***Pak. J. Bot.*, 49(6): 2419-2427, 2017.**

DYNAMIC DISTRIBUTION OF CALCIUM IN THE STIGMA AND STYLE OF LILY AFTER POLLINATION

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Abstract

Lilies are ornamental plants of global importance, yet cross incompatibility remains a major problem in breeding programmes. Calcium plays an essential role in the sexual reproduction of flowering plants. However, the regulatory mechanisms of calcium distribution during the fertilization process are unclear at present. An accurate assessment of calcium ions in pollinated styles can help to substantiate the role of calcium in pollination. In the current study, *Lilium pumilum* DC. and two Asiatic *Lilium* cultivars ('Pollyanna' and 'Prato') were used for crossing. Three cross combinations with different compatibilities were selected. The style of the female parent was collected before and after pollination at different stages. The amount of exudate in the stylar canal was estimated at the same time points. The style was cut into four different segments using an ultramicrotome and calcium ions were localized using potassium pyroantimonate. Calcium precipitates were examined and photographed using transmission electron microscopy. The results showed that the amount of exudate in the style increased prominently following pollination in compatible crosses ('Prato' × 'Pollyanna'). The amount of calcium in the stigma increased after pollination, particularly significantly so in incompatible crosses ('Prato' × *L. pumilum*). The amount of calcium in the canal cells and vascular bundles of the style was greater than in the adjacent parenchyma cells. In the style of compatible crosses, calcium was obviously transferred from the top to the base in the canal cells and vascular bundles, and a rising calcium gradient was ultimately formed. It is speculated that a rapid increase and high concentration of calcium in the stigma of the female parent could negatively impact pollen tube growth. Instead, abundant calcium in the style appears to promote the growth of the pollen tube towards the ovary.

Key words: *Lilium*, *Pumilum*, Calcium, Potassium pyroantimonate, Stigma, Style, Lily.

Introduction

Calcium is a necessary ion in plant growth and development (Hepler, 2005). The role of calcium in sexual reproduction has received particular attention in recent years, including its effect on pollen germination, pollen tube elongation, and induction of gametic fusion (Bednarska, 1989a). The distribution and concentration of calcium ions appears to be involved in important signalling, physiological, and regulatory functions during the fertilization process in flowering plants (Li *et al.*, 2007). Pollensare essential for pollination and fertilization (Erdogan, 2015). Brewbaker & Kwack (1963) discovered that when pollen grains germinated in small numbers *In vitro*, the germination rate was often lower than in larger scale pollen culture. This so called 'population effect' can be overcome by adding pollen or calcium to the medium, demonstrating that pollen absorbs exogenous calcium during germination (Bednarska, 1989a) and that calcium accumulates in the membranes of germinating pollen (Polito, 1983). Feijó *et al.* (1995) noted that pollen hydration maight result in turgidity of the plasma membrane at the site of the germination aperture and activated calcium channels on the membrane, thereby allowing the uptake of exogenous calcium.

The stigma is the landing site for pollen grains. Badnarska *et al.* (1989b) found that the receptive stigmatic surface of *Ruscus aculeatus* was rich in calcium. Furthermore, it was verified that the Ca^{2+} present in the stigma was taken up by the germinating pollen grains (Bednarska, 1991). Elleman & Dickinson (1999) revealed that calcium accumulated at the stigmatic surface following pollination in *Brassica oleracea*, indicating that the accumulation of calcium was caused by the contact of the pollen with the surface of the stigmatic papillae.

