

Short Communication

A heavy-ion beam-induced mutant of *Verbena*×*hybrida* and wild-type *V. peruviana* demonstrate different types of self-incompatibility

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Abstract We previously used heavy-ion beam irradiation to generate a self-incompatible mutant of *Verbena*×*hybrida* ‘Temari Coral Pink’ (SC), which exhibits a late-acting self-incompatibility system. In the present study, the behavior of pollen tubes and seed productivity after self-pollination were comparatively investigated in SC and wild species *Verbena peruviana* (VP), one of the parental species of *Verbena*×*hybrida*. Although reciprocal cross-pollination between VP and SC produced seeds with high frequencies, namely 57.1% for SC×VP and 59.7% for VP×SC, self-pollinated VP flowers produced no seeds. In the latter, almost all of the pollen germinated on the stigma, but further growth of the pollen tube was inhibited at the upper part of the style. These observations of pollen tube behavior may indicate that the SI system of VP was different from that of SC. VP may possess a homomorphic gametophytic SI system, which is characterized by the inhibition of pollen tube growth at style. We propose a set of SC and VP as a novel model plants for genetic analysis of the SI mechanism in the *Verbenaceae* family.

Key words: Heavy-ion beam, self-incompatible mutant, self-incompatibility, *Verbena*×*hybrida*, *Verbena peruviana*.

Self-incompatibility (SI) is the most widespread mechanism preventing inbreeding and promoting outbreeding in hermaphroditic flowering plants. SI is also useful to plant breeders because it provides an efficient pollination control system for the commercial production of hybrid seeds (Gibbs 1986; de Nettancourt 2001). In addition, SI is probably the best defined cellular communication system in the plant kingdom (Dickinson 1990), and it has been identified in numerous plant species (Williams et al. 1994).

The *Verbenaceae* family comprises approximately 90 genera and nearly 2,000 species of trees, shrubs and herbs. Majority of them are tropical plants of economic importance such as Lemon verbena (*Aloysia triphylla*), beautyberry (*Callicarpa* spp.), gmelina (*Gmelina arborea*), teak (*Tectonia grandis*), verbena (*Verbena* spp.), and vitex (*Vitex* spp.). Some studies on SI have been reported previously in woody species such as *G. arborea* (Bolstad and Bawa 1982), *T. grandis* (Tangmitcharoen and Owens 1997a,b) and *Vitex cooperi* (Bawa et al. 1985) of the *Verbenaceae* family.

In these studies, several inbreeding inhibition

mechanisms of woody species of the *Verbenaceae* were described as late-acting SI (Seavay and Bawa 1986). The late-acting SI system, which is characterized by either arrest of self-incompatible pollen tube within the ovary or abortion during self-pollinated zygote development, has been described in a number of monocotyledonous and dicotyledonous herbaceous species (Sage et al. 1994).

We have characterized an SI mutant from the self-compatible cultivar *Verbena*×*hybrida* ‘Coral Pink’ of the Temari® series (Suntory Flowers, Tokyo, Japan) that was isolated after mutation induction using heavy-ion beam irradiation (Kanaya et al. 2008). In this study, since the inhibition of the self-pollen tube growth of the SI-*Verbena* mutant occurred at the lower region of the style or the upper region of the ovary, we classified the self-pollen-rejection system of this mutant as a late-acting SI system similar to those of woody species of the *Verbenaceae* family (Bolstad and Bawa 1982; Bawa et al. 1985; Tangmitcharoen and Owens 1997a, b).

There is no report on the characterization of SI mechanisms in wild species of *Verbena*. Also, it is

unknown whether the SI mechanism(s) of mutants are identical to that of the wild species. In the present study, we have investigated the seed production and behavior of self-pollen tube growth in wild *Verbena* species in order to characterize their SI mechanism.

