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Studies on the Self-Incompatibility of *Petunia hybrida* in Excised-Style Culture. Differences in Self-Incompatibility Reaction Among Four Clones

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Summary. In *Petunia* clones with different S-alleles, self- or cross-pollinated excised-styles of 5, 10, 15, 20 or 25 mm were incubated on a standard agar medium for 24, 48 or 72 h. The length and number of protruding incompatible pollen tubes were compared with those of compatible ones. Throughout the experimental period, the length and number of incompatible pollen tubes of pollen from the S_1S_1-clone were always less than those of compatible ones. In pollen from S_2S_2-, S_3S_3- and S_2S_3-clones the incompatible pollen tube growth was barely arrested in 5 and 10 mm excised-styles during the first 24 h of incubation. However, inhibition of incompatible pollen tube growth was strengthened with the increase of both excised-style length and incubation period: this was clearly evident in 15 mm or longer excised-styles incubated for 48 h. Ratios of incompatible to compatible pollen tube length in excised-styles incubated for 72 h, were for S_3S_3 pollen tubes = 0.28, $S_1S_1 = 0.48$, $S_2S_3 = 0.50$, and $S_2S_2 = 0.60$, and ratios on tube numbers were $S_3S_3 = 0.01$, $S_1S_1 = 0.1$, $S_2S_2 = 0.21$, and $S_2S_3 = 0.21$. These results were in agreement with those of in vivo self-pollination. The incompatibility reaction seemed strongest in S_3S_3-, weaker in S_1S_1- and weakest in S_2S_2- and S_2S_3-clones, and therefore the intensity of S-allele expression would be $S_3 > S_1 > S_2$.

Key words: *Petunia hybrida* – Excised-style culture – Protruding pollen tubes – Self-incompatibility reaction – Allelic expression

Introduction

Self-incompatible clones in *Petunia hybrida* having different S-alleles as selected by Straub (1947) form no seed after self-pollination because pollen tube growth stops in the pistil. However, the intensity of the self-incompatibility reaction is weakened by certain environmental factors (Linskens 1975). This led us to initiate in vitro studies concerning the growth of pollen tubes in the style. A previous paper (Niimi 1982) showed that an improved excised-style culture technique might be helpful not only in studying the interaction between style and pollen tubes during tube growth, but also in detecting the number of tubes at a distance from the stigma.

The present studies were undertaken, firstly, to discover whether the self-incompatibility reaction occurring in vivo in *P. hybrida* could be demonstrated also in vitro with the above technique, and secondly, to find out if pollen from clones having different S-alleles would give incompatible and compatible pollen tubes having different behaviour in the in vitro technique.

Materials and Methods

Self-incompatible clones of *Petunia hybrida* Hort. Vilm. Andr., W43 (S_1S_1), Ka3 (S_2S_2). T2U (S_3S_3), and W166H (S_2S_3) (Linskens and Straub 1978) were used and the plants grew under the conditions described by Linskens (1977).

In vitro pollen germination was determined with a method described by Gilissen (1978). Pollen (about 4 mg) was incubated in 1 ml germination medium in a 25 ml Erlenmeyer flask. The germination medium contained 10% sucrose and 0.01% boric acid dissolved in distilled water. The flask was shaken (120 stroke/min) for 3 h at 25 °C. Pollen germination was measured with a projection microscope. Pollen germination was $S_1S_1 = 14\%$, $S_2S_2 = 18\%$, $S_3S_3 = 8\%$, and $S_2S_3 = 62\%$.

To find out if the S-alleles differed in self-incompatibility, three homozygous (S_1S_1, S_2S_2, and S_3S_3) and one heterozygous clones (S_2S_3) were pollinated in vivo. After flower buds of these clones were emasculated the day before anthesis, they were cross- or self-pollinated in the greenhouse. Number of capsules and mature seeds were determined 30 days after pollination.

Methods used in the present experiments were described in detail in a previous paper (Niimi 1982). The entire style, with pollinated stigma, was removed from the excised-pistil, cut with a sharp razor blade at 5, 10, 15, 20 or 25 mm from the stigma tip. For both cross- and self-combinations, three

excised-styles of each stylar length and of each incubation period were laid directly on 5 ml of a standard medium (Niimi 1982) in a petri dish. The petri dish with three excised-styles were kept in a dark room at 25 °C for 24, 48 or 72 h. Each experimental treatment was replicated 4 times.