Pollen tubes penetrate the stigmatic surface and enter the transmitting tissue of the closed style following pollen germination. In species that possess a hollow style, however, the pollen tube travels from the stigma to the ovule on a transmitting tract epidermis. Loosely bound calcium stores were found in the transmitting tract of *Lilium longiflorum* (Zhao *et al.*, 2004). The pollen tube experiences polar elongation, for which calcium is required (Jaffe *et al.*, 1975; Nobiling & Reiss, 1987; Reiss & Herth, 1978; Reiss & Nobiling, 1986; Steer & Steer, 1989). The distribution of the calcium gradient in the style explains the polar elongation of the pollen tube, and was observed in a number of flowering plants including *Lactuca sativa* L. (Qiu *et al.*, 2009), *Plumbago zeylanica* L. (Ravikumar & Sudha, 2010), *Antirrhinum majus* (Mascarenhas & Machlis, 1964), and *Gladiolus* (Day *et al.*, 1971). In cultured pollen tubes of *L. longiflorum* (Obermeyer & Weisenseel, 1991; Reiss & Herth, 1980), *Tradescantia virginiana* (Picton & Steer, 1983), and *Nicotiana tabacum* (Tian & Russell, 1997), a suitable calcium concentration in the medium is required for tube growth. Pollen tube growth is inhibited by adding calcium channel blockers or antagonists into the medium (Malhó *et al.*, 2004). Significant progress has been made towards understanding the role of calcium in the growth of the pollen tube *In vitro* at the molecular level. The molecular mechanisms upstream and downstream of the calcium signalling system have been studied (Potocký *et al.*, 2012; Yu *et al.*, 2009; Zhu *et al.*, 2013). However, the distribution of calcium in the stigma as well as in the style before and after pollination has not yet been studied comprehensively.

Lilies constitute one of the most important ornamental crops in the world. The genus *Lilium* consists of around 100 species and more than 9,400 cultivars (van Tuyl *et al*., 2011; Yousuf *et al*., 2015). Crossing between cultivars or species from different taxonomic sections in the genus is generally difficult. Cross incompatibility is a major problem in lily breeding and can succeed only through the use of special techniques, such as cut-style pollination, style grafting, ovary culture, and ovary-slice culture (Van Tuyl *et al*., 1991). Previous studies revealed that calcium was related to the incompatibility response of self-incompatible plants (Franklin-Tong *et al*., 2002; Franklin-Tong *et al*., 1993) In this study, potassium pyroantimonate osmium was utilized to localize the calcium ions, with the aim of quantifying the distribution and amount of calcium in the stigma and style of the female parent before pollination and at different stages after pollination in lily. Three cross combinations were used to assess the relationship between calcium distribution and cross compatibility.

Materials and Methods

Plant material: *Lilium pumilum* DC. and two Asiatic *Lilium* cultivars (‘Pollyanna’ and ‘Prato’) were used for crossing. Bulbs of *L. pumilum* were collected in the field from Huzhu, Qinghai, China (E102°46′49.1″, N36°22′34.7″, altitude 2,647m) in 2012. The Asiatic cultivars were introduced from the Netherlands via Beijing Clover Floraculture CO, LTD. All plant materials were maintained vegetatively in a nursery by the Plateau Flower Research Centre of Qinghai University (Xining, China).

Pollination: Pollination was carried out in the nursery at the beginning of July 2014. Based on previous crossing experience, three cross combinations (‘Prato × Prato’, ‘Prato × Pollyanna’, and ‘Prato × *L. pumilum*’) were selected in this study. Pollination was carried out at anthesis between 9:00 and 10:00 am.

Sampling: Materials were collected at five different stages: two h before pollination (stage **b2**), six h after pollination (stage **a6**), 24 h after pollination (stage **a24**), 48 h after pollination (stage **a48**), and 72 h after pollination (stage **a72**). The top 2 mm of four segments (stigma, top of the style, middle of the style, and base of the style; Fig. 1) was sampled from the female parent ‘Prato’ at each stage. Segments were cut with an ultramicrotome (Leica EM UC7). Three replicates were prepared for each segment.

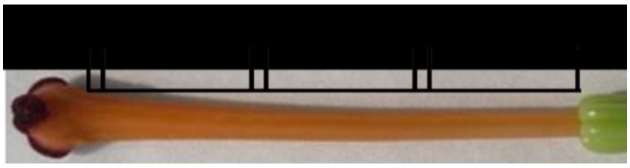

Fig. 1. Sections of the style. A, stigma; B, top segment, C, middle segment; D, basal segment.

