Decreased RNase Activity Under High Temperature Is Related to Promotion of Self-pollen Tube Growth in the Pistil of the Japanese Flowering Cherry, *Prunus × yedoensis* ‘Somei-yoshino’

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**Prunus × yedoensis** Matsum. ‘Somei-yoshino’ is one of the most popular flowering cherry cultivars in Japan. However, because flowering cherries have strict self-incompatibility (SI), it is difficult to develop selfing families or inbred lines for breeding and genetic studies. SI in *Prunus* is achieved through arrest of self-pollen tube growth in the style by ribonuclease S-RNase. To determine a suitable way to overcome SI in *Prunus*, we examined the influence of hot water immersion on RNase activity in the style of ‘Somei-yoshino’. When the styles were immersed in 20°C, 30°C, and 40°C water baths, their RNase activity was not significantly different from that of untreated styles (*P* > 0.05); however, it significantly decreased in styles heated in 50°C water baths (*P* < 0.01). Furthermore, the RNase activity decreased with an increase in the incubation time (e.g., 1, 3, and 5 min). Additionally, heat treatment at 50°C promoted the growth of self-pollen tubes in the style. Some self-pollen tubes even reached the bottom of the style. The high temperature treatment with reduced RNase activity can overcome stylar SI in *Prunus*.

**Key Words:** hot water immersion, inactivation of self-incompatibility, *Prunus*, S-RNase.

**Introduction**

To construct self-fertilized families or inbred lines, self-compatibility (SC) is a crucial characteristic required for breeding and genetic studies. Not surprisingly, the inactivation of self-incompatibility (SI) systems has been attempted in many allogamous plants using various methods, such as bud pollination, stigma excision, and high-temperature treatment (Hopper et al., 1967; Okazaki and Hinata, 1987; Okazaki and Murakami, 1992; Suyama et al., 2013). Of these, hot water immersion, in which styles or flowers are dipped into hot water, has been widely used and has contributed to successful production of selfed individuals in many outbreeding plants. However, the general mechanism underlying SI inactivation by high temperature remains unclear. Only a few studies have inferred the involvement of a disruption of style tissue structures in *Lilium* (Hopper et al., 1967) and Brassicaceae (Okazaki and Hinata, 1987), as well as changes in protein composition in *Pyrus* (Hiratsuka and Tomita, 1989).

Flowering cherry (*Rosaceae, Prunus* subgenus *Cerasus*) is one of the most famous ornamental trees in Japan. *Prunus × yedoensis* Matsum. ‘Somei-yoshino’, the most popular cultivar that is widely planted in Japan, shows SI due to a lack of seed set after self-pollination (Tsuruta et al., 2012; Watanabe and Yoshikawa, 1967). In a previous study, we attempted to overcome SI in ‘Somei-yoshino’ using bud pollinations, stigma excisions, and hot water immersions from 20°C to 50°C (Tsuruta and Mukai, 2016). However, only hot water immersions at > 45°C promoted the growth of the self-pollen tube within the style. Therefore, identifying the physiological changes in the self-style induced by hot water immersion will likely provide a better understanding of *Prunus* SI that may help in overcoming it.

Most Rosaceae species, including *Prunus*, have an S-RNase-based gametophytic SI system (GSI: Sassa, 2016; Tao and Iezzoni, 2010). This GSI system consists of a pollen S determinant, which is an S-locus F-box protein, and a pistil S determinant, which is a glycoprotein S-RNase. When the S allele of the pollen matches either of the two alleles of the pistil, pollen tube growth halts in the style, where S-RNase is secreted. S-RNase has ribonuclease (RNase) activity and can degrade the RNA of the self-pollen. Consequently, S-RNase activity...
is essential for SI system functionality. Indeed, insufficient activation of SI occurs when the amount of S-RNase or its expression level is reduced in some GSI plants, such as *Solanum* (Qin et al., 2006) or *Prunus mume* Sieb. et Zucc. (Watarai et al., 2007). Therefore, in the current study, we focused on measuring the RNase activity in the style following hot water immersion treatment.

**Materials and Methods**

*Plant materials and hot water immersion*

Three ‘Somei-yoshino’ trees planted at Gifu University (the same trees used in our previous study; Tsuruta and Mukai, 2016) were used for the experiments. Branches with flower buds were collected from the field prior to anthesis and blossomed at 20°C in the laboratory. After removing the unbloomed buds and overly-mature flowers, the remaining flowers were immersed in a water bath (F-003D; Tokyo Garasu Kikai Co., Ltd., Tokyo, Japan), after which their pistils were collected, flash-frozen with liquid N₂, and stored at −80°C. The immersion and collection conditions were as follows: treatment temperature (TEM), 20°C, 30°C, 40°C, 45°C, or 50°C for 3 min, followed by immediate collection; treatment duration (DUR), 50°C for 1, 3 minz, 5 minz, or 5 min, followed by immediate collection; treatment restoration (RES), immersing in 50°C for 3 min, followed by pistil collection after 0 (identical to 50°C TEM), 0.5, 1, or 3 h. Non-immersed flowers were used as a control. At least 20 flowers were used for each treatment (Table 1).

