Evaluation of pollen tube growth ability in *Petunia* species having different style lengths

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Abstract Pollen tube growth is essential for the fertilization process in angiosperms. When pollen grains arrive on the stigma, they germinate, and the pollen tubes elongate through the styles of the pistils to deliver sperm cells into the ovules to produce the seeds. The relationship between the growth rate and style length remains unclear. In previous studies, we developed a liquid pollen germination medium for observing pollen tube growth. In this study, using this medium, we examined the pollen tube growth ability in *Petunia axillaris* subsp. *axillaris*, *P. axillaris* subsp. *parodii*, *P. integrifolia*, and *P. occidentalis*, which have different style lengths. *Petunia occidentalis* had the longest pollen tubes after 6h of culture but had a relatively shorter style. Conversely, the pollination experiments revealed that *P. axillaris* subsp. *parodii*, which had the longest style, produced the longest pollen tubes in vivo. The results revealed no clear relationship between the style lengths and the growth rate of pollen tubes in vitro. Interspecific pollinations indicated that the styles affected pollen tube growth. We concluded that, in vitro, the pollen tubes grow without being affected by the styles, whereas, in vivo, the styles significantly affected pollen tube growth. Furthermore, interspecific pollination experiments implied that the pollen tube growth tended to be suppressed in the styles of self-incompatibility species. Finally, we discussed the pollen tube growth ability in relation to style lengths.

Key words: pollen germination, pollen tube growth, self-compatibility, self-incompatibility, style length.

Introduction

In plant breeding, interspecific hybridization is a useful way to produce attractive genotypes by incorporating novel traits of other species; it involves crossing barriers between species as well as between genera. As these barriers contribute to speciation, a knowledge of cross-incompatibility can lead to an understanding of plant evolution. Plant speciation is closely related to morphological changes. Understanding these changes is important because the differences express adaptation to the environment (Barrett 2002). In particular, differences in floral structures as reproductive organs are related to reproductive isolation (Cuevas et al. 2018; Kulcheski et al. 2006). In *Narcissus*, the relationship between style length and the type of pollinators has been investigated (Pérez-Barralés et al. 2006, 2014). However, the relationship between the style length and pollen tube growth rate is not well understood. To investigate ways to surmount the barriers and facilitate breeding, our study focuses on pollen tube growth ability in some *Petunia* species having different style lengths. Williams and Rouse (1988) suggested that the ability to produce hybrids could be related to comparative flower sizes of the parental species in *Rhododendron*. In the comprehensive research for genus *Rhododendron*, the rate of pollen tube growth was faster in the species having longer styles (Williams and Rouse 1990). In the present study, we selected the genus *Petunia* as a model plant, which has several species with apparent different style lengths.

*Petunia* originates in South America (Lorenz-Lemke et al. 2010). The flower shapes vary among species (Galliot et al. 2006). Previously, an expedition to collect the wild genetic resources of *Petunia* was conducted (Ando and Hashimoto 1993; Ando et al. 1995a, 1995b, 2001; Kokubun et al. 1998, 2006); phylogenetic analyses were conducted (Ando et al. 2005; Chen et al. 2007; Kulcheski et al. 2006; Zhang et al. 2008), and cross-compatibility was examined (Watanabe et al. 2001, 1996, 1997). *Petunia* exhibits gametophytic self-incompatibility (Linskens 1975), but a degree of variation in self-incompatibility response occurs in the wild genetic resources (Ando et al. 1998). The current study evaluating pollen tube growth will contribute to the research on the mechanisms of...
In our previous studies, pollen behavior was examined by developing a liquid in vitro pollen culture system that induces normal pollen germination and tube growth in various genera such as *Alstroemeria* (Hirano and Hoshino 2009), *Cyrtanthus* (Hirano and Hoshino 2010) and *Petunia* (Hoshino et al. 2016). The medium is characterized by the addition of a low concentration of yeast extract, which encourages normal and high pollen germination. As an example of the application of pollen culture, assessment of gamma-irradiated pollen grains was conducted for haploid production in *Juglans regia* (Grouh et al. 2011). In the present study, we evaluated pollen tube growth ability using the pollen culture medium developed previously. In the previous study (Hoshino et al. 2016) and the preliminary experiments, the pollen germination ability was confirmed in several *Petunia* species used in this study. Then, we examined the pollen tube growth rate in the styles of several *Petunia* species having different style lengths. Moreover, the effects of the pollen tube growth ability on the pollen tube lengths in the styles were analyzed. In total, we discussed the pollen tube growth ability in vitro, and pollen tube lengths in the styles after cross-pollination or interspecific pollination.

