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Penetration of pollen tubes with accumulated *Raspberry bushy dwarf virus* into stigmas is involved in initial infection of maternal tissue and horizontal transmission

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ABSTRACT

Torenia fournieri (Torenia) plants were infected with *Raspberry bushy dwarf virus* (RBDV) by pollination with RBDV-infected raspberry pollen grains. The infected raspberry pollen grains germinated on Torenia stigmas, and then the pollen tubes penetrated into the stigma, even though the pollen tubes were arrested in the styles. In whole-mount in situ hybridization of germinating infected raspberry pollen grains, RBDV accumulated in the tips of the pollen tubes. Tissue blot hybridization of Torenia plants pollinated with infected raspberry pollen grains revealed that the first virus infection site leading to systemic infection is the stigma. When infected raspberry pollen grains that had lost germination capacity were pollinated on Torenia stigmas, RBDV could not infect the stigmas, and no horizontal transmission occurred. These results indicate that penetration of pollen tubes with accumulated RBDV into stigmas is essential in causing the first viral infection in the stigma to lead to systemic infection.

Introduction

Pollination is an essential step in the reproduction of flowering plants and is also crucial in agriculture in regard to fruit development, seed output, and the creation of new varieties of plants. However, at least 46 plant viruses have been reported as being pollen-transmitted among the over 1000 plant viruses officially recognized by the International Committee on Taxonomy of Viruses (Card et al., 2007; Liu et al., 2013). When a virus is transmitted by pollen, it either infects the mother plant through the pollinated flower (horizontal transmission by pollen), or it infects the seed developed from the fertilized flower, and thereby infecting the seedling growing from that seed (vertical transmission by pollen). While all of these pollen-transmitted viruses cause vertical transmission by pollen, 18 of these viruses can also cause horizontal transmission by pollen (Card et al., 2007). The horizontal transmission by pollen is epidemiologically important for viruses infecting perennial crops. For example, the infection of *Raspberry bushy dwarf virus* (RBDV) in a cultivated field of approximately 900 red raspberry trees can reach 100% in 5–6 years (Bulger and Martin, 1990; Martin, 2002). However, mechanisms by which pollination with virus-contaminated pollen grains cause systemic viral infection to healthy plants have been unknown since 1918, when the possibility that viruses might be spread by pollen from plant to plant was first raised (Mink, 1993; Reddick and Stewart, 1918).

RBDV, the only member of the genus *Idaeovirus*, is one of the most important viral pathogens of red raspberry (*Rubus idaeus* in the family *Rosaceae*). It occurs throughout the world, wherever raspberry is grown. The *Rubus* species, which includes the black raspberry (*R. occidentalis*), blackberry (*R. fruticosus*), loganberry (*R. loganobaccus*), boysenberry (*R. ursinus* × *idaeus*) and arctic bramble (*R. arcticus*), can be naturally infected with RBDV (Jones et al., 1982; Kokko et al., 1996; Strik and Martin, 2003). In Slovenia and Serbia, RBDV has been found to infect grapevines (Jevremović and Paunović, 2011; Mavrič et al., 2003). RBDV is regarded as an integral component of the disease syndrome (Fauquet et al., 2005). The main cause of bushy dwarf disease is *Black raspberry necrosis virus*, and the additional presence of RBDV contributes significantly to the disease symptoms. In addition, RBDV is a causal agent of crumbly fruit disease in some raspberry and blackberry cultivars, and an increase in the severity of the crumbly fruit symptoms is caused by the additional presence of *Raspberry leaf mottle virus* (RLMV) and *Raspberry latent virus* (Jones et al., 1996;

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Quito-Avila et al., 2013; Strik and Martin, 2003). The concentration of RBDV in red raspberry cultivar Meeker, co-infected with RLMV, is enhanced approximately 400-fold (Quito-Avila and Martin, 2012). Murant et al. (1974) showed that RBDV is transmitted through raspberry pollen grains, and that healthy raspberry plants pollinated with RBDV-contaminated pollen grains may become infected with the virus. They also showed that deflowered plants do not become infected with the virus. Thus, the flower is believed to be the avenue for infection. Mink (1992) doubted the relevancy of unassisted horizontal transmission by pollen, since Sdoodee and Teakle (1987, 1988, 1993) concluded that thrips (*Thrips tabaci*) transmit *Tobacco streak virus* associated with pollen grains via its feeding wounds. Bulger and Martin (1990)), however, have reported that *T. tabaci*, which feeds on *Chenopodium quinoa* plants dusted with RBDV-contaminated pollen grains, do not transmit RBDV, suggesting that thrips feeding on pollen grains are not involved in the transmission of RBDV.