**Protein extraction**

Soluble protein was extracted from five pistils (approximately 25 mg fresh weight). Pistils frozen with liquid N₂ were ground into powder using a Multi Beads Shocker (Yasui Kikai Corporation, Osaka, Japan). Then, the powder was suspended in pre-chilled 85% acetone with 0.05% 2-mercaptoethanol and centrifuged (12,000 rpm) at 4°C for 5 min, after which the precipitates were collected. This procedure was repeated three times. Then, the precipitates were dried under reduced pressure and dissolved in 200 μL of an extraction buffer containing 50 mM Tris-HCl (pH 7.8), 10% glycerin, and 0.05% 2-mercaptoethanol. After centrifugation (12,000 rpm, 4°C, 5 min), the supernatants were collected as a soluble protein extract. Total protein concentration in the extract was quantified using the Bradford method with bovine serum albumin as a standard.

**Measurement of RNase activity and statistical analysis**

RNase activity was measured in terms of increasing absorbance at a wavelength of 260 nm (A₂₆₀), the maximum wavelength for nucleotides (McClure et al., 1989). The sample protein extract (20 μL) was mixed with 980 μL of 0.1 M sodium acetate reaction buffer (pH 5.4) containing 5 μg of RNA isolated from Daikon radish sprouts (*Raphanus sativus* L. var. *longipinnatus* L. H. Bailey) using the CTAB method. The mixtures were gently pipetted at 1-min intervals at 20°C, and A₂₆₀ was measured. RNase activity was expressed as the rate change of A₂₆₀ per extraction from a pistil (ΔA₂₆₀·min⁻¹/pistil) and per protein unit (ΔA₂₆₀·min⁻¹·mg⁻¹ protein).

**Table 1.** Protein concentration and RNase activity of pistil extractions in each treatment.

| Treatment          | Pistils | N  | Protein concentration (mg·mL⁻¹) | Change in A₂₆₀·min⁻¹/pistil | Change in A₂₆₀·min⁻¹·mg⁻¹ protein
|--------------------|---------|----|-------------------------------|----------------------------|----------------------------------|
|                    |         |    | mean (SD)                     | mean (SD)                  | mean (SD)                       |
| Control            | 30      | 6  | 1.097 (0.237)                 | 0.054 (0.028)              | 0.241 (0.079)                   |
| **Treatment temperature (TEM)** |         |    |                               |                           |                                  |
| 20°C               | 45      | 9  | 1.047 (0.321)                 | 0.061 (0.013)              | 0.227 (0.061)                   |
| 30°C               | 50      | 10 | 0.930 (0.348)                 | 0.056 (0.012)              | 0.232 (0.072)                   |
| 40°C               | 45      | 9  | 0.892 (0.370)                 | 0.044 (0.014)              | 0.196 (0.065)                   |
| 45°C               | 15      | 3  | 0.685 (0.192)                 | 0.042 (0.034)              | 0.110 (0.066)                   |
| 50°C               | 45      | 9  | 0.559 (0.226)**               | 0.014 (0.009)**            | 0.104 (0.074)**                 |
| **Treatment duration (DUR)** |         |    |                               |                           |                                  |
| 1 min              | 25      | 5  | 0.857 (0.363)                 | 0.027 (0.005)*             | 0.170 (0.052)                   |
| 3 min              | 45      | 9  | 0.559 (0.226)**               | 0.014 (0.009)**            | 0.104 (0.074)**                 |
| 5 min              | 25      | 5  | 0.486 (0.266)**               | 0.009 (0.008)**            | 0.107 (0.094)*                  |
| **Treatment restoration (RES)** |         |    |                               |                           |                                  |
| After 0 h           | 45      | 9  | 0.559 (0.226)**               | 0.014 (0.009)**            | 0.104 (0.074)**                 |
| After 0.5 h         | 20      | 4  | 0.518 (0.184)*                | 0.013 (0.005)**            | 0.130 (0.026)                   |
| After 1 h           | 20      | 4  | 0.840 (0.061)                 | 0.021 (0.004)**            | 0.128 (0.021)                   |
| After 3 h           | 20      | 4  | 0.861 (0.050)                 | 0.020 (0.003)**            | 0.116 (0.013)*                  |

* z data are identical to those of the 50°C TEM treatment.

