice with germinated seedlings from seeds collected from fruits formed after pollination with infected pollen grains.
[d] Vertical transmission rate (%) = (total number of samples containing viroids / total number of samples tested) × 100

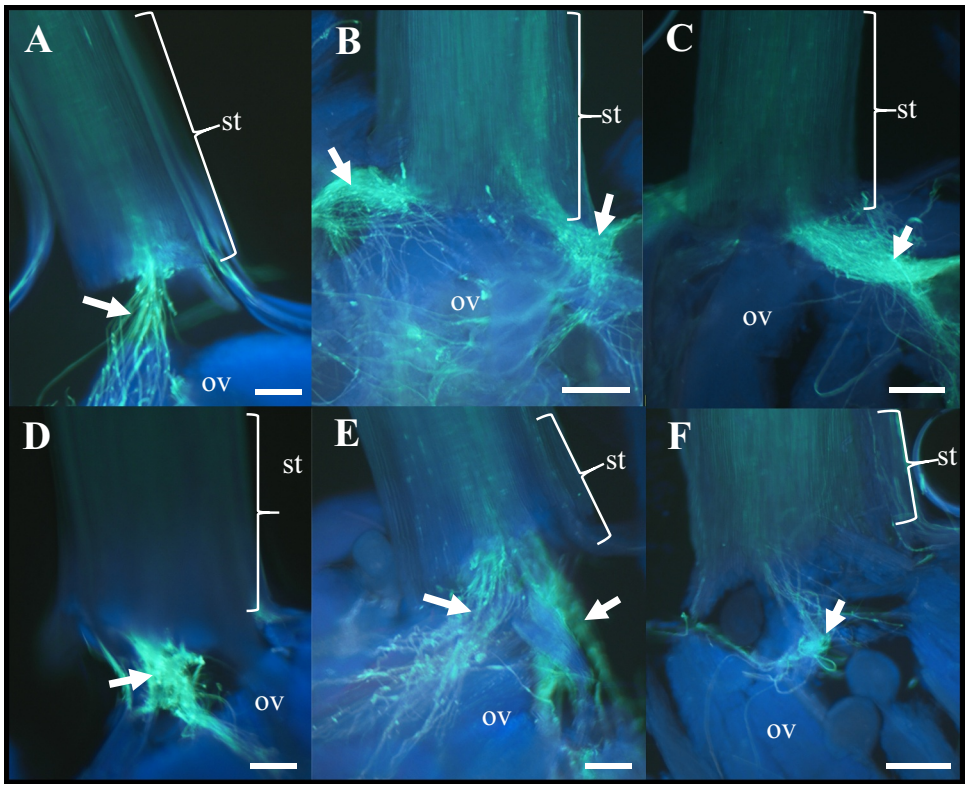

Fig. 3. Aniline blue staining of pollen tubes of *Petunia* ×*hybrida* at 24 h after pollination to the stigma of healthy petunia with pollen grains collected from different petunia plants. (A) Healthy plants, (B) TPMVd-infected, (C) PSTVd-infected, (D) PS/TP-TLP-infected, (E) TP/PS-TL-infected, (F) PS/TP-TL-infected. The pollen tubes are identified with white arrows. st, style; ov, ovary. Bar, 200 μm.

from the style to the ovary during the elongation of the pollen tubes for establishment of horizontal transmission (Yanagisawa and Matsushita, 2018). In our current study, PS/TP-TLP and PS/TP-TL moved at high frequency from the style to the ovary *via* the elongation of the pollen tube within 24 h after pollination (Table 3; Fig. 3), and eventually invaded not only the developing seeds but also the ovary wall, placenta, calyx, and peduncle in mature fruits, indicating the establishment of horizontal transmission. The results obtained for these two chimeric viroids were as identical to those obtained for TPMVd. In contrast, TP/PS-TL was not detected in the ovary even though pollen tubes reached the ovary after pollination (Table 3; Fig. 3), and was also not detected in the ovary wall, the placenta, the calyx, or the peduncle in the mature fruits after fertilization (Table 4). Similarly, PSTVd was not detected in these organs. Considering the result that the infectivity of viroids in 8 mpi pollen grains after mechanical inoculation was not different among the three infectious viroid chimeras or the parental viroids (Tables 1 and 2), the differences found in their ability for horizontal and vertical transmission by pollen suggests that the TL domain has a somewhat significant influence on the establishment of horizontal transmission after pollination; for example, on the replication/accumulation or the stability of the viroid during the elongation of the pollen tube in the floral organ.

