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Pollen tube growth in *Lilium longiflorum* following different pollination techniques and flower manipulations

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SUMMARY

After cut-style pollination of *Lilium longiflorum* Thunb. using compatible pollen, the percentage of ovules with a pollen tube in the micropyle was very low when compared with pollination at the stigma. Pollen tube growth in the ovary, as observed with scanning electron microscopy, did not show any differences between these two pollination methods until the arrival of the pollen tube at the inner integument. After cut-style pollination, the majority of the pollen tubes either grew past the inner integument and ignored it, or grew over but not into the micropyle. Grafting a stigma just above the ovary did not increase the penetration percentage. Possible activation of the ovary, induced by pollination or pollen tube growth in the style or even in the ovary itself, preceding intrastylar or placental pollination did not result in an increase of the penetration percentage. However, the percentage of ovule penetration after cut-style pollination did increase when a longer part of the style was left at the ovary. The basis of the interaction between pollen tube and pistil, which led to ovule penetration, was built up during pollen tube growth through the style.

Key-words: cut-style pollination, *Lilium longiflorum*, ovule, placental pollination, pollen tube growth, style.

INTRODUCTION

Interspecific crosses within the genus *Lilium* are valuable additions to breeding programmes. But the pollen tubes formed after interspecific pollination at the stigma will either cease their growth when they reach the stylar canal, or grow further down without reaching the ovules (Ascher & Peloquin 1968). Reciprocal differences in the absolute and relative length of the pollen tubes in the style were found (Asher & Drewlow 1971). The length of the pollen tubes inhibited at the base of the stigma can be increased by injecting stigmatic exudate of *L. longiflurum* into its style before pollination with pollen of another species, but growth will still stop in the style (Ascher & Drewlow 1975). Interspecific crosses were successfully made in a number of combinations, using cut-style pollination in which the pollen grains were applied at the

cut surface resulting from largely removing the style (Asano & Myodo 1977; Van Tuyl *et al.* 1986, 1991). Hybrid embryos could be obtained in small numbers, but their development was retarded when compared with intraspecific embryos obtained after pollination at the stigma. The endosperm remained liquid or was almost absent, and transfer to tissue culture was necessary to continue the growth of the embryos (Myodo & Asano 1977).

The low number of embryos obtained is partly the result of poor ovule penetration by the pollen tube, as observed after interspecific cut-style pollination in lily. This may be caused by an interspecific barrier in front of the micropyle or the absence of ovule enhancement due to cut-style pollination (Van Roggen *et al.* 1988). When cut-style pollination was carried out with intraspecific compatible pollen the percentage of ovules with a pollen tube in the micropyle was also very low (Janson 1988), indicating inability of the pollen tubes to penetrate or the absence of ovule enhancement. This study reports on intraspecific pollen tube growth after cut-style pollination, and on manipulations carried out with flowers before or during compatible pollination in order to gain more insight into the combined action of both the pollen tube and the ovary.

MATERIALS AND METHODS

Plants of *Lilium longiflorum* Thunb. were grown in pots in a glasshouse with a night-time temperature not lower than 15°C and a day-time temperature sometimes as high as 35°C in the summer. Additional illumination was given in winter. Compatible pollinations were carried out with 'Gelria' as the female parent and 'White American' as the male parent. Flower buds were emasculated 1-2 days before anthesis and the stigma was covered with an aluminium foil cap. When using cut-style pollination, the style with the stigma was cut off and stigmatic exudate was applied to the cut surface, followed by fresh pollen grains. The pollinated surface was subsequently covered by an aluminium foil cap to prevent cross-pollination and to avoid evaporation. The length of the style left at the ovary is either expressed in mm (the term just above the ovary implies 3 mm of style left at the ovary) or as a percentage of the complete length of the style. In the latter case, no part of the style or the stigma was removed at 100%—which is, in fact, stigmatic pollination—whereas 95% results in removal of mainly the stigma. Intrastylar pollination was carried out through a slit made in the style just above the ovary, after which stigmatic exudate was applied followed by the pollen grains. Cut-style pollination just above the ovary was carried out at 4 days after anthesis (DAA). Pollination half-way down the style was carried out at 3 DAA; stigmatic pollination at 2 DAA. In this way the ovules, when reached by the pollen tubes, would be at a comparable stage of maturation. After pollination the flowers were either left on the plant or put in a vase filled with water and transferred to a growth chamber with 16 h of illumination at 24°C.