*,,**,*** Significant difference between control and each treatment at P<0.05, <0.01, and <0.001, respectively (Dunnett’s test). Different letters indicate 5% level of significant difference among treatments (Tukey’s test).
Table 2. Number of flowers and fruit set in artificial self- and cross-pollination of ‘Somei-yoshino’ for the control and 50°C immersed treatments.

| Year | Treatment | Cross type | Bags | Total number of pollinated flowers | Number of fruit set (rate z) | SD |
|------|-----------|------------|------|-----------------------------------|-----------------------------|----|
| 2016 | Control   | Self       | 5    | 45                                | 0                           | —  |
|      | 50°C      | Self       | 9    | 76                                | 0                           | —  |
| 2019 | Control   | Self       | 5    | 18                                | 0                           | —  |
|      | 50°C      | Self       | 5    | 31                                | 0                           | —  |
|      | Control   | Cross      | 2    | 11                                | 5 (0.483)                   | 0.448 |
|      | 50°C      | Cross      | 3    | 18                                | 5 (0.226)                   | 0.215 |

* The fruit set rate was calculated for each pollination bag and averaged.

Statistical analysis of the mean RNase activity among treatments and between the control and each treatment were performed to determine significant differences using Tukey’s test and Dunnett’s multiple comparison test, respectively.

Artificial pollinations and observation of pollen tube growth

A decrease in RNase activity under high temperature was ascertained in vivo. Artificial pollination was performed on ‘Somei-yoshino’ trees planted at Gifu University (March 31 and April 2, 2016) and The University of Tokyo Tanashi Forest (March 27, 2019) (Table 2). Branches with flower buds were isolated by enclosing them in nylon pollination bags until pollination. In the field, a 50°C water bath was prepared in a hot water bath and incubated for 3 min. For complete heating, the stamens and top of the calyx tube were removed in the 2019 pollination. After the stigma was dried, self-pollen (i.e., ‘Somei-yoshino’) or compatible cross-pollen (collected from a Prunus jamasakura Sieb. ex Koidz. tree) were used to pollinate the flowers with cotton-tipped sticks. Then, flowers were re-covered with pollination bags. Untreated pistils pollinated with self- and cross-pollen were used as the control group (we also cut the stamens and top of the calyx tube in 2019). Around 10 days after pollination, an abscission layer formed between the ovary and style, and the style dropped off. At this stage, all the styles were collected and fixed with 5% formaldehyde, 5% acetic acid, and 45% ethanol (FAA). Over a 30-day period following pollination, the number of surviving flowers was recorded every third day.

After replacing FAA with deionized water (DW) using a graded ethanol series, the pistils were softened by immersing them in 5N NaOH overnight. The alkaline solution was then replaced with DW, and the pistils were stained for 4 h with aniline blue buffer (0.75% anilin blue and 45% acetic acid). After washing with 0.1N NaOH, the pistils were pressed under cover glass, and their pollen tube growth was observed under a fluorescence microscope. The length from the stigma surface to the tip of the most elongated pollen tube was measured with ImageJ software (Abrámoff et al., 2004). The mean length of pollen tube growth was compared among treatments using the pairwise t-test.

Results

RNase activity and protein concentrations in each treatment

In the control pistils, the mean RNase activity was 0.054·min⁻¹/pistil (SD = 0.028). Although this activity was similar to that of the pistils treated at 20°C (mean = 0.061·min⁻¹/pistil), 30°C (0.056·min⁻¹/pistil), and 40°C (0.044·min⁻¹/pistil; P > 0.05, Dunnett’s test), the RNase activity in the pistils treated at 50°C was significantly lower than that of the control (0.014 ± 0.009·min⁻¹/pistil, P < 0.001 for both Tukey’s test and Dunnett’s test; Table 1). RNase activity decreased in all 50°C treatments, including the DUR and RES. In the DUR, RNase activity decreased significantly (P < 0.05, Tukey’s test) with increasing immersion time, i.e., 0.027·min⁻¹/pistil, 0.014·min⁻¹/pistil, and 0.009·min⁻¹/pistil for 1, 3, and 5 min treatments, respectively (Table 1). In the RES, low levels of RNase activities were maintained even after 3 h of immersion (P < 0.01, Dunnett’s test) (Table 1). When RNase activity was considered on the basis of per protein units (·min⁻¹·mg⁻¹ protein), a significant difference was found for the 45°C and 50°C TEM, 3 and 5 min DUR, and after 0 and 3 h RES, but not for the 1 min DUR or after the 0.5 and 1 h RES (Dunnett’s test) (Table 1). When RNase activity was compared with the control pollination, cross-pollen tubes grew and almost all of the tubes reached the bottom of the style (Figs. 1 and 2). The mean length...
of the most elongated cross-pollen tube was 10.48 mm (SD = 0.60). Conversely, most self-pollen tube growth halted around the middle of the style (mean, 3.17 mm; SD, 0.95). In 50°C-treated pistils, self-pollen tube growth was enhanced significantly (mean, 6.23 mm; SD, 2.51; \( P < 0.001 \)). We also observed that self-pollen tubes grew to the bottom of the style in some flowers (N = 4/77 flowers in 2016 and 10/23 flowers in 2019). However, no significant difference was observed in cross-pollen tube growth in either treatment (Fig. 2). Considerable fruit set was observed in both the control and 50°C-treated cross-pollination groups with 21.5% of treated and 44.8% of control pollinations setting (Table 2); however, all self-pollinated flowers dropped before maturation, which occurred approximately 10–20 days after pollination.