**Materials and methods**

**Plant material**

The following seven accessions of *Petunia* species were used in this study (Table 1); *Petunia axillaris* (Lam.) Britton. Sterns & Poggenb. subsp. *axillaris* (accession name: A122, B656, U157) (Figure 1A), *P. axillaris* (Lam.) Britton. Sterns & Poggenb. subsp. *parodii* (Steere) Cabrera (accession name: A165, U27) (Figure 1B), *P. integrifolia* (Hook.) Schinz & Thell. (accession name: U106) (Figure 1C), and *P. occidentalis* R. E. Fr. (accession name: A109) (Figure 1D). All these flowers had different style lengths (Figure 1E). The plants were grown from seeds, and eight plants per accession were cultivated as potted plants and grown in a glasshouse at the Experiment Farms, Hokkaido University, Japan. During the flowering season, a liquid fertilizer (Hyponex) was applied in the appropriate concentration and at appropriate intervals.

Style length indicates the length the pollen tube must attain to reach the ovule from the stigma in each species. To analyze the relationship between style length and pollen tube growth ability, the following experiments were designed. Initially, pollen tube growth ability was evaluated in vitro.

**Measurements of the floral organs**

Pistil length, stamen length, flower length, corolla width, and ovary length were measured in at least 20 flowers immediately after flowering. The pistil and stamen lengths were measured using a caliper (CD-S15C, Mitutoyo, Kawasaki, Japan) after cutting the flowers. The style lengths were calculated from the data of the pistil and ovary lengths; the pistil length = the style length + stigma length. In this study, this value was used as the style length because the stigma length was minute. The ovary length data of *P. occidentalis* were obtained

| Species/Accession          | Origin              | Flower color | SC or SI | Reference          |
|----------------------------|---------------------|--------------|----------|--------------------|
| *P. axillaris* subsp. *axillaris* (A122) | Argentine | White | SC | Kokubun et al. (2006) |
| *P. axillaris* subsp. *axillaris* (B656) | Brazil | White | SC | Kokubun et al. (2006) |
| *P. axillaris* subsp. *axillaris* (U157) | Uruguay | White | SI | Ando et al. (1998) |
| *P. axillaris* subsp. *parodii* (A165) | Argentine | White | SC | Kokubun et al. (2006) |
| *P. axillaris* subsp. *parodii* (U27) | Uruguay | Red-purple | SI | Ando et al. (1995c) |
| *P. integrifolia* (U106) | Uruguay | Blue-purple | SC | Ando et al. (1995c) |
| *P. occidentalis* (A109) | Argentine | Red-purple | SC | Ando et al. (1995c) |

Self-compatibility (SC), Self-incompatibility (SI).
Measurement of pollen tube length in vitro

The pollen culture was conducted according to the procedure of Hirano and Hoshino (2009) with minor modifications. Briefly, anthers were harvested immediately after flowering, and each anther was cut into quarters. One of the quarters was put into a 3.5-cm Petri dish containing 1.5 ml of a liquid medium, and pollen grains were released from the anther piece by gentle shaking. The Petri dishes were kept at 22°C. For each Petri dish, the frequency of pollen germination was calculated from five fields of view under an inverted microscope (Axiovert 200, Carl Zeiss, Jena, Germany). Pollen tube lengths were also measured using a digital camera scale (DS-Fi2, Nikon Corporation, Tokyo, Japan) with a camera control unit (DS-L3, Nikon Corporation, Tokyo, Japan). The samples showing more than 70% pollen germination frequencies after 6 h were treated as analytical data to delete data from abnormal pollen grains. Evaluation of pollen germination frequencies and measurement of pollen tube length were performed 1, 3, and 6 h after culture. At least three replicates were examined in each experiment. The ten longest pollen tubes were used for data analysis.

To detect changes in the speed of pollen tube growth for characterizing each species, pollen tube growth rate was calculated at each time point by the following formulas: for characterizing each species, pollen tube growth rate ten longest pollen tubes were used for data analysis. At least three replicates were examined in each experiment. The statistical analysis was performed using R software. The data were analyzed by R Tukey’s Honestly Significant Difference (HSD) test (p < 0.05) with R software.