Pollen tube growth in the style and ovary were monitored using a binocular microscope. After dissecting the pistil the pollen tubes were stained with water diluted cotton blue (Asano 1980). The length of the longest pollen tube in each of the two longitudinal stylar halves was measured and the two figures were averaged. Pollen tube growth in the ovary was studied using scanning electron microscopy (SEM). Therefore, the material from the glasshouse was fixed for 4 h in 5% glutardialdehyde in 0·1 M phosphate buffer, pH 7·2, supplemented with 0·25 M sucrose. This was followed by several rinses in phosphate buffer with sucrose and postfixation for 6 h in 1% osmium tetroxide in demineralized water. Rinsing and dehydration in a graded ethanol series

was followed by critical point drying over CO_2, applying the specimen to a preparation holder and sputtering it with palladium/gold. The material was observed with a JEOL JSM-5200 SEM.

Pollen tube length in the micropyle was determined after destaining ovules while still attached to the placenta in a mixture of water, glycerol and lactic acid (1:2:1) for 1 h at 80°C. Then the material was stained for 2 min at 80°C in a solution of 1% aniline blue in the same mixture. Subsequently, the material was destained briefly at 80°C in the destaining solution (modification of Gerlach 1977). The statistical method used was an analysis of variance (ANOVA).

To determine sperm cell formation the pollen tubes were dissected from the style or the ovary of 35 pistils and stained with an aqueous solution of 50 $\mu g\ l^{-1}$ DAPI (4'6-diamidino-2-phenylindole.2HCl) at pH 4·0.

In experiments using grafted styles the junction was fastened with a drinking straw, the ends of which were filled with vaseline after reconstruction to avoid evaporation. Junctions were also secured with 'cling-film'. The flowers were incubated in a growth chamber.

To achieve ovule enhancement, pollen tubes were blocked in the style or in the ovary by injecting Mowilith (Hoechst) or cyano-acrylate glue at the desired place. The obstruction was always checked at the end of the experiment, using diluted cotton blue as described above to localize the pollen tubes. Another way of activating the ovary in combination with cut-style pollination was to allow pollen tube growth into the style and then cut it beyond the pollen tube tips, after which cut-style pollination could be carried out.

To assess any stimulation by fertilized ovules of ovules in another cavity, which might induce pollen tube penetration of pollen grains applied to the latter cavity, flowers were placentally pollinated. Pollen tubes originating from the stigma were blocked in the top of one of the three cavities by Mowilith or cyano-acrylate glue, allowing access to the two other cavities. The cavity below the barrier, which was deprived of pollen tubes, was opened and placental pollination was carried out. One row of ovules was removed resulting in exposure of the other row of ovules together with the placenta, to which pollen grains (with or without stigmatic exudate) were applied. Unpollinated flowers were used as a control. The flowers were incubated in the growth chamber.

RESULTS

After compatible pollination at the stigma, on average 70–85% of the ovules (Table 1) were penetrated by a pollen tube (Fig. 1a). The percentage of ovules with a pollen tube in the micropyle was lower, however, after cut-style pollination just above the ovary (Fig. 1b), with a highest average percentage of 9 (Table 1, $n=10$, $\sigma_{n-1}=8{\cdot}5$). After germination at the cut surface the pollen tubes grew through the few millimetres of style and entered the ovary. They therefore grew over a zone containing secretory cells, where the pollen tubes spread out when growing towards the placentas of the three cavities of the ovary (Fig. 1c). If a pollen tube tended to grow from the secretory zone it was observed to bend back (Fig. 1d). At the placenta an exudate was secreted (Janson 1992) by secretory cells which cover the surface, even into the slit between the two rows of ovules. Thus, these cells had contact with each other and had a more pointed than spherical shape (Fig. 1e). From the central bundle formed in each of the three cavities, the pollen tubes bent and grew inbetween the ovules towards the micropylar side

