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Pollen tubes introduce *Raspberry bushy dwarf virus* into embryo sacs during fertilization processes

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ABSTRACT

We developed a fertilization method in which pollen tubes entered into embryo sacs without any need to contact surrounding female sporophytic cells by using *Torenia fournieri* (Torenia) plants under the condition of hindering movement of the virus from a stigma, which is the first infection site leading to systemic infection. When RBDV-infected Torenia pollen grains were used for the developed fertilization method, the virus was transmitted to the seeds by pollen tubes germinating from them. On the other hand, no seeds were infected with the virus when Torenia plants were pollinated with healthy Torenia pollen grains in combination with RBDV-infected raspberry pollen grains, which caused the virus infection in the stigma by penetration of their pollen tubes arrested in its style. Our results indicate that vertical transmission of RBDV by pollen occurs in the transport of the virus into embryo sacs by pollen tubes reaching the embryo sacs.

Introduction

In flowering plants, pollen tubes germinating from pollen grains play an essential role in delivering a sperm cell to an egg cell for fertilization. However, at least 46 plant viruses have been known as pollen-transmitted viruses (Card et al., 2007; Liu et al., 2014) and pollen tubes of infected pollen grains lead to a virus infection not only in the seeds but also in the mother plant body (vertical and horizontal transmission of viruses by pollen, respectively). When a virus is vertically transmitted by pollen, it infects the seed developed from the fertilized flower, thereby infecting the seedling growing from that seed. It is generally accepted that seed transmission occurs through infection of the embryo with the exception of tobamoviruses (Amari et al., 2009). Seed transmission of tobamoviruses is the result of contamination of the seed coat with the viruses, resulting in subsequent infection of the germinating seedling by mechanical inoculation due to the stability of the virus particles (Moreno et al., 2004; Taylor, 1962).

Raspberry bushy dwarf virus (RBDV), the only member of the genus *Idaeovirus*, is vertically and horizontally transmitted by pollen (Fauquet et al., 2005; Murant et al., 1974). RBDV naturally infects *Rubus* spp., and is one of the important viral pathogens of red raspberry (*Rubus idaeus*), since the virus causes significant reductions in fruit quality, fruit size, and yield in some cultivars (Daubeny et al., 1982; Jones, 1979, Murant, 1987). In particular, mix-infections of RBDV with other virus species lead to more severe symptoms (Jones, 1980; Jones et al., 1982), and the concentration of RBDV in red raspberry cultivar 'Meeker' plants co-infected with *Raspberry leaf mottle virus* is enhanced approximately 400-fold (Quito-Avila and Martin, 2012). In addition to *Rubus* spp., RBDV naturally infects grapevines (Mavrič et al., 2003). These natural host plants with woody stems are perennial plants and pollination is an essential step in their developing fruit. Thus, an increase in number of infected plants is observed in a cultivated field by pollination with infected pollen grains every year. In fact, RBDV infection can reach 100% in 5–6 years in some cultivars of red raspberry (Bulger et al., 1990; Martin, 2002). In horizontal transmission by pollen, our recent report has shown that penetration of pollen tubes that accumulated the virus into stigmas cause the first viral infection in the stigma to lead to systemic infection in the mother plant body (Isogai et al., 2014). Furthermore, infected pollen grains horizontally transmit RBDV to its host plant beyond plant family level by penetration of infected pollen tubes into stigmas (Isogai et al., 2014). On the other hand, in vertical transmission by pollen, little evidence has been presented to demonstrate how pollen-transmitted viruses, including RBDV, are vertically transmitted by infected pollen grains.

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Torenia fournieri (Torenia) plant is infected with RBDV without symptoms (Barnett and Murant, 1970). Torenia pollen tubes begin to germinate from the pollen grains on the stigma 5 min after pollination and enter into the ovary approximately 9 h after pollination (Higashiyama et al., 1997). The pollen tubes passing through the style can be selectively guided to the unfertilized embryo sac by defensin-like peptide LUREs from the synergid cells (Higashiyama et al., 2001). In addition, the egg cell and the two synergid cells in the Torenia embryo sac are located outside the ovule for the reason that the embryo sac protrudes from the micropyle, although an embryo sac of most angiosperms is located inside the ovule and covered with thick layers of the integument and nucellus (Guilford and Fisk, 1952). Thus, Torenia pollen tubes arrive precisely at the site of the synergid cells and enter into the embryo sac without any need to contact surrounding female sporophytic cells.

In this study, we analyzed whether the pollen tubes emerging from RBDV-infected pollen grains introduce the virus into embryo sacs during the fertilization processes by developing a fertilization method using Torenia plants. Also, we analyzed whether the virus infection of stigma which leads to horizontal transmission is associated with vertical transmission of the virus by pollen.

Results

Stigma infection is not essential for vertical transmission of RBDV by pollen

Our previous report has shown that stigma infection caused by penetration of pollen tubes with accumulated RBDV into a stigma lead to systemic infection of the mother plant (Isogai et al., 2014). The virus infection spreading from the Torenia stigma to its style was observed as soon as one day after pollination with RBDV-infected Torenia pollen grains, however, in some cases the virus did not spread from the pollinated stigmas (Fig. 1a–c; Isogai et al., 2014). To examine whether spread of the virus from the stigma is necessary for vertical transmission of RBDV by pollen, the virus infection of the stigmas and their styles was analyzed by tissue blot hybridization at 3 days after pollination with the infected pollen grains and we chose the ovaries without spread of the virus from the stigmas to their styles (Fig. 1c). The selected ovaries continued to grow on the mother plants for 2–3 weeks after pollination, and then the RBDV infection of the seeds was analyzed by tissue blot hybridization before their seed coats became hard. Interestingly, the RBDV RNAs were detected in the seeds (Fig. 1d and e), even though no spread of the virus from the stigmas to their styles was observed (Fig. 1c). These results indicated that movement of the virus from the stigmas is dispensable for vertical transmission of RBDV by pollen. In addition, it is noteworthy that the viral RNAs were not detected in the placenta, which is connected with the seed by the funiculus (Fig. 1d).