For horizontal transmission of RBDV by pollen, the mechanisms of infection of the mother plant body have not been tested due to the difficulties of applying raspberry plants: they have long generation cycles in which they bloom only once or twice a year. Therefore, to study horizontal transmission of RBDV by pollen, we used Torenia plants (*Torenia fournieri* in the family *Scrophulariaceae*) that are infected with the virus, but are asymptomatic (Barnett and Murant, 1970). Additionally, there is the possibility that the infection route to horizontal transmission by pollen is associated with the infection route of the embryo, leading to vertical transmission (since horizontal transmission of the virus by pollen coincides with vertical transmission). We, therefore, analyze horizontal transmission under separation from vertical transmission by pollination of Torenia plants in cross-incompatibility combination.

Results

Horizontal transmission of RBDV in homogeneous and heterogeneous stigma and pollen combinations

It has been reported that RBDV transmits healthy red raspberry plants by pollen grains of infected red raspberry plants (Murant et al., 1974). To analyze the fact that Japanese RBDV isolates also cause horizontal transmission by pollen, four plants of red raspberry cultivar Glen Moy were hand-pollinated with pollen grains from red raspberry cultivar Autumn Britten infected with the J1 isolate of RBDV (RBDV-J1; Isogai et al., 2012). One year after hand pollination, each raspberry plant was tested by RT-PCR using total RNAs extracted from their leaves. RBDV was detected in one of the four raspberry plants (Table 1). The raspberry plant infected with RBDV was also confirmed by tissue blot hybridization using the leaves (Supplemental Fig. 1). Thus, RBDV-J1 was used in all of the experiments in this paper.

It has been shown that Torenia plants can be infected with RBDV by mechanical inoculation of leaves (Barnett and Murant, 1970). However, it was not clear whether the virus was horizontally transmitted to healthy Torenia plants by pollination with pollen grains from the infected Torenia plants. To examine horizontal transmission of the virus to Torenia plants by pollen, seventeen Torenia plants were hand pollinated with pollen grains from the infected Torenia plants. As a result, ten of the seventeen pollinated plants were infected with the virus; each detected by RT-PCR at one month after pollination (Table 1).

Subsequently, to analyze horizontal transmission of RBDV by pollen in a cross-incompatibility combination, eighteen Torenia plants were hand-pollinated with pollen grains from infected raspberry plants. At one month after pollination, the heterogeneously pollinated Torenia plants were tested by RT-PCR. Interestingly, RBDV was detected in six of the eighteen pollinated plants (Table 1).

Behavior of raspberry pollen grains on Torenia stigma

To determine how raspberry pollen grains behave on Torenia stigma, the grains from infected raspberry plants were pollinated on Torenia stigmas and then observed by aniline blue staining. One day after pollination, pollen grains from infected raspberry plants germinated on Torenia stigmas, and their pollen tubes penetrated into them (Fig. 1a, b). Furthermore, to analyze elongation of the

Table 1
Horizontal transmission of RBDV to healthy plants pollinated with RBDV-infected pollen grains.

RBDV-infected pollen grains (♂)	Pollinated plant (♀)	Transmission of RBDV[b]	
		No. of infected plants	No. of tested plants
Rubus idaeus	*R. idaeus*	1	4
Torenia fournieri	*T. fournieri*	10	17
R. idaeus	*T. fournieri*	6	18
R. idaeus[a]	*T. fournieri*	0	14

[a] Pollen grains treated with 1% sodium dodecyl sulfate as described in Materials and methods.

[b] 16–75 flowers on each *R. idaeus* cultivar Glen Moy plant were pollinated with pollen grains from RBDV-infected *R. idaeus* cultivar Autumn Britten plants. On the other hand, 8–12 flowers on each *T. fournieri* plant were pollinated with pollen grains from RBDV-infected *R. idaeus* cultivar Autumn Britten or *T. fournieri* plants.