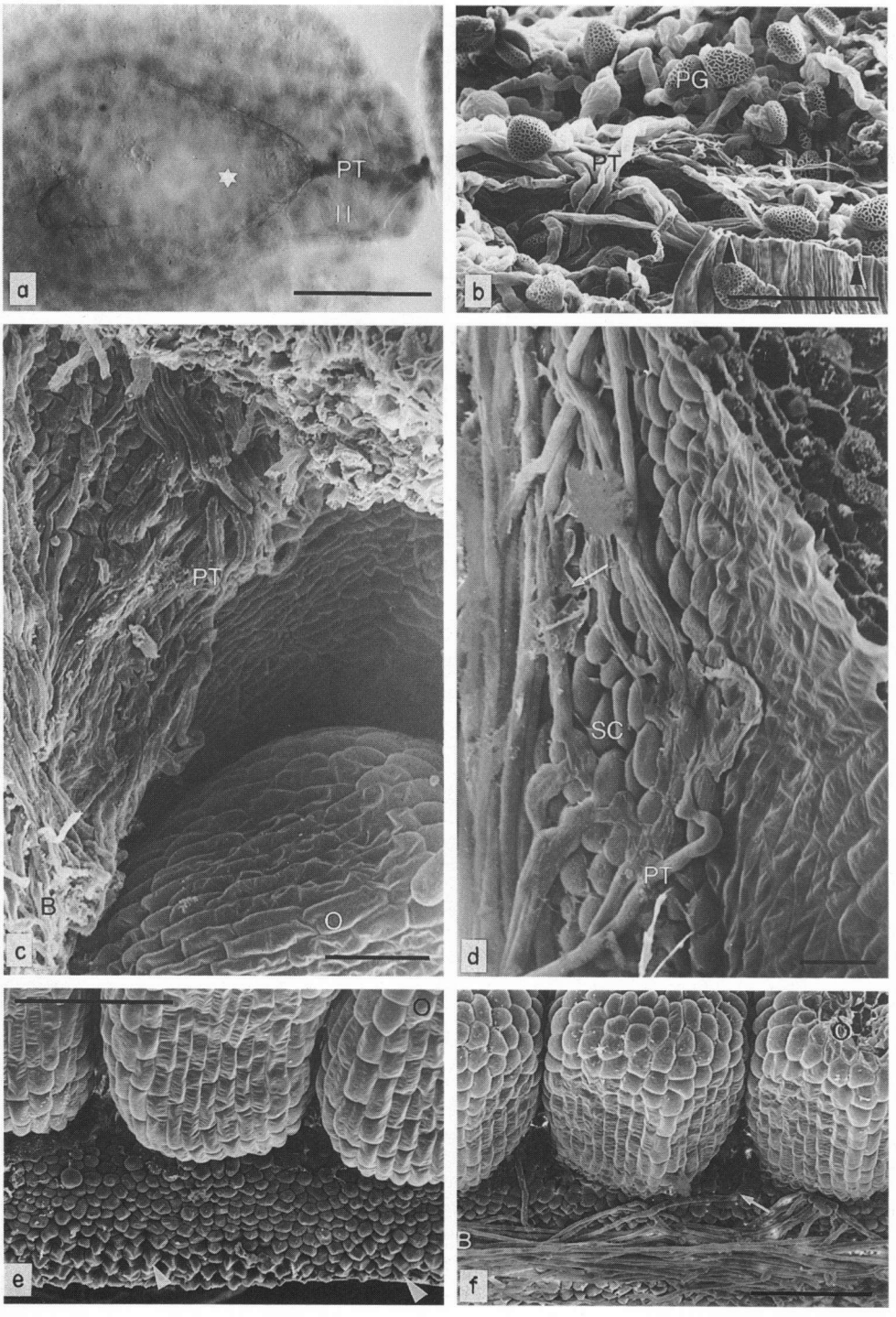
Table 1. A summary of different experiments (I–VI). Average percentages of ovules with a pollen tube in the micropyle (per flower two rows of ovules were counted, values of different flowers were averaged) after compatible pollination at different places in pistils of *L. longiflorum*