When examining the correlation between the replication/accumulation or the transmission of viroids and the predicted molecular structures in mechanically inoculated host plants, the P domain of PSTVd and CEVd was first identified as a pathogenicity determinant of viroids, and the V domain was also shown to have some influence on the initial infection or replication process (Schnölzer et al., 1985; Visvader and Symons, 1985, 1986; Keese, Symons, 1985). Using the chimeras between TASVd and CEVd, multiple structural domains were shown to play important roles in the replication/accumulation and severity of symptom expression; for example, the stunting symptom has been mapped to the TL and P domains, whereas replication/accumulation has been mapped to the variable-terminal right domains (Sano et al., 1992). More recently, using chimeras from severe and mild isolates of TPMVd, a single base pair in the terminal right domain was identified as a virulence determinant factor in tomatoes, but the nucleotides did not affect replication/accumulation (Li et al., 2017). Zhong et al. (2008), by focusing on loop structures in the PSTVd genome, reported that loops in the TL and central domains of PSTVd affected replication ability, and those in all domains affected systemic trafficking. Furthermore, the latest analysis by the same group identified that the RNA motif loop 19 in the variable domain is important for PSTVd to spread from the palisade to the spongy mesophyll in infected leaves (Takeda et al., 2018). In this way, symptom severity was often mapped to the P and/or TL domains, whereas, efficiency of replication/accumulation or trafficking was often mapped to the V or terminal right domains. In contrast, the TL and P domains, but not the V or terminal right, were identified in our experiment as a molecular determinant for the horizontal and vertical transmission of TPMVd. Since previous findings were based on the behavior of viroids in mechanically infected host vegetative tissues, and the results presented here were obtained from the analysis of viroids in floral organs, the present conclusions might therefore be influenced by different factors than those in previous reports.

The vertical transmission rate of pospiviroids depends on the species of pospiviroids and host plants (Matsushita and Tsuda, 2016). We found in the previous study that the vertical transmission rate of an isolate of TPMVd was extremely high (~90%) in petunia plants compared to that of PSTVd (~20%) (Yanagisawa and Matsushita, 2017). In the current study the nucleotides and/or structures of the TL domain are suggested to be involved not only in horizontal transmission but also in vertical transmission. To establish vertical transmission by pollen, viroids need to travel to and replicate in the microspore mother cells before pollen formation (Matsushita and Tsuda, 2014; Matsushita and Yanagisawa, 2018). However, there were no significant differences between the infectivity and concentration of all the viroid chimeras in the infected pollen at 8 mpi, indicating that all viroid chimeras could infect and replicate in the microspore mother cells and were consequently present in the sperm nuclei of the pollen. Accordingly, these findings suggested that the TL domain could have some influence on the establishment of vertical transmission after pollination. On the other hand, the vertical

transmission rate of TP/PS-TL was much lower than that of the other viroid chimeras. Our previous study showed that the PSTVd in pollen grains disappeared with the elongation of pollen tubes in the style and the ovary. Specifically, the PSTVd concentration dropped below the detection limit of the RT-qPCR assay after pollination, and resulted in a decline in the vertical transmission rate (Yanagisawa and Matsushita, 2018). Similar results were obtained in TP/PS-TL in this analysis (Table 3). Thus, factor(s) in the TL domain of PSTVd might be involved in the disappearance of viroids in pollen grains during the elongation process and the inability of viroids to be transmitted vertically. Further studies are needed to investigate the mechanism behind PSTVd's disappearance from the style during the elongation of pollen tubes.

The time elapsed since viral infection affects the seed infection rate, and seed transmission is difficult in the early stages of infection (Mink, 1993; Wang, Maula, 1992). Thus, to determine the influence of the time since inoculation, vertical and horizontal transmission tests were conducted using pollen grains at different times (months) post infection. PS/TP-TLP and PS/TP-TL more efficiently reached the ovary *via* the style when pollinated with 8 and 10 mpi pollen than with 4 mpi pollen (Table 3). Similarly, when pollinated with 8 and 10 mpi pollen, the viroid chimeras invaded the ovary wall, placenta, calyx, and peduncle at higher frequencies (Table 4). Moreover, the vertical transmission rate of 8 mpi pollen was significantly higher than that of 4 mpi pollen (Table 5). Since their nucleotide sequence did not change before and after vertical transmission, it was apparent that the increase in the vertical transmission rate over time was not likely to be derived from the introduction of mutations. Therefore, the horizontal and vertical transmission rates of viroids by infected pollen rise seems to depend on of the time elapsed since infection. When PSTVd infected *N. benthamiana*, it did not invade the floral and vegetative meristems in the initial stage of infection but did late in the infection (Di Serio et al., 2010). Considering that the viroid infection of pollen grains requires the invasion of the floral meristems before gametogenesis, a sufficient infection period may be necessary for invasion into the floral meristems. Accordingly, further studies are needed to compare the concentration of each viroid chimera in pollen grains at different time points after infection.