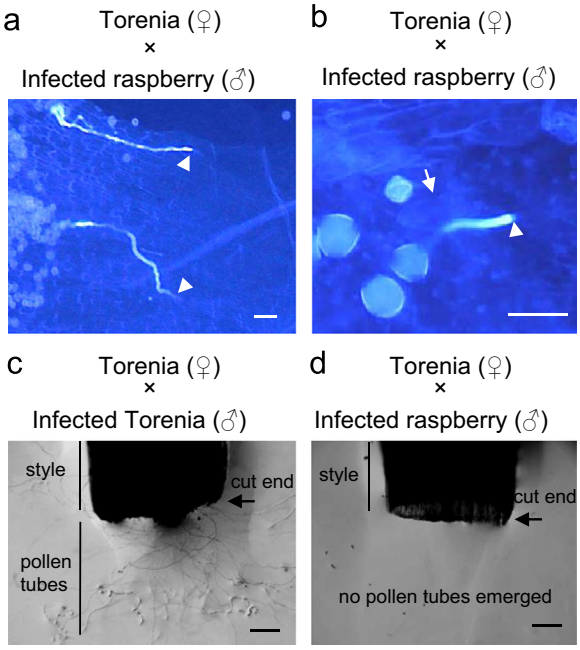

Fig. 1. Pollen tube growth of pollen grains from RBDV-infected raspberry plants on Torenia stigmas. (a) Fluorescent image of pollen grains and pollen tubes at Torenia pistils stained with aniline blue. (b) shows the raspberry pollen grain was germinated, and the pollen tube penetrated into the Torenia stigma at the base of the stigmatic papillar cell. The pollen tubes penetrating into the stigma are marked with white arrowheads. The stigmatic papillar cell is marked with a white arrow. (c) Elongation of pollen tubes from infected Torenia pollen grains in a Torenia style that was approximately 1.0 cm in length from the stigma. Torenia pollen tubes are emerged from the cut end of the style. (d) Elongation of pollen tubes from infected raspberry pollen grains in a Torenia style that was approximately 1.0 cm in length from the stigma. No raspberry pollen tubes are emerged from the cut end of the Torenia style. Bars: (a) and (b): 50 μm; (c) and (d): 200 μm.

raspberry pollen tubes in Torenia styles, Torenia stigmas were hand-pollinated with pollen grains from infected raspberry plants. Their styles were cut at 1.0 and/or 2.0 cm in length from the stigmas, and then the styles were placed in the pollen tube growth medium. However, no raspberry pollen tubes emerged from the cut ends of Torenia styles that were 1.0 and 2.0 cm in length, even two days after pollination (Fig. 1d). As a reference for homogeneous pollination, Torenia pollen tubes emerged from the cut end of Torenia styles that were 1.0 and 2.0 cm in length, when observed at a 12 h incubation period (Fig. 1c). These results indicated that raspberry pollen tubes were arrested in less than 1.0 cm of length of the Torenia styles, even though the raspberry pollen tubes penetrated into Torenia stigmas.

Localization of RBDV in germinating pollen grains

To determine localization of RBDV in germinating pollen grains from infected Torenia and raspberry plants, whole-mount in situ hybridization (whole-mount ISH) analysis was performed. The pollen grains were incubated in a germination medium for 1 h, and then processed by whole-mount ISH. Hybridization with the DIG-labeled probe specifically showed strong positive reactions to the tips of the pollen tubes from infected Torenia and raspberry plants (Fig. 2a, e), while no RBDV RNA was found to react with the pollen tubes of healthy Torenia and raspberry (Fig. 2b, f). RBDV RNA was also observed in germination apertures of the pollen grains from infected Torenia and raspberry plants (Fig. 2c, g), while no positive reactions were observed in the apertures from healthy Torenia and raspberry pollen grains (Fig. 2d, h). These results indicated that Torenia and raspberry pollen grains from infected plants contain RBDV, with higher accumulations in the tips of pollen tubes and the germination apertures.