Experiment		I	II	III	IV	V	VI
Pollinated at stigma*		78	73	70			
Style damaged			85				
Cut-style†							
Unactivated	3 mm	9	1		2	5	
	15 mm	11					
Activated	1 day	4			3	5	
	2 days	5	10		3		
Intrastylar pollination‡			6	2			4
Intrastylar + activation			8	4			2
Intrastylar after stigmatic							1
Total no. of flowers		50	24	20	10	12	16
Total no. of analysed ovules		4734	4591	3760	1113	1522	1652

*Pollination at the stigma of an intact pistil and of a pistil of which the style was damaged by some longitudinal cuts which were made just before pollination.
†Cut-style pollination just above (3 mm) and 15 mm above the ovary. The ovary was activated by pollinating the stigma. After 1–2 days the style with the pollen tubes was removed after which cut-style pollination at 3 mm was carried out.
‡Intrastylar pollination just above the ovary. Pollen grains were applied through a slit in the style. In case of intrastylar + activation the pollen tubes from pollen applied at the stigma were blocked in the style by injecting a drop of Mowilith or glue just above the basis of the style beyond which intrastylar pollination was carried out. + indicates a simultaneous pollination of stigma and style, 'after' indicates intrastylar pollination carried out 2 days later than pollination of the stigma.

(Fig. 1f). After passing the ovules they bent again to grow over the placenta, and along the funiculi and the inner integuments (Fig. 2a) of the anatropous ovules. On this side the pericarp was only partly covered with secretory cells. In a later stage, when more exudate had been produced (Janson 1992), the pollen tubes on the micropylar side bent farther away from the exudate secreting placenta, but they were still in contact with the other pollen tubes (Fig. 2b). On the micropylar side the pollen tubes grew both towards the base and towards the top of the ovary and some even passed a few ovules without reacting to the micropyles (Fig. 2c). To reach the micropyle, the pollen tube had to leave