Results

Measurement of floral organs in Petunia species

The sizes of the floral organs (pistil length, stamen length, flower length, corolla width, and ovary length) were measured, and the style lengths were calculated (Table 2). Petunia axillaris subsp. parodi (A165) had a maximum style length at an average of 59.0 mm, whereas P. integrifolia had the shortest style length at an average of 11.1 mm. Pistil lengths of P. axillaris subsp. axillaris and P. axillaris subsp. parodi were longer than the stamen lengths of these species, meaning that the stigmas were positioned above the anthers in both species. In P. integrifolia and P. occidentalis, the stigmas were positioned below the anthers.

Evaluation of the pollen tube length as “the pollen tube growth ability” using in vitro pollen culture system

We used an in vitro pollen culture system to evaluate

| Species/Accession | Pistil length (mm) | Stamen length (mm) | Flower length (mm) | Corolla width (mm) | Ovary length (mm) | Style length (mm) |
|-------------------|--------------------|--------------------|--------------------|--------------------|--------------------|------------------|
| P. axillaris subsp. axillaris (A122) | 49.2 ± 1.32 | 47.8 ± 1.46 | 56.2 ± 3.02 | 26.3 ± 1.91 | 8.6 ± 0.80 | 40.6 |
| P. axillaris subsp. axillaris (B656) | 49.4 ± 2.67 | 47.7 ± 2.3 | 52.4 ± 3.82 | 23.8 ± 2.46 | 7.2 ± 1.35 | 42.2 |
| P. axillaris subsp. axillaris (U157) | 44.5 ± 2.59 | 42.8 ± 2.58 | 50.5 ± 5.31 | 22.6 ± 1.79 | 7.1 ± 1.18 | 37.4 |
| P. axillaris subsp. parodi (A165) | 65.9 ± 2.76 | 63.6 ± 3.46 | 74.7 ± 6.47 | 24.5 ± 5.75 | 6.9 ± 1.42 | 59.0 |
| P. axillaris subsp. parodi (U27) | 63.0 ± 4.23 | 61.8 ± 3.95 | 68.0 ± 5.18 | 19.0 ± 2.50 | 5.9 ± 0.73 | 57.1 |
| P. integrifolia (U106) | 14.8 ± 0.93 | 16.7 ± 0.91 | 23.9 ± 1.92 | 16.0 ± 2.41 | 3.7 ± 0.55 | 11.1 |
| P. occidentalis (A109) | 17.1 ± 0.39 | 17.7 ± 0.72 | no data | no data | 3.47 ± 1.1 | 13.6 |

Data were obtained from at least 20 flowers. a) The value in Ando et al. (1995c) was used. b) The style length was calculated by the pistil length—the ovary length. In this study, this value included stigma length.

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the development of the pollen tube. In the preliminary experiments, we measured pollen tube length after culture periods of 6, 9, 12, or 24 h. The growing pollen tubes became entwined after a 9 h culture, making measurement difficult. Therefore, we used a 6 h culture period (Figure 2) to evaluate the speed of pollen tube growth by measuring pollen tube length in time.

The pollen tube lengths were compared in each species 6 h after the in vitro pollen culture (Figure 3A). As a result, their pollen tube growth ability differed in each species. For instance, *P. occidentalis*, which has a shorter style length, had maximum pollen tube length. The data indicate that pollen tube growth ability is species-specific, independent of style lengths. Thus, the pollen tube growth in vitro did not correlate with the style lengths of the flowers in these *Petunia* species (Figure 3A).

Temporal changes in pollen tube growth rates were detected between 0 to 1 h, 1 to 3 h, and 3 to 6 h in the culture medium (Figure 3B). There was no common pattern of pollen tube growth among all the species; in particular, *P. occidentalis* showed a remarkable pollen tube growth rate between 3 to 6 h in the culture.

In vitro pollen culture was not affected by the styles, which are the in vivo environmental factors. To examine pollen tube growth in the styles, cross-pollination experiments were conducted.