Tissue blot hybridization analysis of Torenia pistil pollinated with infected pollen grains

It is predicted that a Torenia plant pollinated with infected raspberry pollen grains become the first to be infected with the virus at a site between the stigma and its style during the raspberry pollen tubes elongation. The raspberry pollen tubes penetrate into Torenia stigmas, but arrest in the Torenia style before reaching a length of 1.0 cm (Fig. 1). To analyze the first infection site between the stigma and its style, infected raspberry pollen grains were pollinated on healthy Torenia stigmas, and then the pollinated stigmas and their styles were analyzed by tissue blot hybridization. Tiny spotted positive signals were detected in stigmas pollinated with infected raspberry pollen grains one day after pollination. The positive signals at the stigma proceeded strongly, and some of their styles showed the positive signals two days after pollination (Fig. 3c–e). Although pollen tubes from infected raspberry pollen grains were arrested in Torenia styles before reaching a length of 1.0 cm (Fig. 1d), the positive signal was detected at the bottom of the style, which was 2.0 cm in length from the stigma (Fig. 3d, e), indicating that the virus infection spread from the stigma to its style. On the other hand, the tiny spotted signals at the stigmas did not grow stronger, and no positive signal was observed in the styles even three days after pollination (Fig. 3f–h), when infected raspberry pollen grains which lost germination capacity (Supplemental Fig. 2) were used for pollination on Torenia stigmas. In addition, no horizontal transmission to Torenia plants occurred by pollination with infected raspberry pollen grains that had lost germination capacity (Table 1). From these, the tiny spotted signals in the stigmas pollinated with infected raspberry pollen grains which lost their germination capacity were derived from the virus in the infected pollen grains. The stigmas pollinated with infected raspberry pollen grains one day after pollination also showed the same tiny spotted positive signals as those in the stigma pollinated with infected raspberry pollen grains that had lost their germination capacity, suggesting that the stigmas pollinated with infected raspberry pollen grains one day after pollination mainly originated from the virus in the infected pollen grains (Fig. 3c, f, g, h). These results indicated that Torenia stigmas became the first to be infected with the virus by pollination with infected raspberry pollen grains that had germination capacity.

To analyze whether the stigma is the first infection site in homogeneous stigma and pollen combinations, healthy Torenia stigmas pollinated with infected Torenia pollen grains were analyzed. The stigma and its style showed positive signals starting one day after pollination, as in the case of the heterogeneous combination (Fig. 3i–k). It is noteworthy that the detection of RBDV in the stigma and its style in the homogeneous combination was one day sooner than that of the heterogeneous combination (Fig. 3d, j).

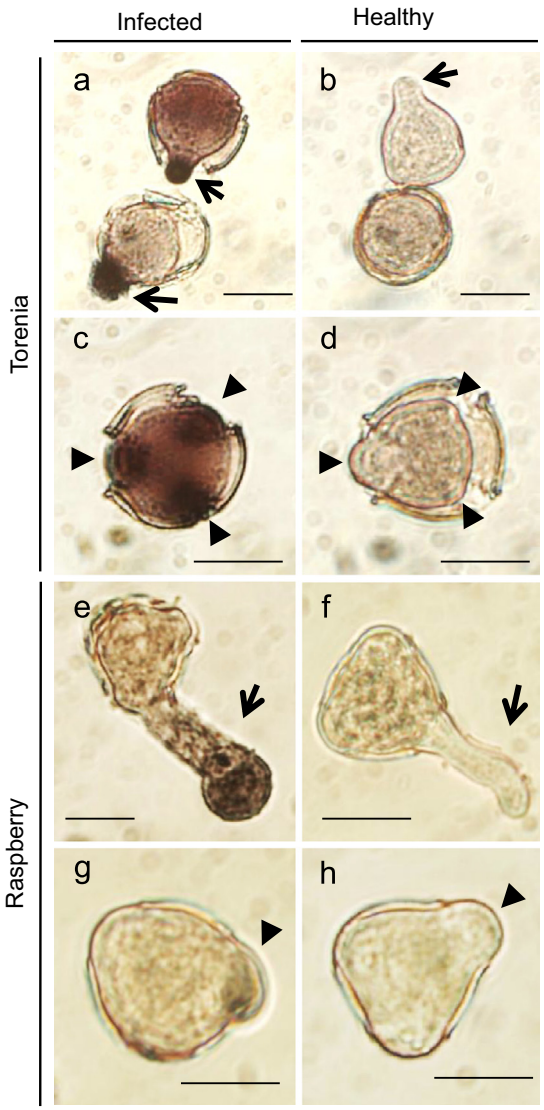

Fig. 2. Localization of RBDV in germinating pollen grains from infected Torenia and raspberry plants by whole-mount in situ hybridization. (a, and c) Localization of RBDV in germinating infected Torenia pollen grains. (b, and d) Negative control of germinating healthy Torenia pollen grains. (e, and g) Localization of RBDV in germinating infected raspberry pollen grains. (f, and h) Negative control of germinating healthy raspberry pollen grains. Pollen tubes and germinating apertures are marked with arrows and arrowheads, respectively. Bars = 20 μm.