Fig. 1. (a) A pollen tube present in the micropyle, as observed with light microscopy 9 days after pollination at the stigma. Nucellus with embryo sac (*). Space bar: 50 μm. (b) Germinated pollen grains at the cut surface (arrowheads) after cut-style pollination, as observed by SEM. The stigmatic exudate which was applied before pollination was washed away during the preparation procedure. Five days after cut-style pollination (DACP), space bar: 100 μm. (c) A SEM micrograph of pollen tubes growing from the style (upper side of the picture), into one of the three cavities, spreading out but form a central bundle just before the first ovule. The bundle continues inbetween the two rows of ovules in one cavity. 3 DACP, space bar 50 μm. (d) While entering the ovary (the style is at the top and the base of the flower is beyond the bottom of the picture) pollen tube growth is restricted to the area with the secretory cells and return to this zone is observed if a pollen tube happens to leave it. Note some exudate covering a pollen tube and secretory cells (arrow). SEM, 1 DACP, space bar: 10 μm. (e) At the place where the two sides of the placenta are firmly pressed together, the secretory cells are more pointed (arrowheads) than spherical, as observed at places closer to the ovules. SEM, 7 days after anthesis, space bar: 50 μm. (f) One day after cut-style pollination a cental bundle is formed at the placenta in the ovarian cavity and tubes bend (arrow) to grow inbetween the ovules towards the micropylar side. A SEM observation, space bar: 50 μm. B, central pollen-tube bundle; II, inner integument; O, ovule; PG, pollen grain; PT, pollen tube; SC, secretory cells.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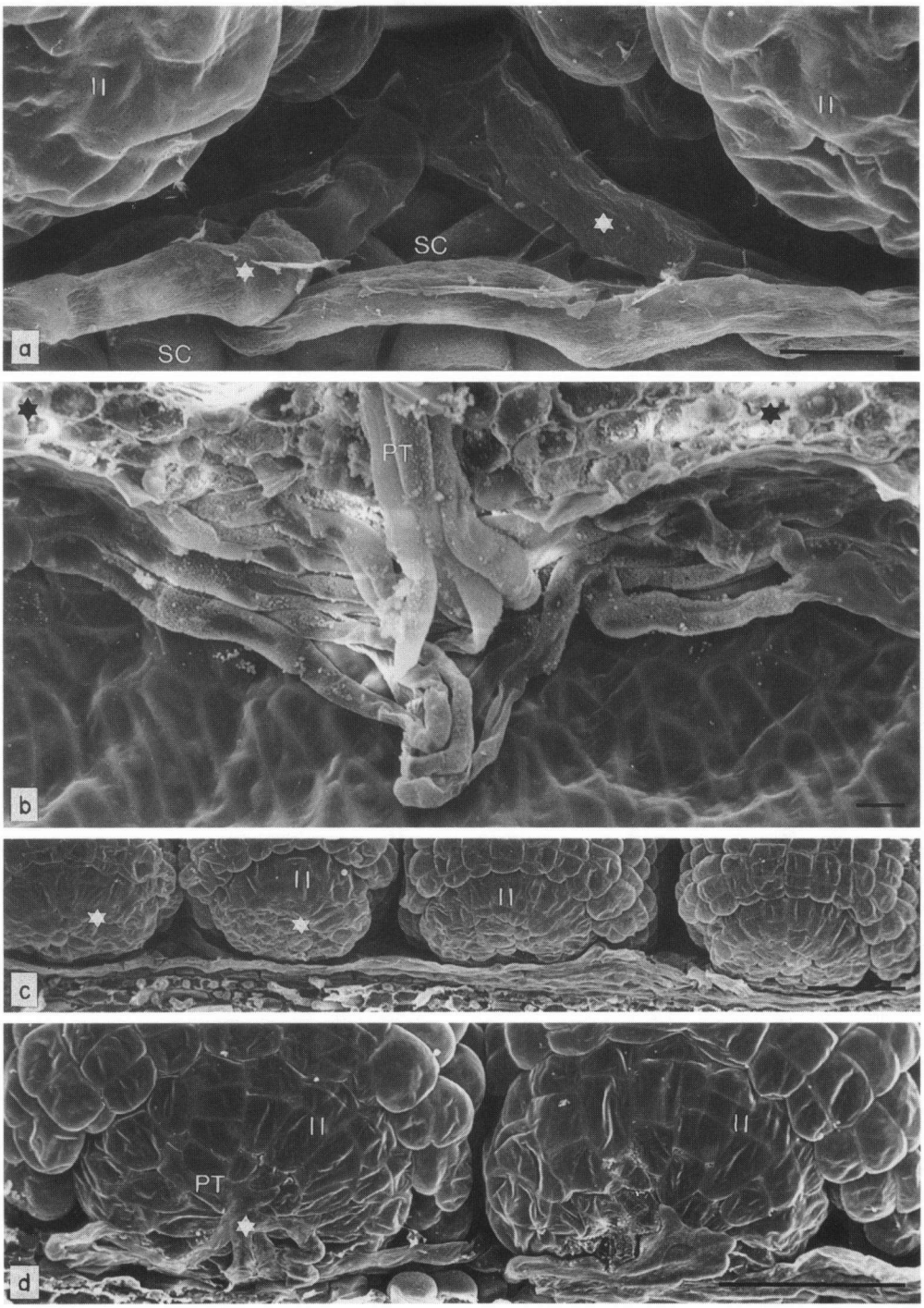
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the placenta and grow inbetween the inner integument andthe ovary wall. Frequently, pollen tubes were observed to grow over the inner integument, thereby flattened and in close contact with the underlying cells (Fig. 2d). Penetration could only be confirmed by the staining procedure used in Fig. 1a, because part of the pollen tubes in close contact with the inner integument did not enter the micropyle and stopped their growth. Pollen tubes regularly grew towards the micropyle without penetrating and continued their growth by returning to the small bundle formed on the micropylar side of the ovules. Occasionally, the pollen tubes returned from the micropylar side of the ovules to the central pollen tube bundle. Pollen tube growth inbetween the outer integument and the pericarp did not occur.

Manipulation of the style

Sperm cells were formed both after cross-pollination at the stigma and after cut-style pollination. This not only occurred when cut-style pollination was carried out just above the ovary, but also when the style was cut off at 25, 50 or 75% of its length. Sperm cell formation took place from 18 h after cut-style pollination, independent of the length of the style. After cross-pollinating the stigma, the first sperm cell formation was observed about 2 h earlier compared with cut-style pollination at the lengths mentioned above. After 20 h of incubation the pollen tubes derived from stigmatic pollination were longer compared with the cut-style originating pollen tubes.