![Figure 2. Typical pollen tube growth of *P. axillaris* subsp. *axillaris* (A122) in the liquid culture medium 6 h after incubation. The lengths of the pollen tubes are measured using a digital camera with an inverted microscope.](Image)

![Figure 3. Comparison of pollen tube growth and pollen tube growth rates in *Petunia* species having differing style lengths. (A) The relationship between the pollen tube length in vitro 6 h after incubation and their own-species style length is shown in Table 2. No significant difference was detected in the pollen tube length and the style length (*p* > 0.05). (B) Changes in the pollen tube growth rates in *Petunia* species during 6 h of culture in the liquid medium.](Image)
The pollen tube growth in the styles after pollination

The pollen tube growth in the styles was evaluated by measuring the lengths detected with aniline blue staining after interspecific pollinations. In the preliminary experiment, the measurements took place 6 and 18 h after intraspecific pollination. The pollen tubes arrived at the near end of the style 18 h after intraspecific pollination in *P. axillaris* subsp. *parodii* (Figure 4A). To detect differences between the species and compare the pollen tube lengths in vitro for the same time period, 6 h after pollination was selected as the appropriate time. The pollen tube lengths were measured in the styles 6 h after intraspecific pollinations (Figure 4B). Consequently, the style lengths in each species were correlated with the pollen tube lengths in the pistils (Supplementary Figure S1). Among them, *P. axillaris* subsp. *parodii*, which had the longest styles, also had the longest pollen tubes, approximately 9.2 mm on average. In *P. occidentalis*, which had the greatest pollen tube growth rate in vitro (Figure 3B), the pollen tube length was the shortest, approximately 3.1 mm on average (Figure 4B). The pollen tube length in *P. axillaris* subsp. *parodii* was significantly greater than that in the other species.

The pollen tube lengths in the styles (Figure 4B) were compared as the ratio of the pollen tube length to style length (Figure 4C). The ratios, indicating the degree of pollen tube achievement in the styles, showed that the values of *P. axillaris* subsp. *parodii* and *P. occidentalis* were similar, and there was no obvious difference in this experiment.

To compare the pollen tube growth in the styles of other species, the pollen tube lengths were measured in the styles after interspecific pollinations. Through this test, the effects of the pollen grains from another species on the ovary parent became apparent (Figure 5A). It was found that pollen grains of *P. occidentalis* had different pollen tube lengths in different species and showed a longer pollen tube length in the styles of *P. axillaris* subsp. *parodii* (interspecific pollination) than in *P. occidentalis* (intraspecific pollination). Similarly, the pollen tubes of *P. integrifolia* became longer in the styles of *P. axillaris* subsp. *parodii* (interspecific pollination) than that of *P. integrifolia* (intraspecific pollination). In the styles of *P. axillaris* subsp. *parodii*, the pollen grains from the other three species could grow pollen tubes similar to those they would grow in styles of their own species. In the styles of *P. integrifolia*, which harbors self-incompatibility, the pollen tube growth seemed to be suppressed. In the styles of *P. occidentalis*, the pollen tubes of *P. axillaris* subsp. *parodii* were longer than that of *P. occidentalis* (intraspecific pollination). Thus, the pollen tube lengths were altered by the styles of different species.

To analyze the results of cross-pollination experiments, the data of interspecific pollinations in Figure 5A were aligned in the order of the pollen tube lengths (Figure 5B). This exercise showed that the styles in the species showing self-incompatibility suppressed pollen tube growth in the interspecific cross-combinations.

The pollen tube growth ability in vitro was not related to pollen tube lengths in the styles after intraspecific pollinations. Also, it was not related to the style lengths of their own species in the genus *Petunia*.

**Discussion**

Our results indicated that the pollen tube growth ability in vitro was not a factor controlling the pollen tube growth rate in the styles. This study suggests that pollen tube lengths in the styles were not determined by the pollen tube growth ability but rather by the control mechanism of pollen tube growth in cooperation with the styles. The results of this study implied the following...
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points: 1) a species retains a species-specific pollen tube growth ability in vitro, 2) the pollen tube growth ability does not have a direct relationship to the style length of the species, and 3) the pollen tube growth changed in different-species ovary parents indicating the effects of the styles on the speed of pollen tube growth.

Accounts of the interaction between pollen tube growth and styles have been published in research about gametophytic self-incompatibility (Meng et al. 2011; Sun et al. 2018). In comparison with the present study, we should note a case study of heteromorphic self-incompatibility showing that flowers of different style lengths (pin and thrum) are compatible with each other. In this heteromorphic self-incompatibility, the pollen grains from the thrum flowers are compatible with the pin flowers and vice versa. In the genus *Fagopyrum* (buckwheat) showing heteromorphic self-incompatibility, Hirose et al. (1994) reported that the pollen tubes from the thrum flowers could grow longer in various interspecific pollinations. Morris (1951) observed that the pollen tubes from thrum flowers stopped on the thrum flower stigmas, whereas the pollen tubes from the pin flowers stopped on the pin flower styles. Thus, pistil styles have a significant effect on pollen tube growth. In the present study, the pollen tube length was positively correlated with the style length in the same species within the genus *Petunia*. However, the pollen tube growth ability evaluated using in vitro culture did not correlate with style length.