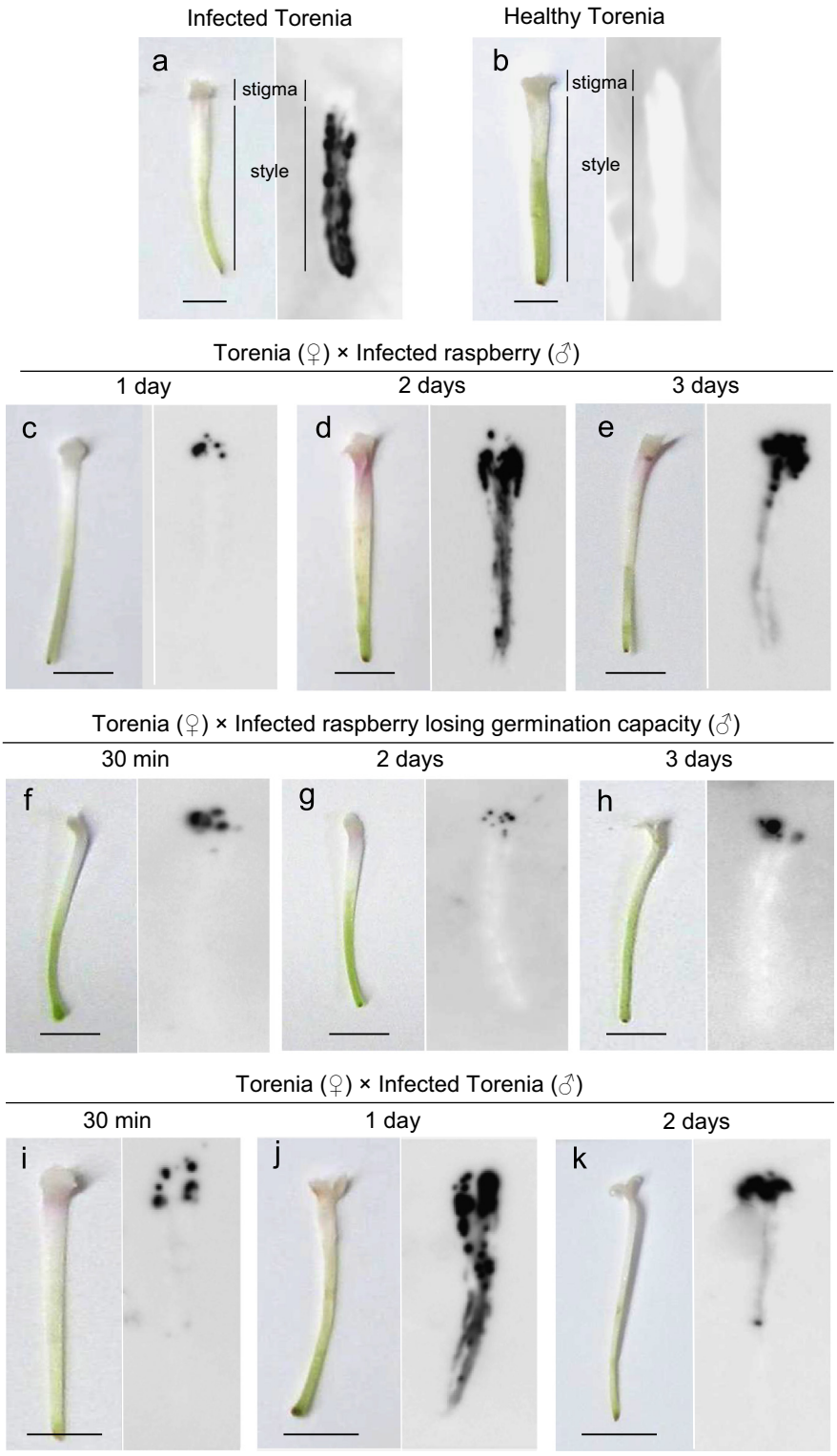

Fig. 3. Detection of RBDV in Torenia stigmas and their styles pollinated with infected pollen grains from Torenia and raspberry plants by tissue blot hybridization. (a, b) Stigmas and their styles from RBDV-infected (a) and healthy (b) Torenia plants. (c–e) Stigmas and their styles from healthy Torenia plants pollinated with RBDV-infected raspberry pollen grains at 1 day (c), 2 days (d), and 3 days (e) after pollination. (f–h) Stigmas and their styles from healthy Torenia plants pollinated with RBDV-infected raspberry pollen grains losing germination capacity at 30 min (f), 2 days (g), and 3 days (h) after pollination. (i–k) Stigmas and their styles from healthy Torenia plants pollinated with RBDV-infected Torenia pollen grains at 30 min (i), 1 day (j), and 2 days (k) after pollination. The samples (stigmas and their styles) cut at the end of styles (the left column of a–k) were subjected to tissue blot hybridization assay (the right column of a–k). Bars=0.5 cm.

Discussion

In this study, using Torenia plants pollinated with the infected raspberry pollen grains, we showed that the stigma is the first RBDV infection site leading to horizontal transmission of the virus by pollen. If the infection of the Torenia stigma was caused by mechanical inoculation of the virus associated with the method of hand pollination on the stigma, it is thought that the infection at

the stigma takes about the same amount of time, when compared between pollination with infected raspberry and Torenia pollen grains. However, the infection of the stigma in the homogeneous combination was one day sooner than that in the heterogeneous combination (Fig. 3d, j). Infected raspberry pollen grains rarely started germinating on the Torenia stigmas even 4 h after pollination, although Torenia pollen grains began to germinate 5 