In cases of cut-style pollination, the percentage of ovules penetrated by a pollen tube was higher when the style was kept longer (Fig. 3). Also, the percentage of seeds with an embryo increased in the same way, although less drastically than the percentage of penetrated micropyles. Removing just the stigma and applying stigmatic exudate before pollination did not influence the percentage of penetrated ovules when compared with pollination of the intact pistil. Damaging the style by a longitudinal cut did not influence the penetration percentage (Table 1). In a vertically positioned pistil with the stigma downward, the pollen tubes still reached the ovules.

Grafting a stigma with a few mm of style just above the ovary and subsequently applying pollen, hardly raised the percentage of penetration when compared with cut-style pollination just above the ovary. When these few mm were raised to a total of 25% of the normal style, including the stigma, the percentage increased to 20. Cut-style pollination carried out leaving a quarter of the style at the ovary gave a micropyle penetration of 9% (average of five flowers each), which was lower but not significantly different from the 20% mentioned above, due to the large variation.

Fig. 2. (a) At the micropylar side of the placenta the pollen tubes bend again (*) to stay in the proximity of the secretory cells and grow along the inner integuments (II). The pericarp was broken away through the last non-secretive cells. SEM, 3 DACP, space bar: 10 μm. (b) Five days after pollination more pollen tubes have grown inbetween the ovules, which have broken away during preparation (*); the tubes bend to grow along the inner integuments in a later stage (compare with (a)), thereby leaving the zone with secretory cells. SEM, space bar 10 μm. (c) A SEM micrograph of pollen tubes growing along the inner integuments of several ovules at 3 DACP without responding to the micropyles (*). Space bar: 50 μm. (d) After cut-style pollination just above the ovary, intensive contact between the inner integument and the pollen tube can be observed. From this image it is impossible to state whether the pollen tube enters the micropyle. A pollen tube arriving later (*) also grows towards the micropyle but turns and grows back towards the bundle formed at the side of the inner integument. A SEM micrograph, 3 DACP, space bar: 50 μm. II, inner integument; PT, pollen tube; SC, secretory cells.

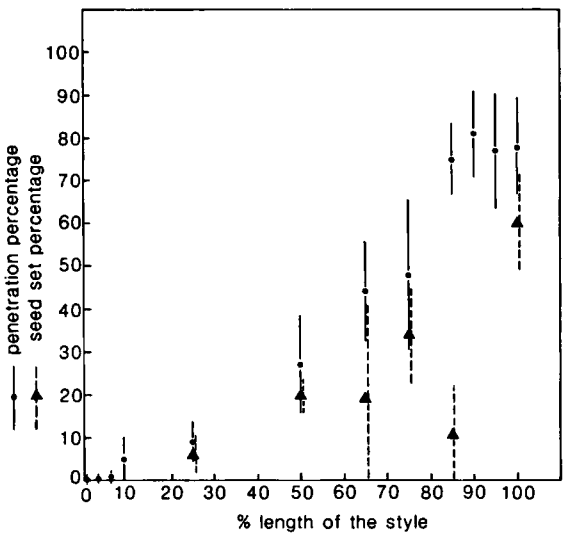

Fig. 3. The percentage of ovules with a pollen tube in the micropyle (●, experiment with 69 flowers, lines represent the standard deviation, σ_{n-1}) and the percentage of seeds with an embryo (▲, experiment with 30 flowers, dotted lines represent σ_{n-1}) achieved after cut-style pollination carried out with compatible pollen and at different style lengths, with a stigma present only at 100%.

Manipulation of the ovules

To overcome the absence of a pollination-signal in the cut-style method, ovaries were pretreated by pollination and pollen tube growth in the style; the style was then cut off before the pollen tubes had reached the ovary. Subsequently, cut-style pollination was carried out. It was also possible to block the pollen tubes at about 1 cm above the ovary by injecting a drop of Mowilith or glue, beyond which intrastylar pollination was carried out inbetween the site of the block and the ovary. This allowed the presence of the pollen tubes in the style during which intrastylar pollination was carried out. Neither treatments, with various incubation times, had any influence on the low percentage of ovule penetration by the pollen tubes after cut-style or intrastylar pollination (Table 1).