Evaluation of exudates in the style: Exudates are produced on the surface of the stigma and in the stylar canal when the flower is mature. Style samples were stained with toluidine blue and cut into 3-μm segments. The exudate quantity in the stylar canal was estimated as the ratio of the proportion of the exudate to the transverse section area of the stylar canal.

Evaluation of calcium in the style: *Reagent preparation*: Firstly, four stock solutions were prepared. Stock A was 4% potassium pyroantimonate solution; stock B was 2% osimic acid solution; stock C was 25% glutaraldehyde solution; and stock D was 0.2 mol/L phosphate buffer (pH 7.4). The prefix solution contained 2.5% glutaraldehyde and 1% fresh potassium pyroantimonate at pH 7.4. It was made up as follows: A:C:D:ddH_2O= 25:1:50:24. The postfix solution contained 1% osimic acid and 1% fresh potassium pyroantimonate at pH7.4. It was made up as A:B:D:ddH_2O=1:1:2. The rinse solution contained 1% fresh potassium pyroantimonate at pH 7.4 and was made up as A:D:ddH_2O=1:2:1. For the control, double distilled H_2O was used to replace stock A.

Sample preparation for microscopy: Samples were prepared in four steps (Chaubal & Reger, 1990), including, fixation, dehydration, infiltration, and embedding (Table S1). Firstly, samples were prefixed for 4 h in prefix solution at 10°C-18°C immediately after division. The prefixed samples were washed with rinse solution four times (30 min each time). The samples were then post-fixed using postfix solution for 3 h at 4°C, and then rinsed thoroughly with the rinse solution. Following fixation, the samples were dehydrated in a graded acetone solution. Dehydration treatment was performed as follows: 30% acetone for 20 min, 50% acetone for 20 min, 70% acetone for 20 min, 80% acetone for 20 min, 90% acetone for 20 min, 95% acetone for 20 min, 100% acetone for 15 min, 100% acetone for 15 min, and 100% acetone for 15 min. Following this, the samples were infiltrated with acetone and resin solution. The ratio of acetone to resin and infiltration time for each step was 3:1 (4 h), 1:1 (12 h), 1:3 (12 h), 0:1 (24 h), and 0:1 (24 h). The samples were then embedded using Spurr low-viscosity embedding resin. The embedding temperature and processing time were 37°C (12 h), 45°C (12 h), and 60°C (24 h). The embedded blocks were kept in a vacuum dryer until use. The embedded stigma and style were sliced into 70 nm sections using an ultramicrotome and then dyed with 2% uranyl acetate. The sliced samples were examined and photographed using transmission electron microscopy (HITACHI HT7700).

The quantity of calcium precipitates was counted using the program Image-Pro Plus (IPP, MEDIA CYBERNETICS). As the stigma is a complex structure, only the calcium precipitates on the surface were studied. The structure of the style is clear and is uniform from the top to the base. In this study, the transverse section of the style was further separated into four parts: canal cells (CC), parenchyma cells adjacent to the canal cells (PACC), parenchyma cells adjacent to the vascular bundle (PAVB), and vascular bundle (VB; Fig. 2).

Table S1. Steps of sample preparation for microscopy.

	Step 1-fixation	Step 2-dehydration	Step 3-ınfiltration	Step 4-embedding
1.	Pre-fix 4 hours	30% acetone 20min,	3:1 (4h)	37°C (12h)
2.	Rinse 4 times (30min each time)	50% acetone 20min	1:1 (12h)	45°C (12h)
3.	Post-fix 3 hours	70% acetone 20min	1:3 (12h)	60°C (24h)
4.	Rinse thoroughly	80% acetone 20min	0:1 (24h)	
5.		90% acetone 20min	0:1 (24h)	
6.		95% acetone 20min		
7.		100% acetone 15min		
8.		100% acetone 15min		
9.		100% acetone 15min		