min after pollination (Higashiyama et al., 1997; Fig. 1a, b). Additionally, the virus accumulates in the raspberry and Torenia pollen tubes (Fig. 2). Thus, it is preferable to think that the first infection at the stigma would have been caused by penetration of pollen tubes that had accumulated the virus in the stigmas. This is supported by the evidence that RBDV does not cause the stigma infection and the horizontal transmission to a mother plant body, when a healthy Torenia plant was pollinated with infected raspberry pollen grains that had lost germination capacity (Table 1 and Fig. 3f–h). Each pollen tube typically penetrates into the cuticle of the stigmatic papilla, enters the outer layer of the cell wall, grows through the cell wall and/or between the cell wall and the plasma membrane, and then enters the extracellular matrix of the underlying secretory cells in the style (Cheung, 1996; Elleman et al., 1992; Kandasamy et al., 1994). During elongation of a pollen tube growing by tip extension, exocytosis of new plasma membrane and cell wall materials occurs adjacent to the pollen tube apex (Zonia and Munnik, 2008), and enzyme modification of the cuticle and cell wall of the stigmatic papilla is required to allow further pollen tube growth (Kandasamy et al., 1994). We show that RBDV accumulates in the tips of the pollen tubes (Fig. 2a, e). Also, Amari et al. (2007) reported that *Prunus necrotic ringspot virus* (PNRSV), which is horizontally transmitted by pollen, is detected in the pollen tubes of infected apricot pollen grains. Thus, if the exocytotic vesicles of the plasma membrane and cell wall materials included the virus, the virus would exit from the pollen tube by exocytosis, and then enter the cytoplasm of the stigmatic papilla under favor of enzyme modifications and/or minor wounds by pollen tube penetration and elongation.