Brewbaker and Kwack (1963) noted the importance of calcium for pollen germination and proposed a useful culture medium. Based on the composition of this medium, we developed a liquid pollen culture medium containing yeast extract, which can be used for a wide range of plant species such as *Alstroemeria* (Hirano and Hoshino 2009), *Cyrtanthus* (Hirano and Hoshino 2010), and *Petunia* (Hoshino et al. 2016). In these studies, we focused on binucleate pollen species and analyzed

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**Figure 5.** Evaluation of the pollen tube growth in the styles after interspecific pollinations. (A) Comparison of the pollen tube lengths 6 h after interspecific and intraspecific pollinations. (B) The data of Figure 5A are aligned in the order of pollen tube lengths without the data of intraspecific pollinations. The traits of self-incompatibility and self-compatibility are indicated.
the timing of sperm cell formation (the division of generative cells) during pollen tube development using flow cytometry. In the pollen germination medium, the pollen grains of these species showed sperm cell formation with timing unaffected by extrinsic factors, suggesting that this culture system would be suitable for investigating pollen tube growth ability. In the previous study, the addition of pistil extracts into the culture medium promoted pollen tube growth in Nicotiana (Wu et al. 2000). In the present study, the suppressive effect of the styles on pollen tube growth in some species was examined by comparing the pollen tube growth ability in vitro and the pollen tube length in the styles. To explain the suppressive effect, we propose two hypotheses: 1) the influence of self-incompatibility by the styles, and 2) coordination of pollen tube growth by the styles to control sperm cell formation, which is required for normal fertilization.

In the self-incompatibility of the genus Petunia (Solanaceae), S-RNase as an inhibitory agent affects pollen tubes in the styles (Kubo et al. 2015). Incompatible pollen tubes cannot decompose S-RNase, and the compatible pollen tubes need to grow while decomposing the S-RNase in the pistils with self-incompatibility. In Lilium, self-incompatible stylar extracts inhibited pollen tube growth in vitro (Dickinson et al. 1982). In the present study, the pollen tubes may grow slowly in the styles with self-incompatibility (Figure 5B). Conversely, the pistils of self-compatible species affected the pollen tube growth promotively even in interspecific pollination when P. occidentalis was used as an ovary parent (Figure 5A). Notably, the pollen tubes of P. axillaris subsp. parodi were the longest in P. occidentalis ovaries suggesting the presence of compatible and promotive combinations between pollen grains and styles.

Regarding sperm cell formation, we showed that the timing of generative cell division was 12–15h in a Petunia cultivar using the same pollen culture system as in the present study by flow cytometry (Hoshino et al. 2016). This indicates that the division of the generative cells occurs independently without the assistance of the styles. However, the pollen tubes have to stay in the styles for an appropriate time to form sperm cells before fertilization. For example, P. occidentalis, which has small pistils, grew the longest pollen tubes in vitro but had the shortest pollen tubes in the styles. Interestingly, pollen tube growth rates changed as faster or slower when compared to the self-styles after interspecific pollinations in Rhododendron (Williams and Rouse 1990). We concluded that the styles control pollen tube growth to ensure sperm cell formation followed by normal fertilization in the ovules.

This study revealed differences between pollen tube growth ability and the pollen tube length in styles of different lengths after intraspecific or interspecific pollinations. Although the medium has high availability on different species, the results of this study should be considered for the differences in the medium and the species affinity. A further study is required to optimize the culture medium for each species. In addition, we will focus on the analysis of the relationship between pollen tube behaviors and self-incompatibility on a molecular level.

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Conflicts of interest/Competing interests
The authors declare that they have no competing interest.

Authors’ contributions
MK and YH conceived and designed the research. MK performed experiments. HW provided the plant materials and essential suggestions for research plan. MK and YH wrote manuscript. All authors read and approved the manuscript.