An SI mutant of verbena cultivar *Verbena*×*hybrida* ‘Temari Coral Pink’ (SC) (Kanaya et al. 2008) and wild-type VP, which is one of the original species of *Verbena*×*hybrida* (Griffiths 1994; Maberley 1997), were used. Plants were cultivated at 25°C under a 16/8 h (light/dark) photoperiod in a growth chamber.

To determine whether VP exhibits SI, artificial self- as well as cross-pollinations using VP and SC were conducted. Ten clusters from five plants (two clusters per plant) for each of VP and SC were used, and opened flowers and small buds (below 2 mm) were removed from inflorescences before pollination tests. Corollas and anthers of mature buds within one day after opening were also removed. Whole inflorescences with emasculated flowers were washed with distilled water and covered with paper bags for one day. Fresh pollen grains harvested at anthesis were applied to pistils of emasculated flowers, and then inflorescences were covered with paper bags again. Seed production was observed within one month after pollination.

Pollen germination on stigmas and pollen-tube growth in pistils after self-pollinations were analyzed to determine the type of self-pollen-rejection system. Pistils with ovaries from each line were collected at 4, 8 and 16 h after self-pollination, and were fixed in FAA solution (70% ethanol:glacial acetic acid:formaldehyde in a v/v/v ratio of 90 : 5 : 5) for more than 24 h at room temperature. After rinsing with distilled water, pistils were cleared in 1N KOH for one day at room temperature. They were then rinsed twice with water and stained for 2 h with 0.1% aniline blue solution prepared in 0.1 M K₃PO₄ (Singh 2003). Samples were squashed gently in a drop of 50% glycerin under a cover glass and observed under ultraviolet illumination using an Olympus IX70 light fluorescence microscope, and three pistils per sample time for each of VP and SC were observed.

Pollination tests of the wild species of *Verbena* were conducted between the SC mutant and VP to investigate the SI system. Seed production after hand pollination is shown in Table 1. In SC, open-pollination and self-pollination when conducted by hand at blooming period produced few seeds within one month after flowering. On the other hand, wild-type VP flowers produced no seeds after open-pollination and self-pollination. However, cross-pollination between SC and VP showed high percentage of seed setting (57.1% for SC×VP, 59.7% for VP×SC), which indicates that both SC and VP have fertile male and female gametophytes, and furthermore that SC and VP exhibit SI.

To characterize the post-pollination events of self-

Table 1. Seed production following pollination involving a *Verbena*×*hybrida* sterile mutant SC and wild-type *V. peruviana*.

	No. of florets producing seeds/ No. of florets pollinated (%)
<i>Verbena</i> × <i>hybrida</i> mutant (SC)	
Open-pollination	2/156 (1.3)
Artificial self-pollination	1/156 (0.6)
Artificial crossing	72/126 (57.1)
<i>V. peruviana</i>	
Open-pollination	0/120 (0)
Artificial self-pollination	0/113 (0)
Artificial crossing	80/136 (58.8)

Artificial pollinations were carried out at anthesis.