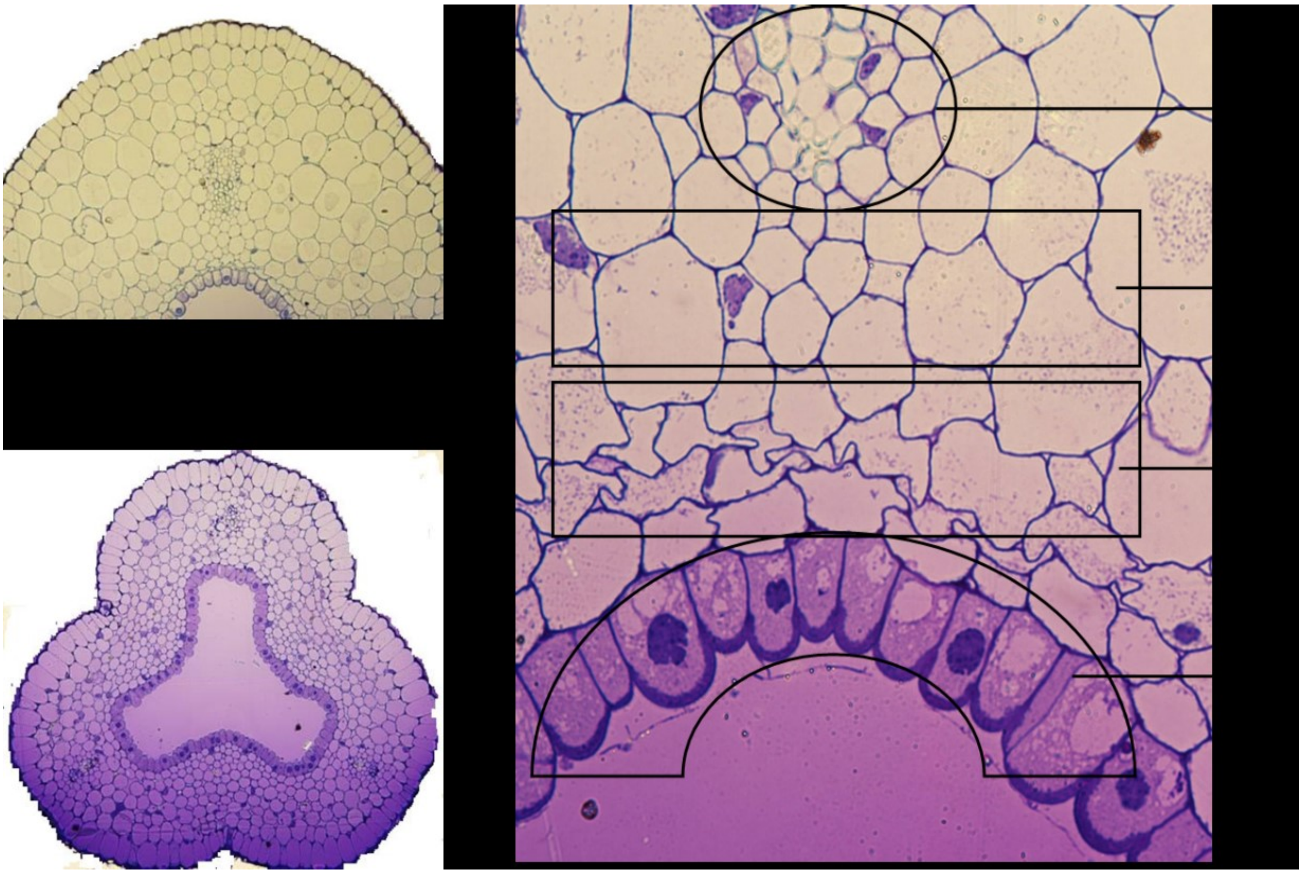

Fig. 2. Transverse sections of the style. A, canal cells; B, parenchyma cells adjacent to canal cells; C, parenchyma cells adjacent to vascular bundle; D, vascular bundle.

Results

Evaluation of cross compatibility in three cross combinations: Of the three cross combinations used in this study, cultivar 'Prato' × 'Pollyanna' exhibited the best compatibility (Table 1). In this cross, the percentage of obtained capsules was the highest (100%) and all the capsules were inflated. The average number of seeds per capsule reached up to 241, in which 77.17% constituted plump seeds. For the cross 'Prato × Prato', only four capsules (8.16%) were obtained from 49 pollinated flowers and only two were inflated. The seeds were largely shrivelled in this cross. The percentage of plump seeds per capsule was 22.14%, which was far lower than the cross 'Prato × Pollyanna'. The cross between the Asiatic cultivar 'Prato' and *L. pumilum* was incompatible, and no capsules were obtained from 177 pollinated flowers.