After incubation, each excised-style with protruding pollen tubes was fixed and stained with a 0.08% lactophenol-cotton blue solution. The length of the ten longest protruding pollen tubes was measured as previously described (Niimi 1982), and after the bundle of pollen tubes was cut free of the excised-style, all protruding pollen tubes were counted under a dissecting microscope by teasing each pollen tube from the bundle with a forceps.

Results

Seed Setting After In Vivo Pollination

Differences in the incompatibility reaction expressed as number of capsules and mature seeds per capsule are shown in Table 1. All cross-pollinated pistils formed capsules. The S_1S_1- and S_3S_3-clones formed no seeds in self-combinations. In the S_2S_2- and S_2S_3-clone, however, the incompatibility reaction was not sufficiently strong to completely block seed formation after self-pollination. The incompatibility reaction of the homozygous combination $S_2S_2 \times S_2S_2$ was stronger than that of the heterozygous combination $S_2S_3 \times S_2S_3$ (Table 1).

Table 1. Number of capsules and mature seeds formed after in vivo pollination in different clones. ± indicates standard error of the mean

Combination	Pistils pollinated	Capsules formed	Seed number per capsule
Self-pollination			
♀ ♂			
$S_1S_1 \times S_1S_1$	40	0	0
$S_2S_2 \times S_2S_2$	40	12	52± 5
$S_3S_3 \times S_3S_3$	40	0	0
$S_2S_3 \times S_2S_3$	40	22	48± 8
Cross pollination			
♀ ♂			
$S_2S_2 \times S_1S_1$	40	40	125±14
$S_1S_1 \times S_2S_2$	40	40	253±14
$S_1S_1 \times S_3S_3$	40	40	189±15
$S_1S_1 \times S_2S_3$	40	40	209±13

Differences in Length of Protruding Compatible and Incompatible Pollen Tubes

Growth pattern of pollen tubes was different among the four clones (Fig. 1). The length of pollen tubes protruding from the excised-styles in the compatible combination ($S_2S_2 \times S_1S_1$) was always longer than in

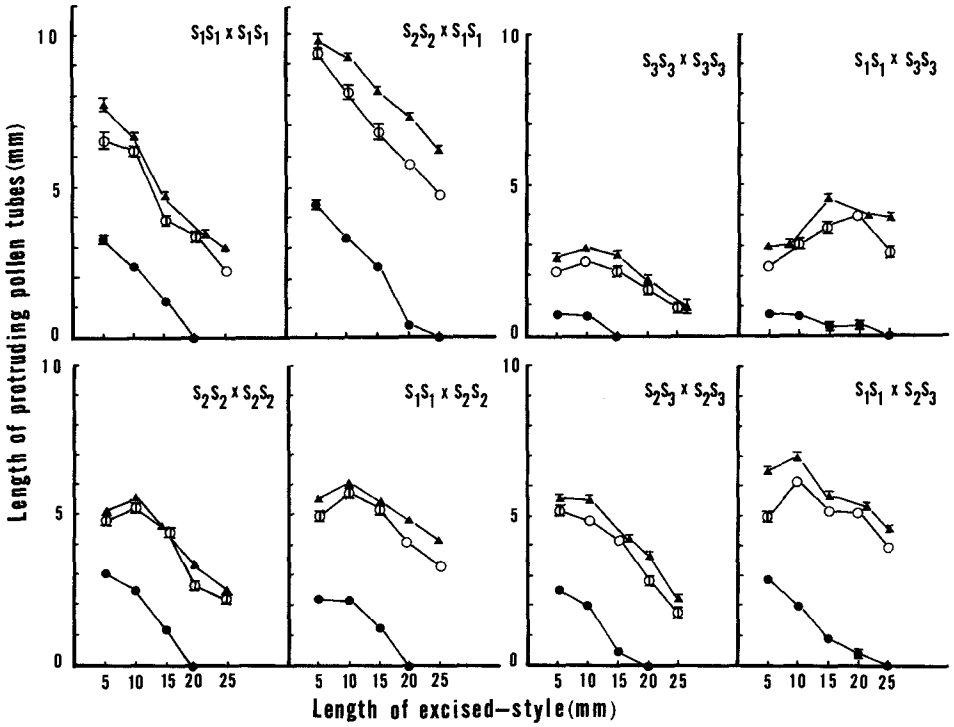
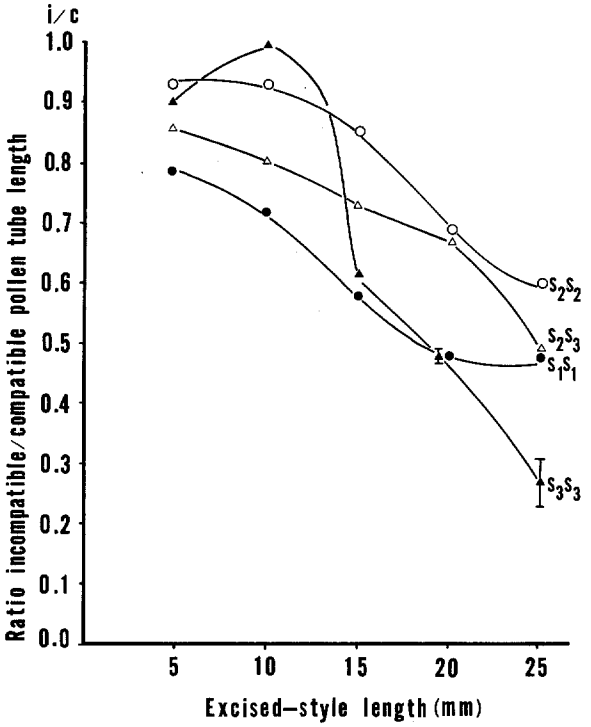