References
Ando T, Hashimoto G (1993) Two new species of Petunia (Solanaceae) from southern Brazil. Bot J Linn Soc 111: 265–280
Ando T, Iida S, Kokubun H, Ueda Y, Marchesi E (1995a) Distribution of infraspecific taxa of Petunia axillaris (Solanaceae) in Uruguay as revealed by discriminant analyses. APG 45: 95–109
Ando T, Iida S, Kokubun H, Ueda Y, Marchesi E (1995b) Distribution of Petunia axillaris sensu latu in Uruguay as revealed by discriminant analysis of the live plants. J Jpn Soc Hortic Sci 64: 381–391
Ando T, Kokubun H, Watanabe H, Tanaka N, Yukawa T, Hashimoto G, Marchesi E, Suárez E, Basualdo IL (2005) Phylogenetic analysis of Petunia sensu Jussieu (Solanaceae) using chloroplast DNA RFLP. Ann Bot 96: 289–297
Ando T, Kurata M, Sasaki S, Ueda Y, Hashimoto G, Marchesi E (1995c) Comparative morphological studies on infraspecific taxa of Petunia integrifolia (Hook.) Schinz et Thell. (Solanaceae). Shokubutsu Kenkyu Zasshi 70: 205–217
Ando T, Nomura M, Tsukahara J, Watanabe H, Kokubun H, Tsukamoto T, Hashimoto G, Marchesi E, Kitching JI (2001) Reproductive isolation in a native population of Petunia sensu Jussieu (Solanaceae). Ann Bot 88: 403–413
Ando T, Tsukamoto T, Akiba N, Kokubun H, Watanabe H, Ueda Y, Marchesi E (1998) Differentiation in the degree of self-incompatibility in Petunia axillaris (Solanaceae) occurring in Uruguay. APG 49: 37–47
Barrett SCH (2002) The evolution of plant sexual diversity. Nat Rev Genet 3: 274–284
Brewbaker JL, Kwack BH (1963) The essential role of calcium ion in pollen germination and pollen tube growth. Am J Bot 50: 859–865
Chen S, Matsubara K, Omori T, Kokubun H, Kodama H, Watanabe H, Hashimoto G, Marchesi E, Bullrich L, Ando T (2007) Phylogenetic analysis of the genus Petunia (Solanaceae) based on...
the sequence of the Hf1 gene. *J Plant Res* 120: 385–397

Cuevas E, Espino J, Marques I (2018) Reproductive isolation between *Salvia elegans* and *S. fulgens*, two hummingbird-pollinated sympatric sages. *Plant Biol* 20: 1075–1082

Dickinson HG, Moriarty J, Lawson J (1982) Pollen-pistil interaction in *Lilium longiflorum*: The role of the pistil in controlling pollen tube growth following cross-and self-pollinations. *P Roy Soc Lond B Bio* 215: 45–62

Galliott C, Hoballah ME, Kuhlemeier C, Stuurnan J (2006) Genetics of flower size and nectar volume in *Petunia* pollination syndromes. *Planta* 225: 203–212

Grouh MSH, Vahdati K, Lofit M, Hassani D, Biranvand NP (2011) Production of haploids in Persian walnut through parthenogenesis induced by gamma-irradiated pollen. *J Am Soc Hort Sci* 136: 198–204

Hirano T, Hoshino Y (2009) Detection of changes in the nuclear phase and evaluation of male germ units by flow cytometry during in vitro pollen tube growth in *Alstroemeria aurea*. *J Plant Res* 122: 225–234

Hirano T, Hoshino Y (2010) Sperm dimorphism in terms of nuclear shape and microtubule accumulation in *Cytanthus mackenii*. *Sex Plant Reprod* 23: 153–162

Hirose T, Ujihara A, Kitabayashi H, Minami M (1994) Interspecific cross-compatibility in *Fagopyrum* according to pollen tube growth. *Ipom J Breed* 44: 307–314

Hoshino Y, Eiraku N, Ohata Y, Komai F (2016) Dynamics of nuclear phase changes during pollen tube growth by using in vitro culture in *Petunia*. *Sci Hortic (Amsterdam)* 210: 143–149

Hoshino Y, Murata N, Shinoda K (2006) Isolation of individual egg cells and zygotes in *Alstroemeria* followed by manual selection with a microcapillary-connected micropump. *Ann Bot* 97: 1139–1144

Kokubun H, Ando T, Kohyama S, Watanabe H, Tsukamoto T, Marchesi E (1998) Distribution of intermediate forms of *Petunia axillaris* subsp. *axillaris* and subsp. *parodi* (Solanaceae) in Uruguay as revealed by discriminant analysis. *APG* 48: 173–185