In conclusion, our study showed that structural factors that determine the efficiency of horizontal and vertical transmission of viroids are mapped to the TL and P domains. The TL and P domains (especially TL) of TPMVd are indispensable not only for horizontal but also for vertical transmission through pollen.

4. Materials and methods

4.1. Construction of infectious TPMVd-chimera cDNA clones

To create chimeric viroids from TPMVd and PSTVd, gene sequences were substituted between TPMVd (accession no. GQ131573, 359 nucleotides; Verhoeven et al., 2011) and PSTVd (EU862231, 358 nucleotides; Matsushita et al., 2010). PS/TP-TLP was converted from the TL and P domains (1−72, 286−358) of PSTVd to those domains (1−72, 285−359) of TPMVd (Fig. 1). TP/PS-TLP was converted from the TL and P domains (1−72, 285−359) of TPMVd to those domains (1−72, 286−358) of PSTVd. Additionally, TP/PS-TL was converted from the TL domain (1−46, 314−359) of TPMVd to that domain (1−44, 314−358) of PSTVd, and likewise, PS/TP-TL was converted from the TL domain (1−44, 314−358) of PSTVd to that domain (1−46, 314−359) of TPMVd. In addition, the original TPMVd and PSTVd gene sequences with no modifications were used as controls. The gene sequence of the T7 promotor was added at the 5′-end. To amplify the cDNA including the gene sequences of the chimera viroids, a gene sequence (TCCGATCTGAT) derived from a cloning vector (pUCFk, FASMAC CO., LTD, Japan) was added at the 5′-end, and likewise a gene sequence (GGAAGAGCACACGTCTGAACTC) was added at the 3′-end. These six viroid gene sequences, including four chimeras, were synthesized by gBlocks gene fragments amplification technology (Integrated DNA Technologies, Iowa, USA). RNA was transcribed from these synthesized cDNAs and used as the inoculum for planting, according to the method of Matsushita and Penmetcha (2009).

4.2. The inoculation of parent and viroid chimeric transcripts onto petunia

Petunia × *hybrida* Vilm. 'Vacara' plants were inoculated with *in vitro* transcribed RNAs of each of the viroid constructs by mechanical inoculation (Yanagisawa et al., 2017). We inoculated three petunia seedlings at the four-leaf stage with 5 μg *in vitro* transcribed RNAs. Inoculated plants were cultivated in a growth chamber at 25–27 °C with a 16:8-h light:dark cycle for more than 4 months after inoculation.

4.3. Confirmation of infection by viroid chimeras

To confirm whether each of the viroid chimeras had infected the petunia seedlings, we collected the uppermost leaves from the inoculated petunia seedlings at 2 months after inoculation, and then extracted total RNAs as the templates for the RT-qPCR method using a RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each of the viroid chimeras and parent viroids was detected using the RT-qPCR method with a universal primer pair (6Pospi-F/R) from the RNA extracted by the above method (Yanagisawa et al., 2017). At least 4 months after inoculation, we collected pollen grains from the petunia plants infected with the viroid chimeras and used them for pollination (Yanagisawa and Matsushita, 2018). Additionally, to monitor pollen tube elongation, the mRNA sequence of *P. hybrida* putative monosaccharide transporter 1 (pmt1; accession no. AF061106; Ylstra et al., 1998) as an internal control for pollen grains was detected by RT-PCR using an MT-1/2 primer set, according to the methods of Yanagisawa and Matsushita (2018).

4.4. Cloning and sequencing the viroid chimeras infecting petunia plants

RT-PCRs were performed using two primer sets for sequence analysis. The first primer set was P1/P2 (Gross et al., 1978) and the secondary primer set was designed as follows: PV87:5′-GGATCCCCGGGG AAACCTGGAGCG-3′ and PV68; 5′-GGATCCCTGAAGCGCTCCTCC GAG-3′. Using these primer sets, the PCR products containing a full-length viroid genome of each of the viroid chimeras were amplified by One step RT-PCR kit (Qiagen, Germany) and purified by QIAquick PCR Purification Kit (Qiagen, Germany). The purified PCR products were cloned into pUC118 vectors included in the Mighty Cloning Reagent Set (Blunt End) (Takara, Japan). The competent cells used *E. coli* DH5α cells (Takara, Japan). Sequence analysis was performed using an ABI 3130 Genetic Analyzer (Thermo Fisher Scientific, USA).