Fig. 1. The length of pollen tubes protruding from the cut end of excised-styles of different lengths after incubation for 24 (●), 48 (○) or 72 h (▲). Vertical bars indicate standard error (SE) of the mean where the SE is not too small



the incompatible one ($S_1S_1 \times S_1S_1$) throughout the experimental period, irrespective of differing lengths of excised-styles. When pollen grains of the S_2S_2- or S_3S_3-clones were used in cross- and self-combinations, however, the lengths of protruding incompatible pollen tubes from 5 and 10 mm excised-styles were almost equivalent to those of compatible pollen tubes. The inhibition of the incompatible pollen tube growth became visible for the first time when excised-styles of greater than 10 mm were cultured for 48 h or more. A similar situation was found in S_2S_3-clone, but there seemed to be a small difference in length between the compatible and incompatible pollen tubes in 5 mm excised-style throughout the experimental periods.

The inhibition of incompatible pollen tube growth in each clone was strengthened with the increase of

Fig. 2. The ratios of incompatible to compatible pollen tube length at 72 hr as influenced by various lengths of excised-style and clone. Vertical bars indicate standard error (SE) of the mean where the SE is not too small

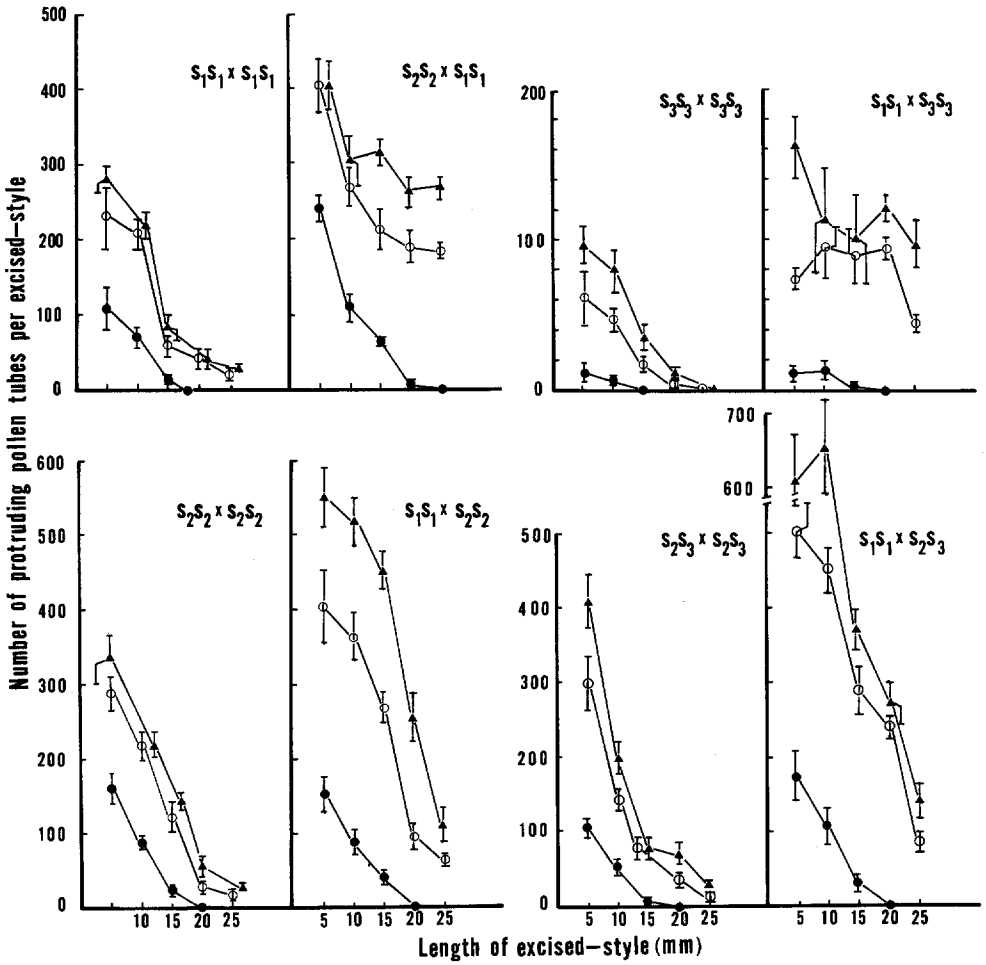


Fig. 3. The number of pollen tubes protruding from the cut end of excised-styles of different lengths after incubation for 24 (●), 48 (○) or 72 h (▲). Vertical bars indicate standard error (SE) of the mean where the SE is not too small

excised-style length. The rate of inhibition was shown by ratios of incompatible to compatible pollen tube lengths (Fig. 2). When excised-styles were incubated for 72 h, the ratios of pollen tube lengths for the three clones S_1S_1, S_2S_2, and S_2S_3 gradually decreased with the increase of length of excised-styles, except for the pollen of the S_3S_3-clone in which the ratios declined sharply in excised-styles of greater than 10 mm. The ratios obtained in the 25 mm excised-styles were $S_3S_3 = 0.28$, $S_1S_1 = 0.48$, $S_2S_3 = 0.50$, and $S_2S_2 = 0.62$.