**Discussion**

As with many previous studies, self-pollen tube growth in the style was promoted after short high temperature treatments (> 45°C) with water immersion in flowering cherry (Fig. 2; Tsuruta and Mukai, 2016). We also observed that the pollen tubes reached the bottom of the style in some of the heat-treated pistils. In this study, we demonstrated that RNase activity was significantly lower than that of the control at high temperature (Table 1). Furthermore, we found that the amount of extracted soluble protein significantly decreased in the 50°C treatment (Table 1).

S-RNase, which is essential for SI, is a member of the T2 RNase family. Although RNase T2 tolerated heating at 90°C for 5 min (Uchida, 1966), many T2 family RNases have a low optimal temperature around 50°C (Deshpande and Shankar, 2002). S-RNases have also been shown to be stable up to 50°C in *Petunia* (Singh et al., 1991) and *Nicotiana* (McClure et al., 1989), but above 50°C, their activity is lost rapidly. These findings lead us to believe that the high temperature exposure caused a decrease in S-RNase activity, which enabled self-pollen tube growth in the 50°C-treated styles of ‘Somei-yoshino’. It should be noted that a reduction in RNase activity does not directly indicate a decrease in actual S-RNase activity, since protein extracts from the style also included other RNases, including so-called S-like RNases (Norioka et al., 2007; Sassa et al., 1992; Tao et al., 1997). In future studies, optimized methodologies that provide a more accurate quantification of S-RNases will improve the ability to make these determinations.

Protein yield was also reduced in the high temperature-treated styles. If we assume that the protein extraction manipulations were free of any artifacts, many proteins, including S-RNase, may have been lost from the soluble fraction via catabolism and denaturation. As a result, RNase activity was significantly reduced to approximately one-fourth (per pistil) or one-third (per protein unit) of that of the control (Table 1). This value was consistent with that of SI potato, in which a one-third reduction in the amount of S-RNase resulted in SC (Qin et al., 2006).

Bud pollination and old-flower pollination also
suggested that overcoming SI is driven by low levels of S-RNase activity. Although S-protein and RNase activities were shown to be sufficiently decreased in flower buds 2–8 days before anthesis in Japanese pear (Hiratsuka et al., 1999; Zhang and Hiratsuka, 2000), bud pollination is not suitable for flowering cherry without promotion of self-pollen tube growth (Tsuru and Mukai, 2016). In this study, we showed that when hot water immersion is applied, it can control RNase activity depending on the bath temperature and duration of immersion (Table 1). However, a small reduction in S-RNase activity in flower buds of flowering cherry may not be enough to overcome SI. Therefore, we conclude that hot water immersion at high temperatures has the potential to overcome Prunus SI through a significant reduction in RNase activity.

Our hot water immersion treatment overcame selfing barriers in styles. Although high-temperature treatment led to self-pollen tube growth to the bottom of the style in some flowers (Figs. 1 and 2), seed set was not observed after self-pollination (Table 2). Because only the tip of the style was heat-treated, the flower necrosis that occurred in our previous study (Tsuru and Mukai, 2016) was not observed in the current study (data not shown). Full seeds were obtained from cross-pollination after 50°C heat treatment (Table 1). This indicates that physiological functions likely remained intact. Indeed, the protein concentration also increased after 3 h of immersion (Table 2). With the goal of overcoming SI and producing selfed populations of flowering cherry, evaluating pollen tube growth beyond the style, determining whether fertilization occurs, and elucidating the reason for the absence of self-seed set are research avenues that should be considered in future studies.

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