4.5. Infectivity and concentration of each viroid chimera in pollen grains

To confirm the infectivity of pollen infected with each of the viroid chimeras, and the original TPMVd and PSTVd, we collected 50 mg of pollen grains from petunia seedlings infected by each of them. The collected pollen grains were ground with 1 mL 0.1 M phosphate buffer (pH 7.5). The resultant solution was diluted 10 times with the same phosphate buffer and similarly diluted 100 and 10,000 times. Using these solutions, we inoculated three tomato plants (*Solanum lycopersicum* 'Rutgers'). Inoculated tomato plants were cultivated in a growth chamber at 25–27 °C with a 16:8-h light: dark cycle. We confirmed viroid-like symptoms and conducted RT-qPCR assays as described above for viroid infection.

To compare the concentration of the viroid present in pollen grains, 50 mg of pollen grains were collected from petunia plants infected with each viroid or chimera. Pollen grains were collected 8 months after inoculation. RNA was extracted from these pollen grains according to the methods of Yanagisawa and Matsushita (2018). In the extracted

RNA, 100 ng RNA was used as template for the detection of viroids by RT-qPCR assay using a 6Pospi-F/R primer set (Yanagisawa et al., 2017), and was then detected by RT-qPCR using actin 11 mRNA as an internal control and the primer set for action 11 of petunias (Mallona et al., 2010).

4.6. Horizontal transmission of each of the viroid chimeras by pollen in petunia plants

Healthy petunia plants were pollinated with pollen grains collected from each parental viroid or chimera (PS/TP-TLP, TP/PS-TL, PS/TP-TL, TPMVd and PSTVd) to examine their distributions in the carpels and fruits after pollination. The pollination procedure was carried out according to the methods of Yanagisawa and Matsushita (2018). In addition, the pollen grains used for pollination were collected 4, 6, 8 and 10 months after inoculation. Healthy petunia seedlings grown for 2 months after seeding were pollinated with infected pollen grains. The pollinated carpels were collected 24 h after pollination, and separated from the stigma, styles, and ovaries. The analysis was repeated three times using nine petunia seedlings per trial per viroid chimera.

Fruits formed after pollination were divided into the calyx, ovary wall, placenta, developing seed, and peduncle and we confirmed whether the viroid could be detected in these tissues by RT-qPCR, as described above.

When viroid chimeras were detected in the placenta or calyx of formed fruits, we inoculated healthy tomato plants with these tissues to confirm the infectivity of the viroid chimera which infected these tissues. Additionally, each viroid chimera was detected in these tissues by RT-PCR using both the P1/P2 and PV87/PV68 primer sets. The obtained PCR products were cloned to produce the complete sequence of each viroid, as mentioned above, and the nucleotide sequence of the viroid chimeras infecting the petunia plants were compared with the obtained sequences from these tissues. The analysis was repeated twice using six petunia seedlings per trial per viroid chimera.

4.7. Observation of pollen tube elongation in petunia plants after pollination

To assess which part of the pollen tube of petunia extended at 24 h after pollination, pollen grains were placed on the stigma of healthy petunia plants. For pollination, we used pollen grains collected 10 mpi pollen grains from each of three chimeric viroid-, TPMVd-, and PSTVd-infected petunia plants, as well as pollen grains from healthy petunia plants. The carpels were collected at 24 h after pollination and, fixed in FAA solution (formalin, 80% ethanol, acetic acid, 1:8:1 (v/v)). The subsequent steps were carried out according to Yumoto and Ichimura (2006). Ten petunia carpels were observed per test group.

4.8. Vertical transmission of each viroid chimera by pollen in petunia plants

To determine the seed transmission rate of each viroid chimera by pollen, we pollinated the pistils of healthy plants with pollen grains collected from infected plants 4, 6, and 8 months after inoculation. Healthy petunia plants grown for 2 months after seeding were used for pollination as described above. After maturation, seeds were collected and then sown according to the method of Yanagisawa and Matsushita (2018). The presence of each viroid was assessed in the germinated seedlings to investigate the vertical transmission rate, following the method described above. In addition, sequencing analysis was performed to confirm the sequence of the viroids that infected the progeny plants, using the same method as a described above. 200 seeds per viroid chimera were sown each time and infection of viroid was examined individually using RT-qPCR. The experiment was repeated twice.

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Author contributions

HY, TS, SH, and MY conceived the study and designed the experiments. HY and MY performed the experiments, and HY, TS, and MY analyzed the data. TS analyzed the secondary structure of each viroid chimera. HY and SH drafted the manuscript, and MY revised and approved the manuscript for publication.

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