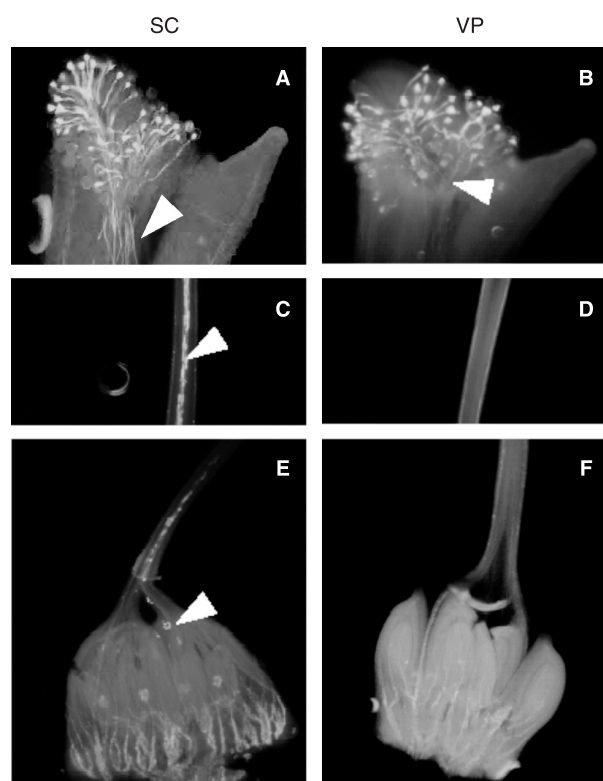


Figure 1. Fluorescence micrographs of SC and VP flowers after self-pollination. (A) SC, 4 h, stigma. (B) VP, 4 h, stigma. (C) SC, 8 h, style. (D) VP, 8 h, style. (E) SC, 16 h, ovary, (F) VP, 16 h, ovary. Pistils were stained with decolorized aniline blue to visualize pollen tube growth. Arrowheads show pollen tubes.

pollinated VP and SC florets, pollen germination on stigma and pollen tube growth in pistils were evaluated. In both self-pollinated SC and VP, numerous pollen tubes grew into pistils 4 h after self-pollination (Figure 1A, B). Pollen germination on self-pollinated stigma surface of VP was similar to that of SC. However, different behavior of pollen tube growth between SC and VP was observed 8 h after self-pollination. Although many pollen tubes were observed in the middle of the style of SC (Figure 1C), there were no pollen tubes in the VP style (Figure 1D). These results indicate that the pollen tube growth of self-pollinated VP is arrested at the upper region of the style. In contrast, almost all SC

pollen tubes reached at the point of lower part of the style or the upper regions of the ovary 16 h after pollination (Figure 1E, 1F). Ovules of both SC and VP remained unfertilized.

SI is a recognition system that reduces the frequency of seed set through the rejection of self-pollens in flowering plants. In the present study, we have demonstrated for the first time that a herbaceous plant of the *Verbenaceae* family, *V. peruviana* (VP), has a functional SI system.

The SI phenotype of VP is different to those of the *Verbenaceae* woody plants *G. arborea*, *T. grandis* and *Vitex cooperi*, and that of the verbena cultivar mutant SC. Although self-pollinated SC and woody plants can produce a few seeds (Hedegart 1973), no seed of VP was presently obtained by self-pollination. The different frequencies of self-seed production among teak, SC and VP may be caused by differences in their SI system. Most of the SC self-pollen tubes can reach regions near the ovary (Kanaya *et al.* 2008) and zygotes of self-pollinated teak aborted during seed development (Tangmitcharoen and Owens 1997a,b). Therefore, the SC and VP seed production after self-pollination may be the result of a very low fertilization rate and zygote development.

In most SI plants, self-incompatible pollens are selectively inhibited at a species-dependent specific stage before fertilization such as immediately after pollination on the surface of the stigma or during pollen tube growth in the style (Franklin-Tong and Franklin 2003; Hiscock and McInnis 2003). Inhibition of self-pollen tube growth in plant species with a well-described gametophytic SI generally occurs at the upper region of the style (Franklin-Tong and Franklin 2003), which coincides with the arrest of pollen tube growth VP. In species with gametophytic SI, pollens tend to be binucleate and the stigma is usually classified as wet based on the presence of fluid secretions on the stigma surface during the pollen receptive period (Shivanna 2003). Teak pollens (Siripatanadilox 1974) and SC pollens (data not shown) are binucleate. In addition, Heslop-Harrison and Shivanna (1977) classified the stigma of *Verbenaceae* plants as wet. These results may suggest that VP has a general homomorphic gametophytic SI system. Further detailed investigations of the SI mechanism of *Verbena* are needed.