Evaluation of exudate in the stylar canal: The amount of exudate in the style of the maternal parent 'Prato' was estimated for each cross combination at different stages. In all three cross combinations, the exudate differed significantly in the three segments as well as at the different stages (Table S2). No exudate was observed in all the crosses before pollination, while the quantity of exudate differed after pollination (Table 2). When 'Prato' was pollinated with pollen from 'Pollyanna', the amount of exudate in the three segments of the stylar canal differed distinctly. The exudate first appeared in the top segment at six h after pollination and increased with time. Forty-eight hours later, a small amount of exudate was observed in the middle (3.48%) and basal (1.38%) segments. The exudate increased in all three segments at 72 h after pollination, and presented a clear top-to-base descending gradient. In the cross combinations 'Prato

× Prato' and 'Prato × *L. pumilum*', the exudate did not obviously vary. The exudate was only observed in the top segment of the stylar canal. Although exudate accumulated in the top section as time progressed, the total quantity remained at a very low level.

Morphology, existence form, and location of calcium precipitates in the style: This study observed the morphology, existence form, and location of calcium precipitates in the style of the maternal parent 'Prato'. Four different forms of calcium precipitates were primarily identified in the style (Fig. 3), and included scattered granular precipitates, scattered fine granular precipitates, scattered flocculent precipitates, and dense flocculent precipitates. The scattered granular precipitates were mainly attached to the inner side of the cell wall, and the granules were large. The scattered fine granular precipitates were found in the intercellular space and were small, while the scattered flocculent precipitates were dispersed in the vacuole and cytoplasmic matrix. The shape of the precipitate was irregular. The shape of the dense flocculent precipitates was also irregular and they were distributed continuously on the tonoplast, the inner side of the plasmalemma, and on the surface of the canal cells.

Calcium in the stigma: In all three cross combinations, the total amount of calcium in the stigma of the female parent 'Prato' increased after pollination. In the cross 'Prato × Pollyanna', it increased slowly and steadily following pollination. The calcium precipitates increased significantly until 48 h. No significant variation was observed between stage a48 and a72 (Table 3). In the cross 'Prato × Prato', no obvious variation in calcium was observed in stage a6 and a24, but a significant increase was observed at 48 h after pollination. In the cross 'Prato × *L. pumilum*', calcium in the stigma increased significantly in the first 6 h and continued to accumulate until 48 h (18.23‰), and then decreased.

Evaluation of calcium in the style: The variation in calcium in the style was assessed separately through four transverse sections (canal cells, parenchyma cells adjacent to canal cells, parenchyma cells adjacent to vascular bundle, and vascular bundle). For each section, the vertical distribution of calcium precipitate from the top to the basal segment was determined in order to ascertain the transportation of calcium in the style.

In all three cross combinations, the amount of calcium in the canal cells (CC) of different stylar segments increased after pollination (Table 4). However, differences among the three cross combinations were also observed. In the cross 'Prato × Pollyanna', the calcium precipitate in the canal cells of all three stylar segments increased sharply after pollination. In the top and middle segments, the precipitate began to decrease at 24 h and 48 h after pollination, respectively, but continued to increase in the basal segment. At 72 h after pollination, the amount of calcium precipitate in the basal segment was the highest (13.04‰), but was lowest (8.18‰) in the top. In this cross combination, a clear top-to-base calcium gradient was observed in the style at 72 h after pollination. In the cross 'Prato × Prato', the precipitate in the canal cells of the top and middle segments started decreasing at 48 h, but increased slowly in the basal segment. At 72 h, the total amount of precipitate in the middle (7.18‰) and basal segments (6.88‰) was less than that in the top (8.00‰). In the cross 'Prato × *L. pumilum*', precipitate in the canal cells of the top and middle segments decreased at 24 h and 48 h, respectively. There was no significant variation in calcium precipitate in the basal segment (Table 4). Seventy-two hours after pollination, the amount of calcium was highest in the top segment (8.15‰) and lowest in the basal segment (4.72‰).