Fertilized ovules did not induce a higher penetration percentage in the placental pollinated cavity of the flowers. In untreated flowers only a few micropyles were penetrated, resulting in a penetration percentage of the ovules of 1–2% after 5–6 days incubation of the placenta-applied pollen. This percentage did not increase when the stigma was pollinated simultaneously with the placenta or when stigmatic pollination was carried out for up to 8 days before pollinating the placenta. The pollen tubes from the style did grow into the other cavities and here on average 68% of the ovules were penetrated. The barrier did block the pollen tubes in the placental pollinated cavity in 77 flowers, which was the total number used in these experiments.

DISCUSSION

Pollen tube pathway

The pathway of the pollen tubes in pistils is predetermined because of the stigma position or, in the case of incomplete pistils, the place of application of stigmatic

exudate, i.e. a source of nutrition and a medium for germination and growth. Both after pollination at the stigma (Janson 1992) and cut-style pollination the pollen tubes followed a pathway which was lined with secretory cells. The pathway followed seemed thus to depend on the surrounding nutrition and growth medium. The pattern of pollen tube growth in the ovary after cut-style pollination also did not seem to differ much from that observed after stigmatic pollination. In both cases the pollen tubes entering the ovary followed the zone with secretory cells and a central bundle was formed in each of the three cavities. Pollen tubes bent from this bundle towards the micropylar side of the ovules and made a second curve to grow along the inner integuments. Thereafter, a difference in the growth pattern of the pollen tubes occurred depending on the pollination method. In cases of cut-style pollination the micropyles were penetrated in low numbers; in cases of stigmatic pollination the majority of the ovules were penetrated. Because of the use of compatible instead of interspecific pollen a barrier was not expected.

In contrast with the pollen grains of the *Lilium* hybrid 'Enchantment' in which starch disappears in a late stage of pollen development (Willemse & Reznickova 1980) the pollen grains of *L. longiflorum* still contain a small amount of starch at the time of anthesis (J. Janson, unpublished). Pollen tubes at 2 days after stigmatic pollination (J. Janson, unpublished) and the degenerated plasma of the pollen tube and the synergid in the embryo sac (Janson 1992), however, contain a large amount of starch grains. During pollen tube growth in the style of *L. longiflorum*, reserves such as starch, proteins, short-chain polysaccharides and the few lipid droplets in the stylar cells are depleted (Dickinson *et al.* 1982). Pollen tubes take up substances from the stylar exudate (Labarca & Loewus 1973). In the pollen grain or tube, starch and probably other substances are formed after pollination and during growth through the style. From previous studies (Janson 1992) it is known that pollen tubes from stigmatic pollination entering the ovary continue to grow but not as fast as in the style. The exudate produced by the ovary is probably not as rich as that in the style. For this reason pollen tubes developed after cut-style pollination may have only a limited chance to take up growth substances, and subsequently neglect the micropyles which offer even less nutrients than the ovarian exudate.

Communication between the pollen tube and the pistil also may not have built up during the short growth after cut-style pollination. This results in circling of the pollen tubes over the inner integument without penetration and either general cessation of pollen tube growth or growth along more than one ovule without responding to the micropyles. Some pollen tubes probably penetrated the micropyle by chance while growing over the inner integument.

It is also possible that the ovules were receptive but that the pollen tubes formed after pollinations carried out just above or in the ovary were not prepared to penetrate the micropyles. In support of this idea was the observation that the longer the style was left prior to cut-style pollination, the higher the percentage of penetration. So, during growth through the style the pollen tube was prepared for penetration. When the style was left longer, the pollen tubes could take up more substances from the stylar exudate, which were used in the assembly of the pollen tube, but also contributed to the preparation of the pollen tube. There was no sudden rise in the penetration percentage with increased style length, which led to the conclusion that there was no critical length. The pollen tubes reacted solely to this 'ageing' with a large variation between the different pollen grains and tubes. When pollination was carried out just above the ovary,

at least a part of the pollen tubes was able to form sperm cells but, as with the presence of a pollen tube, this did not necessarily mean that the pollen tubes were ready for penetration.