Kokubun H, Nakano M, Tsukamoto T, Watanabe H, Hashimoto G, Marchesi E, Bullrich L, Basualdo JL, Kao TH, Ando T (2006) Distribution of self-compatible and self-incompatible populations of *Petunia axillaris* (Solanaceae) outside Uruguay. *J Plant Res* 119: 419–430

Kubo KI, Paape T, Hatakeyama M, Entani T, Takara A, Kajiha K, Tsukahara M, Shimizu-Inatsugi R, Shimizu KK, Takayama S (2015) Gene duplication and genetic exchange drive the evolution of S-RNase-based self-incompatibility in *Petunia*. *Nat Plants* 1: 14005

Kulcheski FR, Muschauer VC, Lorenz-Lemke AP, Stehmann JR, Bonatto SL, Salzano FM, Freitas LB (2006) Molecular phylogenetic analysis of *Petunia* Juss. (Solanaceae). *Genetica* 126: 3–14

Linskens HF (1975) Incompatibility in *Petunia*. *P Roy Soc Lond B Bio* 188: 299–311

Lorenz-Lemke AP, Togni PD, Mäder G, Kriedt RA, Stehmann JR, Salzano FM, Bonatto SL, Freitas LB (2010) Diversification of plant species in a subtropical region of eastern South American highlands: A phylogeographic perspective on native *Petunia* (Solanaceae). *Mol Ecol* 19: 5240–5251

Meng X, Sun P, Kao TH (2011) S-RNase-based self-incompatibility in *Petunia inflata*. *Ann Bot* 108: 637–646

Morris MR (1951) Cytogenetic studies on buckwheat: Genetic and cytological studies of compatibility in relation to heterostyly in common buckwheat. *Fagopyrum sagittatum*. *J Hered* 42: 35–89

Pérez-Barrales R, Simón-Porcar VI, Santos-Gally R, Arroyo J (2014) Phenotypic integration in style dimorphic dailodils (*Narcissus*, Amaryllidaceae) with different pollinators. *Philos Trans R Soc Lond B Biol Sci* 369. 20130258

Pérez-Barrales R, Vargas P, Arroyo J (2006) New evidence for the Darwinian hypothesis of heterostyly: Breeding systems and pollinators in *Narcissus sect. Apodanthi*. *New Phytol* 171: 553–567

Sun L, Williams JS, Li S, Wu L, Khatri WA, Stone PG, Keebaugh MD, Kao TH (2018) S-Locus F-box proteins are solely responsible for S-RNase-based self-incompatibility of *Petunia* pollen. *Plant Cell* 30: 2959–2972

Watanabe H, Ando T, Iida S, Buto K, Tsukamoto T, Kokubun H, Hashimoto G, Marchesi E (1997) Cross-compatibility of *Petunia pubescens* and *P. pygmaea* with native taxa of *Petunia*. *J Jpn Soc Hortic Sci* 66: 607–612

Watanabe H, Ando T, Iida S, Suzuki A, Buto K, Tsukamoto T, Hashimoto G, Marchesi E (1996) Cross compatibility of *Petunia* cultivars and *P. axillaris* with native taxa of *Petunia* in relation to their chromosome number. *J Jpn Soc Hortic Sci* 65: 625–634

Watanabe H, Ando T, Tsukamoto T, Hashimoto G, Marchesi E (2001) Cross-compatibility of *Petunia exserta* with other *Petunia* taxa. *J Jpn Soc Hortic Sci* 70: 33–40

Williams EG, Rouse JL (1988) Disparate style lengths contribute to isolation of species in *Rhododendron*. *Aust J Bot* 36: 183–191

Williams EG, Rouse JL (1990) Relationships of pollen size, pistil length and pollen tube growth rates in *Rhododendron* and their influence on hybridization. *Sex Plant Reprod* 3: 7–17

Wu HM, Wong E, Ogdaol J, Cheung AY (2000) A pollen tube growth-promoting arabinogalactan protein from *Nicotiana alata* is similar to the tobacco TTS protein. *Plant J* 22: 165–176

Zhang X, Takahashi H, Nakamura I, Mii M (2008) Molecular discrimination among taxa of *Petunia axillaris* complex and *P. integrifolia* complex based on PolA1 sequence analysis. *Breed Sci* 58: 71–75