Table 1. Development of the ovary and seed setting of three crossing combinations.

	Prato × Prato	**Prato × Pollyanna**	**Prato × *L. pumilum***
Number of crosses	49	19	177
Total number of capsules	4 (8.16%)	19 (100%)	0 (0%)
Number of inflated capsules	2	19	0
Average number of seeds per capsule	140	241	0
Average number of plump seeds per capsule	31(22.14%)	186(77.17%)	0(0%)

Table 2. Percentages of exudates in the stylar canal at different stages.

Cross combinations	**Stage**	**Exudates in different stylar segment (%)**		
		Top	**Middle**	**Basal**
Prato × Pollyanna	b2	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
	a6	2.73 ± 0.44 b	0.00 ± 0.00 a	0.00 ± 0.00 a
	a24	2.82 ± 0.42 b	0.00 ± 0.00 a	0.00 ± 0.00 a
	a48	15.28 ± 0.37 c	3.48 ± 0.67 b	1.38 ± 0.45 b
	a72	32.97 ± 0.58 d	15.25 ± 0.60 c	9.74 ± 0.64 c
Prato × Prato	b2	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
	a6	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
	a24	3.52 ± 0.75 b	0.00 ± 0.00 a	0.00 ± 0.00 a
	a48	7.31 ± 0.53 c	0.00 ± 0.00 a	0.00 ± 0.00 a
	a72	6.7 ± 0.66 c	0.00 ± 0.00 a	0.00 ± 0.00 a
Prato × *L. pumilum*	b2	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
	a6	3.88 ± 0.33 c	0.00 ± 0.00 a	0.00 ± 0.00 a
	a24	2.75 ± 0.37 b	0.00 ± 0.00 a	0.00 ± 0.00 a
	a48	3.21 ± 0.33 bc	0.00 ± 0.00 a	0.00 ± 0.00 a
	a72	5.87 ± 0.46 d	0.00 ± 0.00 a	0.00 ± 0.00 a

Note. b2=2 hours before pollination; a6= 6 hours after pollination; a24= 24 hours after pollination; a48= 48 hours after pollination; a72= 72 hours after pollination

Table S2. Analysis of variance for amount of exudate in the style.

Cross combination	Source	Sum of squares	df	Mean square	F
Prato × Pollyanna	Segment	0.287	2	0.143	55.091**
	Stage	1.199	4	0.300	115.173**
	Error	0.099	38	0.003	
	Total	2.707	45		
Prato × Prato	Segment	0.209	2	0.105	26.481**
	Stage	0.074	4	0.018	4.687**
	Error	0.150	38	0.004	
	Total	0.537	45		
Prato × *L. pumilum*	Segment	0.249	2	0.124	67.172**
	Stage	0.035	4	0.009	4.671**
	Error	0.070	38	0.002	
	Total	0.478	45		

**Indicates significant at 1% level of significance

Table 3. Percentages of calcium precipitates in stigma of the three cross combinations.

Cross	Stage	Calcium precipitates (‰)
Prato × Pollyanna	b2	4.3 ± 0.60 a
	a6	8.42 ± 0.97 b
	a24	8.63 ± 0.58 c
	a48	11.67 ± 0.50 d
	a72	12.49 ± 0.68 d
Prato × Prato	b2	4.3 ± 0.60 a
	a6	3.97 ± 0.49 b
	a24	7.64 ± 0.73 b
	a48	10.95 ± 0.36 c
	a72	12.3 ± 0.75 c
Prato × *L. pumilum*	b2	4.3 ± 0.60 a
	a6	14.27 ± 1.16 bc
	a24	17.58 ± 0.70 c
	a48	18.23 ± 0.89 bc
	a72	16.51 ± 0.97 b