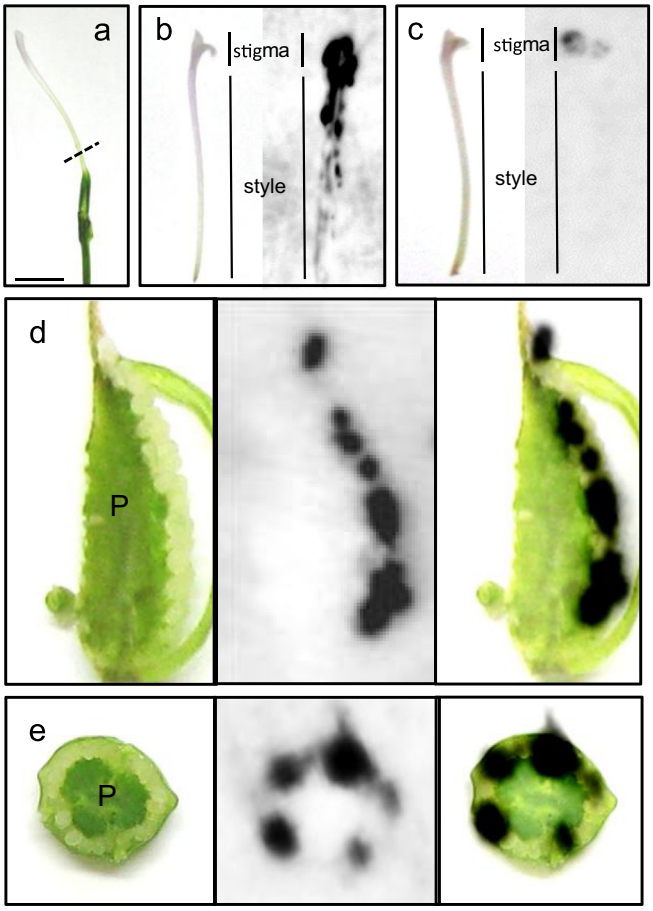

Fig. 1. Detection of RBDV in Torenia pistils pollinated with RBDV-infected pollen grains by tissue blot hybridization. (a) Cutting site of Torenia pistil at the bottom of the style (dotted line position) for analyzing virus infection in the stigma and its style by tissue blot hybridization using RBDV DIG-labeled antisense RNA probe. Bar = 1.0 cm. (b) Spread of the virus infection from the stigma to its style by pollination on healthy Torenia stigmas with RBDV-infected Torenia pollen grains. (c) The style showing no virus infection from the stigma in spite of pollination with the infected pollen grains. (d) Longitudinal and (e) cross-sections of Torenia ovaries in the absence of spreading the virus infection from the stigma to its styles. In the ovary cut lengthwise (d), the seeds on the left side of the placenta (P) were removed for observation of the virus infection in the placenta. The samples (the left columns) were subjected to tissue blot hybridization analyses (the center columns). The right columns represent overlay of the left and center columns to visualize their corresponding positions. Bar = 0.5 cm.

Pollen tubes transmit the virus to a female gametophyte

It is predicted that pollen tubes germinating from RBDV-infected Torenia pollen grains were involved in the transport of the virus to female gametophytes during the fertilization process. Therefore, we developed a fertilization method using Torenia plants, where the Torenia stigma and its style (stigma-style) were separated from the ovary by cutting near the top of the ovary (Fig. 2a, dotted line position), and then placed above the cut ovary with a distance of approximately 0.5 mm (Fig. 2b), hindering movement of the virus from the stigma, which was the first infection site leading to systemic infection. When the stigma-style was pollinated with the infected Torenia pollen grains, the pollen tubes grew into the cut ovary (Fig. 2c), and the ovary was grown on the mother plant for 2–3 weeks after pollination (Fig. 2d). One hundred forty-seven seeds, which were produced from the six mother plants in this method, were blotted on nylon membranes before their seed coats became hard, and were subjected to hybridization analyses for detection of the RBDV RNAs (Fig. 3). As a result, 63 of the 147 seeds showed positive signals, of which 35 seeds gave obvious positive signals as shown in Fig. 3, numbered 1, 2, 4, 5, 12, 13, 16–20, 22, 27–29, 31, 32, 39–42, 44, 46–53, and 55 and the other 28 seeds showed tiny spotted positive signals as shown in Fig. 3, numbered 6, 7, 10, 21, 26, 33, 35, 36, 38, and 54. In contrast, no positive signals were detected in the seeds, when the stigma-styles were pollinated with the healthy pollen grains (Fig. 3, numbered 57–72). These results indicated that the pollen tubes germinating from the infected pollen grains transmitted the virus to the seeds in the cut ovaries. Subsequently, to examine horizontal transmission of the virus to the six mother plants, these plants were tested by RT-PCR using total RNAs extracted from their leaves. RBDV was not detected in the six mother plants. These results indicated that no horizontal