The results of the present study indicate the different types of SI mechanisms in SC and VP. This may indicate that the SC induced by heavy-ion beam irradiation is not a revertant that regains the SI phenotype of its parental species. The life cycle of ornamental, herb verbenas is short compared with woody plant species and it blooms 4 to 5 months after sowing. We propose a set of SC and VP as a novel model plants for genetic analysis of the SI mechanism in the *Verbenaceae* family. To confirm

detailed mechanism of SI systems involved in these *Verbena* species more evident, further examination of the inhibition mechanism of self-pollen tube growth and genetic inheritance analyses are in progress.

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References

- Bawa KS, Perry DR, Beach JH (1985) Reproduction biology of tropical lowland rain forest trees. I. Sexual systems and incompatibility mechanisms. *Amer J Bot* 72: 331–345
- Bolstad PV, Bawa KS (1982) Self-incompatibility in *Gmelina arborea* L. (*Verbenaceae*). *Silvae Genetica* 31: 19–21
- de Nettancourt D (2001) *Incompatibility and incongruity in wild and cultivated plants*. Springer-Verlag, Berlin, Germany
- Dickinson HG (1990) Self-incompatibility in flowering plants. *Bioessays* 12: 155–161
- Franklin-Tong Noni (VE), Franklin FCH (2003) Gametophytic self-incompatibility inhibits pollen tube growth using different mechanisms. *Trends Plant Sci* 8: 606–613
- Gibbs PE (1986) Do homomorphic and heteromorphic self-incompatibility systems have the same sporophytic mechanism? *Plant Syst Evol* 154: 285–323
- Griffiths M (1994) *Index of garden plants*. The Macmillan Press, London, UK
- Hedegart T (1973) Pollination of teak (*Tectonia grandis* L.). *Silvae Genetica* 22: 124–128
- Heslop-Harrison Y, Shivanna KR (1977) The receptive surface of the angiosperm stigma. *Ann Bot* 41: 1233–1258
- Hiscock SJ, McInnis SM (2003) Pollen recognition and rejection during the sporophytic self-incompatibility response: *Brassica* and beyond. *Trends Plant Sci* 8: 598–605
- Kanaya T, Saito H, Hayashi Y, Fukunishi N, Ryuto H, Miyazaki K, Kusumi T, Abe T, Suzuki K (2008) Heavy-ion beam-induced sterile mutants of verbena (*Verbena* × *hybrida*) with an improved flowering habit. *Plant Biotechnol*: 91–96
- Mabberley DJ (1997) *The Plant-Book Second Edition*. Cambridge University Press, Cambridge, UK
- Sage TL, Bertin R, Williams EG (1994) Ovarian and other late-acting self-incompatibility. In: Williams EB, Knox RB, Clarke AE (eds) *Genetic control of self-incompatibility and reproductive development in flowering plants*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 116–140
- Seavey SR, Bawa KS (1986) Late-acting self-incompatibility in Angiosperms. *Bot Rev* 52: 195–219
- Shivanna KR (2003) *Pollen biology and biotechnology*. Science Publishers, Inc., NH, USA
- Singh RJ (2003) *Plant Cytogenetics Second Edition*, CRC Press LLC, New York, USA

Siripatanadilox S (1974) Development of teak (*Tectonia grandis* Linn f.). *Forest Research Bulletin* 31: Kasetsart Univ., Thailand.

Tangmitcharoen S, Owens JH (1997a) Floral biology, pollination, pistil receptivity, and pollen tube growth of teak (*Tectonia grandis* Linn. f.). *Ann Bot* 79: 227–241

Tangmitcharoen S, Owens JH (1997b) Pollen viability and pollen-

tube growth following control pollination and their relation to low fruit production in teak (*Tectonia grandis* Linn. f.). *Ann Bot* 80: 401–410

Williams EG, Clarke AE, Knox RB (1994) *Genetic control of self-incompatibility and reproductive development in flowering plants*, Kluwer Academic Publishers, Dordrecht, The Netherlands