Calcium precipitate increased in the parenchyma cells adjacent to the canal cells (PACC) in all three stylar segments as the flower was pollinated (Table 4). It was most prominent in the cross 'Prato × Pollyanna'. The amount of precipitate in the PACC of the top segment increased significantly until stage a24. In the middle segment the precipitate began decreasing 24 h after pollination, and was obviously lower at a72 (5.03‰) in comparison to a24 (6.16‰), but continued increasing in the basal segment until 48 h (8.17‰). At 72 h after pollination, the calcium precipitate in the basal segment (7.83‰) was far greater than in the middle (5.74‰) and top (5.03‰) segments. In the cross 'Prato × Prato', the total calcium precipitate in the PACC of the style increased slowly after pollination, and continued to increase obviously in the basal segment. However, in the top segment, the precipitate increased until 48 h (6.1‰) and then started declining. In the middle segment, calcium increased significantly between a6 (2.11‰) and a24 (3.08‰), and there was no obvious variation in calcium at a24, a48, and a72. Seventy-two hours after pollination, the total amount of precipitate in the basal segment was highest (4.55‰). In the cross 'Prato × *L. pumilum*', precipitate in the PACC of both the top and middle segments declined significantly from 48 h, but continued to increase in the basal segment. At 72 h, calcium in the basal segment (3.69‰) was higher than in the top (2.89‰) and middle (2.82‰) segments.

In un-pollinated flowers, the amount of calcium in the parenchyma cells adjacent to the vascular bundle (PAVB) of the basal segment of the style was higher (4.68‰) than in the top (3.01‰) and middle (2.99‰) segments (Table 4). When the cultivar 'Prato' was pollinated with 'Pollyanna', the calcium precipitate in the PAVB of all three segments increased significantly between a6 and a24. In the top segment, no obvious variation was observed at a24, a48, and a72. The amount of calcium precipitate in the middle segment decreased from a24. In the basal segment, it continued increasing until 72 h, but the variation in calcium precipitate was not significant. The total amount of calcium in the basal segment (7.93‰) was higher than that in the top (4.46‰) and middle (6.62‰) segments at 72 h. In 'Prato × Prato', calcium accumulated slowly after pollination in the top segment, but increased sharply at24 h after pollination in the middle segment and no obvious decrease was observed. Precipitate in the basal segment increased sharply and was highest at a72 (6.64‰); which was more than in the middle (4.25‰) and top segments (4.04‰). In 'Prato × *L. pumilum*', calcium precipitate accumulated significantly in the top segment within 24 h after pollination, and then decreased. In the middle and basal segments, the accumulation of calcium was not obvious within 48 h after pollination, but increased significantly thereafter, peaking at 72 h.

Calcium in the vascular bundles (VB) of the different stylar segments increased significantly within 24 h after pollination (Table 4). The variation in calcium between 'Prato × Prato' and 'Prato × *L. pumilum*' was similar. Calcium precipitate in the basal segment increased continuously until 72 h, but increased only until 48 h in the top and middle segments.

Table 4. Percentages of calcium precipitates (‰) in different stylar segments in the three cross combinations.