A previously proposed mechanism for horizontal transmission of plant viruses by pollen has been that viruses infect the embryo, and move from the infected embryo to its maternal body due to the plant viruses transmitted by pollen (46 species) causing vertical transmission. However, only 18 of these viruses can also cause horizontal transmission (Card et al., 2007). In other words, it has been predicted that horizontal transmission by pollen is derived as a continuation of embryo infection leading to vertical transmission. However, if horizontal transmission of viruses by pollen required movement of the viruses from the infected embryo to its maternal tissue, a mechanism would be required to enable the viruses to move without direct vascular connection, since the embryo is separated physically from the mother plant by a callose layer. Previous studies on seed transmission of *Pea seed-born mosaic virus* showed that the virus infects embryos from infected mother tissues using a contact point between the testa and suspensor (Wang and Maule, 1994; Roberts et al., 2003). With respect to the reports, it has been predicted that viruses horizontally transmitted by pollen use this contact point to move from an infected embryo to its mother tissues. However, this explanation for movement of viruses from an infected embryo into maternal tissues has remained controversial (Card et al., 2007). In this paper, we clearly demonstrated that vertical transmission is dispensable for horizontal transmission of RBDV by pollen. Additionally, we suggest that the stigma is the first to be infected with the virus by the penetration of infected pollen tubes into it. The virus then spreads to the style and afterwards to the mother plant body (Table 1 and Fig. 3). In vertical transmission of PNRSV by pollen, Amari et al. (2009) reported that the virus is detected in embryos as well as in the outer part of apricot fruits, when infected apricot pollen grains were homogeneously pollinated on a virus-free plant. Thus, there is a possibility that RBDV uses the ovary wall as the route for moving from the infected style to its mother plant body. When infected Torenia pollen grains were homogeneously pollinated on virus-free stigmas, the stigma and its styles were also infected with RBDV as in the case of the Torenia stigma and raspberry pollen combination (Fig. 3i–k). Thus, it is preferable to think that the horizontal transmission of the virus in the homogeneous combination is available for the same route to invasion of a mother plant body as that in the heterogeneous combination use.

Pollen is the only method of field transmission of RBDV from infected raspberry plants to healthy raspberry plants (Murant et al., 1974). In this paper, we demonstrated that infected raspberry pollen grains can transfer the virus to healthy Torenia plants. Therefore, it might be possible that RBDV spreads its infection in its host plant species beyond the plant family level by pollen through wind and/or pollinating insects, if the virus infection was established at stigmas by penetration of infected pollen tubes into these stigmas.

Materials and methods

Plant materials

Torenia fournieri 'Blue and White' (Torenia) plants were grown in plant pots in regulated chambers, MLR-351H (SANYO Electric, Osaka, Japan), at 25 °C with a 16 h photoperiod. Control of aphids, mites, and thrips was achieved by application of insecticides. Pollen grains from infected Torenia pollen grains were collected from Torenia plants infected with RBDV-J1 (Isogai et al., 2012). Red raspberry cultivar Glen Moy plants were grown in plant pots in a green house. Pollen grains from infected raspberry plants were collected from red raspberry cultivar Autumn Britten plants that were infected with RBDV-J1.

Pollen grains from Torenia and raspberry plants

Anthers from flowers of Torenia and raspberry plants were dried by silica gel, and then dried pollen grains were collected from these anthers by a sieve.

To lose the ability of raspberry pollen grains to germinate, 5 mg of dried raspberry pollen grains were suspended in 500 µl of 1% sodium dodecyl sulfate (SDS), gently shaken for 1 min, and centrifuged at 3000 rpm for 1 min. After treatment of 1% SDS, the pollen grains were rinsed by PBS five times, followed by gently shaking in acetone for 1 min. The pollen grains were dried by splashing acetone at a draft chamber. The loss of germination capacity of the SDS-treated raspberry pollen grains on Torenia stigmas was confirmed by aniline blue staining (Supplemental Fig. 2).

Hand pollination

In terms of the hand pollination of raspberry plants, sixteen to seventy five flowers on each raspberry tree had their anthers removed. All were then hand pollinated, and each pollinated flower was covered with a pollen bag to prevent visits by pollinators. The pollinated raspberry plants were grown in plant pots in a green house and the newly emerged floral buds were removed until tests for the assay of the virus by RT-PCR could be conducted. For the hand pollination of Torenia plants, eight to twelve flowers on each healthy Torenia plant had their anthers removed and all were pollinated with pollen grains from infected Torenia or raspberry plants. The pollinated Torenia plants were

grown in plant pots in regulated chambers, MLR-351 H (SANYO Electric, Osaka, Japan), and the occurrence of aphids, mites, and thrips was controlled.