Changes in Numbers of Protruding Compatible and Incompatible Pollen Tubes

In each clone the number of compatible and incompatible pollen tubes increased more rapidly between 24 h and 48 h (Fig. 3). The number of compatible pollen tubes from each length of excised style increased throughout the experimental period, but the number of incompatible ones increased to 48 h and then barely increased. For the pollen of S_1S_1- and S_2S_3-clones, the number of compatible pollen tubes in $S_2S_2 \times S_1S_1$ and $S_1S_1 \times S_2S_3$ was always higher than that of the respective incompatible pollen tubes throughout the experimental period. However, when the S_2S_2- or S_3S_3-clone was used as a pollen donor in self- and cross-combinations ($S_1S_1 \times S_2S_2$ or $S_1S_1 \times S_3S_3$), the number of incompatible pollen tubes was equivalent to or greater than the number of compatible pollen tubes at the first 24 h, and clear-cut inhibition of the incompatible pollen tubes was observed for the first time at 48 h. Hence a distinct reduction in number of incompatible pollen tubes was observed in each length of excised-style at 48 h and 72 h, with one exception. For the 5 mm excised-style in the combinations using pollen of the S_3S_3-clone, the number of incompatible pollen tubes at 48 h was almost the same as in the compatible combination ($S_1S_1 \times S_3S_3$).

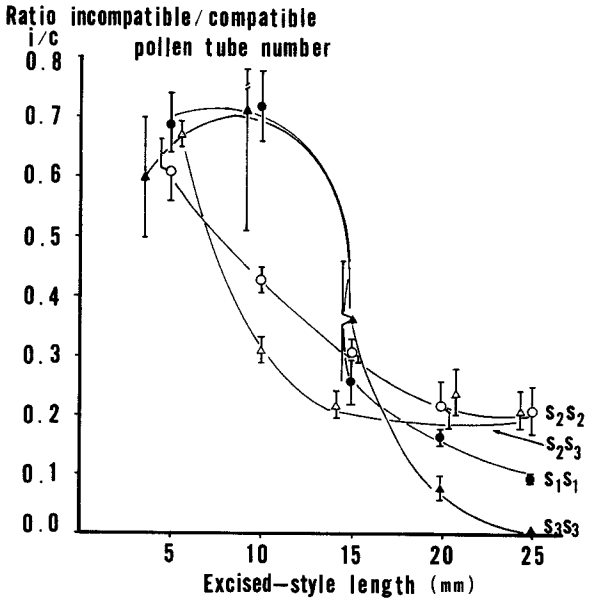


Fig. 4. The ratios of incompatible to compatible pollen tube number at 72 hr as influenced by various lengths of excised-style and clone. Vertical bars indicate standard error (SE) of the mean