Manipulations of the ovules

The attempts to activate the ovules by allowing pollen tubes to grow in the style or even in the ovary preceding cut-style, intrastylar or placental pollination, did not result in a higher percentage of ovules with a pollen tube in the micropyle.

There are several observations in the literature that point towards communication between different flower organs. In cotton, pollination or pollen tube growth induces processes in the ovules, e.g. synergid degeneration, which do not occur in unpollinated flowers (Jensen *et al.* 1983). In *L. longiflorum* the degeneration of a synergid is not observed before pollen tubes enter the embryo sac (Janson 1992). Pollinating the stigma of *Nicotiana alata*, removing the surface of the stigma after 10 min and pollinating the cut end, raises the seed set in comparison with just pollinating the cut surface (Bergamini-Mulcahy & Mulcahy 1988). After pollination, but before the arrival of the pollen tubes in the ovary, the carpels of *Petunia hybrida* are stimulated to synthesize proteins and an increase in rRNA is observed (Linskens 1973). Also in the ovary of *Petunia*, before the arrival of the pollen tubes, there is a difference between cross- and self-pollination in protein metabolism (Deurenberg 1976) and in influx of organic constituents (Linskens 1974). The ovary, in its turn, can influence pollen tube growth. The presence of the ovary of *P. hybrida* (Mulcahy & Mulcahy 1985, 1986b) and *Nicotiana alata* (Mulcahy & Mulcahy 1986a) influences the length reached by the pollen tubes in the style. However, in *N. sylvestris* and *N. tabacum* (Kandasamy & Kirsten 1987) and in *L. longiflorum* (Janson 1992) no such interaction between pollen tube growth and ovary was observed.

It seems that activation of the ovules in *L. longiflorum* did not trigger processes which induced pollen tube entrance into the micropyle, even when fertilized ovules were present in the same flower. After using the cut-style method in interspecific crosses between different lily species, a high percentage of seed pods abort very early. Using the mentor pollen technique, in which non-functional (by gamma irradiation) intraspecific compatible pollen is applied to the stigma together with the pollen of another species, fewer seed pods abort (Van Tuyl *et al.* 1988). This points to a post-fertilization role of intraspecific pollination of pollen tube growth. After different stigmatic treatments of *L. longiflorum*, different bioelectric potentials are registered in the style (Spanjers 1981), so communication through the style is possible. According to Amaki & Yamamoto (1988), compatible pollination of *L. longiflorum* influences the growth medium of the pollen tubes further down the base of the style and enhances pollen tube growth even before the tubes reach this area. In *N. alata*, compatible stigmatic pollination reduces the growth of mid-style applied pollen towards the stigma (Mulcahy & Mulcahy 1986a) and enhances growth towards the ovary (Bergamini-Mulcahy & Mulcahy 1988). In styles of the same species, Bredemeijer & Blaas (1975) observed increasing activity of a peroxidase as a result of pollination or penetration of the pollen tubes into the stigma rather than of pollen tube growth itself in the style.

Adjustments of the pollen tube pathway

Our experiments show that the stigma could be omitted prior to pollination as long as the pollen tubes can reach a certain length in the style. Prior to cut-style pollination at

different style lengths, stigmatic exudate was applied at the cut end as a germination medium and as extra fluid for hydration. This led to better and more repeatable pollen tube growth in the style. Without this exudate, pollen grains can still germinate in the style (Iwanami 1953). Gladding & Paxton (1975) pollinated *L. longiflorum* flowers after stigma removal without applying stigmatic exudate which resulted in a fluctuating seed set, as determined by the swelling of the ovary. This pod set was comparable with pollinating a cut-style 1 cm above the ovary. In our experiments there was a large difference in ovule penetration between the two lengths—just stigma removal and cut-style pollination carried out at 1 cm above the ovary—indicating a loss of interaction between the pollen tube and the micropyle.

Grafting a style with stigma so that the length of the style was reduced to 25% did not have a significantly better result than cut-style pollination at the same height, although there was a slight tendency towards improved penetration. If the stigma was present the surface for germination was larger, as was the amount of exudate. More pollen grains probably germinated and, in contrast with cut-style pollination where not all micropyles were covered by a pollen tube, extra invasion was possible.

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