Rt-PCR assay

Total RNAs were extracted from Torenia and red raspberry leaves as described by Isogai et al. (2012). Reverse transcription (RT) was carried out using a random hexamer and ReverTra Ace (Toyobo, Osaka, Japan). The RT products were amplified by Takara Z-taq™ (Takara, Shiga, Japan) using a sense primer, 5'–CGCAGCT-CAAAAGGCTGGT-3', and an antisense primer, 5'–GGGGTTTGC-TCAGCAAACAACGGCTATG-3'. The PCR reaction was performed as follows: one cycle of initial denaturation at 94 °C for 2 min, 40 cycles of denaturation for 1 s at 98 °C, annealing for 10 s at 58 °C, extension for 10 s at 72 °C and a final extension for 2 min. After PCR amplification, the reaction mix was analyzed by 1% TAE agarose (w/v) gel electrophoresis with ethidium bromide staining.

Pollen tube observation stained by aniline blue

Aniline blue staining of raspberry pollen tubes in Torenia pistils was conducted as described by Kanaoka et al. (2011). Torenia stigmas were hand-pollinated with pollen grains from infected raspberry and Torenia plants. The pistils were collected 24 h later and fixed in the fixation solution (ethanol: acetic acid = 9: 1) overnight. Then, they were rehydrated with 90% ethanol and 70% ethanol, respectively, for 20 min. After rehydration, they were kept in 1 N NaOH solution overnight, then stained with 1% aniline blue solution dissolved in 0.1 M K_3PO_4 (pH 12.4) for 1 h. They were observed under Axio Imager A1 using FS38 filter (Carl Zeiss, Gottingen, Germany) and photographed with an AxioCam MRc5 (Carl Zeiss).

Elongation of raspberry pollen tubes in Torenia styles

Hand-pollinated Torenia pistils were cut at the styles that were 1.0 and 2.0 cm from the stigmas, and then the cut styles were placed on the pollen tube growth medium described by Higashiyama et al. (1998). Pollen tubes emerging from the cut end of the style were observed under a LEICA MZ12 microscope (Leica Microsystems, Wetzlar, Germany) and photographed with an Olympus DP70 (Olympus, Tokyo, Japan).

Whole-mount in situ hybridization

Pollen grains were germinated in liquid medium (12% sucrose, 0.03% $CaCl_2$, 0.01% H_3BO_3 and 0.2% DMSO) for one hour, and then processed by whole-mount ISH as described by Torres et al. (1995). Fixation and pretreatment of the pollen grains were as described by Heuer et al. (2000). The digoxigenin (DIG)-labeled antisense RNA probe corresponding to a coat protein region of the virus was prepared from a cDNA clone corresponding to nucleotide 1326 to 2150 of the RBDV RNA2 genome (RBDV-J1; accession AB698499) after transcription with T7 RNA polymerases in the presence of DIG-11-UTP (Roche Applied Science, Penzberg, Germany). Prehybridization, hybridization, and colorigenic detection were performed as described by García-Castillo et al. (2001). Subsequently, the processed pollen grains were observed under a LEICA DMLB microscope (Leica Microsystems) and photographed with an Olympus DP70 (Olympus).

Tissue blot hybridization

Torenia stigmas and styles were printed onto a Hybond™-N+ membrane (Amersham, Buckinghamshire, UK) using a roller. The membranes were treated with 0.05 N NaOH (30 min), 20 × SSC (30 min), and irradiated with 0.25 J/cm2 of UV light (BLX-254; BIO-LINK, Tokyo, Japan). The membranes were used for hybridization to detect RBDV as described by Yamagishi and Yoshikawa (2009). Prehybridization was performed at 68 °C for at least 2 h. Hybridization was performed overnight at 68 °C using a DIG-labeled antisense cRNA probe corresponding to a coat protein region as described above. After hybridization, the membrane was washed twice for 10 min at room temperature in 2 × SSC, 0.1% SDS at room temp, followed by washing twice for 20 min at 68 °C in 50 ml of 0.1 × SSC, 0.1% SDS. Chemiluminescent detection was conducted by anti-digoxigenin-AP, Fab fragments and CDP-Star Chemiluminescent substrate according the manufacturer's protocol (Rosh Diagnostics, Mannheim, Germany). The chemiluminescent reaction was detected by Image Quant LAS 4010 (GE Healthcare, Life Sciences, NJ, USA).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.02.001.

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