Figure 4 shows ratios of incompatible to compatible pollen tube numbers. The ratios of S_1S_1- and S_3S_3-clones declined rapidly in the excised-styles of greater than 10 mm. The ratios in the S_2S_3-clone decreased sharply in the 10 mm excised-style and then became almost constant in the 15 mm or longer excised-styles. The ratios for the pollen of the S_2S_2-clone gradually decreased with the increase of excised-style lengths. The ratios obtained in the 25 mm excised-styles were $S_3S_3 = 0.01$, $S_1S_1 = 0.1$, $S_2S_2 = 0.21$, and $S_2S_3 = 0.21$.

Discussion

The results (Figs. 1 to 4) show that the entire style can be considered an incompatibility barrier since the behavior of the incompatible pollen in respect of its length and number was gradually arrested in the style and its growth was not inhibited in a distinct stylar zone. The longer the incompatible tubes are in contact with the stylar tissue, the more the growth of the tubes is arrested. The results show, furthermore, that the in vitro technique of excised-style culture used in the present study is able to demonstrate the incompatibility reaction. This is in agreement with earlier suggestions (Straub 1946; Brewbaker and Majumder 1961; Higuchi 1969) that the technique of semi-vitro culture is useful for investigation of the self-incompatibility reaction of *Petunia*.

When each length of excised-style was cultured for 48 h or more, differences in behavior in respect of pollen tube length and number became clear, with a few exceptions. The differences were more obviously seen in tube numbers than in tube lengths. These results mean that in excised-style culture the number of protruding pollen tubes is a somewhat better criterion for determining the incompatibility reaction in vitro than the length of the tubes although as a criterion for detecting the behavior of incompatible pollen tubes in the style in vivo, the length of pollen tubes has been generally used in a U.V.-light microscope.

Differences in the growth of incompatible pollen tubes among the four clones is shown in Figs. 1 and 3. Distinct differences in numbers between compatible and incompatible pollen tubes were observed in each length of excised-style of S_1S_1-clone throughout the experimental period, but the differences between com-

patible and incompatible pollen tube numbers in the other three clones remained small at 5 and 10 mm excised-styles. As other representative differences in the behavior of incompatible pollen tubes among four clones, when the longest excised-styles (25 mm) were incubated for 48 h and 72 h, incompatible pollen tubes in numbers greater than 20 emerged in S_1S_1-, S_2S_2-, and S_2S_3-clones (Fig. 3). Figures 2 and 4 also show that the inhibition of incompatible pollen tube growth took place at different zones of the style with different intensity among four clones. These results indicate that by means of the excised-style culture technique it is possible not only to detect the behavior of incompatible pollen tubes in the style, but also to obtain information on S-alleles.

When the number and length of protruding pollen tubes (Figs. 1 and 3) and the incompatibility reaction in vivo as expressed by the number of capsules (Table 1) are compared, a correspondence between them is found, indicating that the in vitro technique of excised-style culture is able not only to demonstrate the incompatibility reaction, but also to predict the strength of S-alleles. Therefore, it may be concluded that the intensity of incompatibility reaction is strongest in S_3S_3, weaker in S_1S_1, and weakest in S_2S_2 and S_2S_3, and therefore the strength of S-allele expression is $S_3 > S_1 > S_2$.

Another question still remains. The pollen quality expressed as the number of protruding compatible pollen tubes (Fig. 3) and pollen germination (see Materials and Methods) is best in $S_2S_3 > S_2S_2 > S_1S_1 > S_3S_3$, and in contrast, the intensity of self-incompatibility reaction is strongest in $S_3S_3 > S_1S_1 > S_2S_3 \geqq S_2S_2$. This inverse suggests that there are some relations between the intensity of self-incompatibility reaction and the pollen quality in respect of pollen tube length and numbers. More work will be needed to answer the present question.

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