Cross	Stage	CC			PACC			PAVB			VB		
		Top	Middle	Basal	Top	Middle	Basal	Top	Middle	Basal	Top	Middle	Basal
1	b2	4.29±0.52 a	4.11±0.59 a	4.34±0.36 a	2.27±0.57 a	1.93±0.42 a	1.83±0.16 a	3.01±0.38 a	2.99±0.14 a	4.68±0.32 a	4.53±0.85 a	4.65±0.22 a	5.73±0.90 a
	a6	6.41±0.33 b	6.34±0.27 b	5.35±0.46 a	4.71±0.39 b	4.45±0.91 b	4.23±0.34 b	3.69±0.32 ab	4.33±0.47 b	4.79±0.92 a	8.81±1.10 b	5.09±0.53 a	7.42±0.65 b
	a24	9.88±0.25 c	8.07±0.08 c	7.00±0.84 b	6.16±0.58 c	7.76±0.67 d	6.32±0.40 c	5.08±0.59 c	8.16±0.31 d	6.91±0.70 b	12.86±0.78 c	14.27±0.78 b	13.34±0.72 c
	a48	8.85±0.18 d	12.46±0.64 d	9.84±0.87 c	6.15±0.423 c	7.23±0.63 cd	8.17±0.36 d	5.44±0.46 c	7.41±0.45 cd	7.42±0.49 b	10.88±0.97 bc	13.48±0.71 b	14.1±0.90 c
	a72	8.18±0.21 e	10.80±0.88 e	13.04±0.75 d	5.03±0.62 bc	5.74±0.37 bc	7.83±0.66 cd	4.46±0.64 bc	6.62±0.73 c	7.93±0.23 b	8.94±0.72 b	9.87±0.74 c	14.1±0.74 c
2	b2	4.29±0.52 a	4.11±0.59 a	4.34±0.36 a	2.27±0.53 a	1.93±0.73 a	1.83±0.09 a	3.01±0.66 a	2.99±0.07 a	4.68±0.43 a	4.53±0.68 a	4.65±0.55 a	5.73±0.50 a
	a6	4.78±0.25 a	4.63±0.39 a	4.82±0.11 a	2.35±0.20 a	2.11±0.25 a	1.87±0.11 a	3.31±0.62 a	3.29±0.15 a	4.29±0.16 a	9.21±0.28 b	5.89±0.38 b	10.52±0.50 b
	a24	6.98±0.35 b	6.93±0.55 b	5.59±0.44 b	4.48±0.44 b	3.08±0.58 bc	2.86±0.22 b	4.08±0.36 ab	3.55±0.36 ab	4.99±0.36 a	10.52±0.87 bc	11.33±0.33 c	11.08±0.25 b
	a48	8.75±0.20 c	7.56±0.40 b	6.47±0.40 c	6.1±0.62 c	3.67±0.73 c	3.63±0.37 c	4.62±0.23 b	4.34±0.38 c	4.8±0.36 a	15.66±0.35 d	12.6±0.63 c	12.22±0.22 c
	a72	8.00±0.71 bc	7.18±0.49 b	6.88±0.21 c	3.63±0.64 b	3.07±0.69 bc	4.55±0.09 d	4.04±0.35 ab	4.25±0.55 bc	6.64±0.43 b	11.29±0.62 c	12.5±0.20 c	13.65±0.59 d
3	b2	4.29±0.52 a	4.11±0.598 a	4.34±0.36 a	2.27±0.25 a	1.93±0.20 a	1.83±0.51 a	3.01±0.19 a	2.99±0.32 a	3.10±0.44 a	4.53±0.18 a	4.65±0.32 a	5.73±0.38 a
	a6	9.14±0.14 b	5.57±0.16 b	5.47±0.29 b	4.81±0.20 b	2.97±0.10 b	2.2±0.15 a	4.25±0.21 b	3.28±0.14 a	3.19±0.22 a	10.47±0.49 b	10.5±0.32 b	10.99±0.18 b
	a24	9.48±0.47 b	5.27±0.12 b	5.11±0.46 bc	4.97±0.45 b	2.97±0.45 b	2.42±0.39 bc	6.1±0.12 c	3.42±0.11 a	2.33±0.77 a	11.14±0.43 b	10.58±0.61 b	11.02±0.15 b
	a48	8.35±0.80 c	8.4±0.18 c	4.99±0.12 bc	4.49±0.46 b	3.84±0.18 c	3.09±0.15 cd	4.82±0.44 d	3.16±0.27 a	2.59±0.35 a	13.21±0.30 c	14.18±0.30 c	14.22±0.33 c
	a72	8.15±0.27 b	7.23±0.29 d	4.72±0.53 bc	2.89±0.43 a	2.82±0.31 b	3.69±0.38 d	3.82±0.08 b	4.09±0.12 b	5.33±0.39 b	8.62±0.36 d	10.55±0.42 b	15.19±0.48 d

Note. Cross 1, 2 and 3 represents 'Prato×Pollyanna', 'Prato×Prato' and 'Prato×*L. pumilum*'. b2, 2h before pollination; a6, 6h after pollination; a24, 24h after pollination; a48, 48h after pollination; a72, 72h after pollina

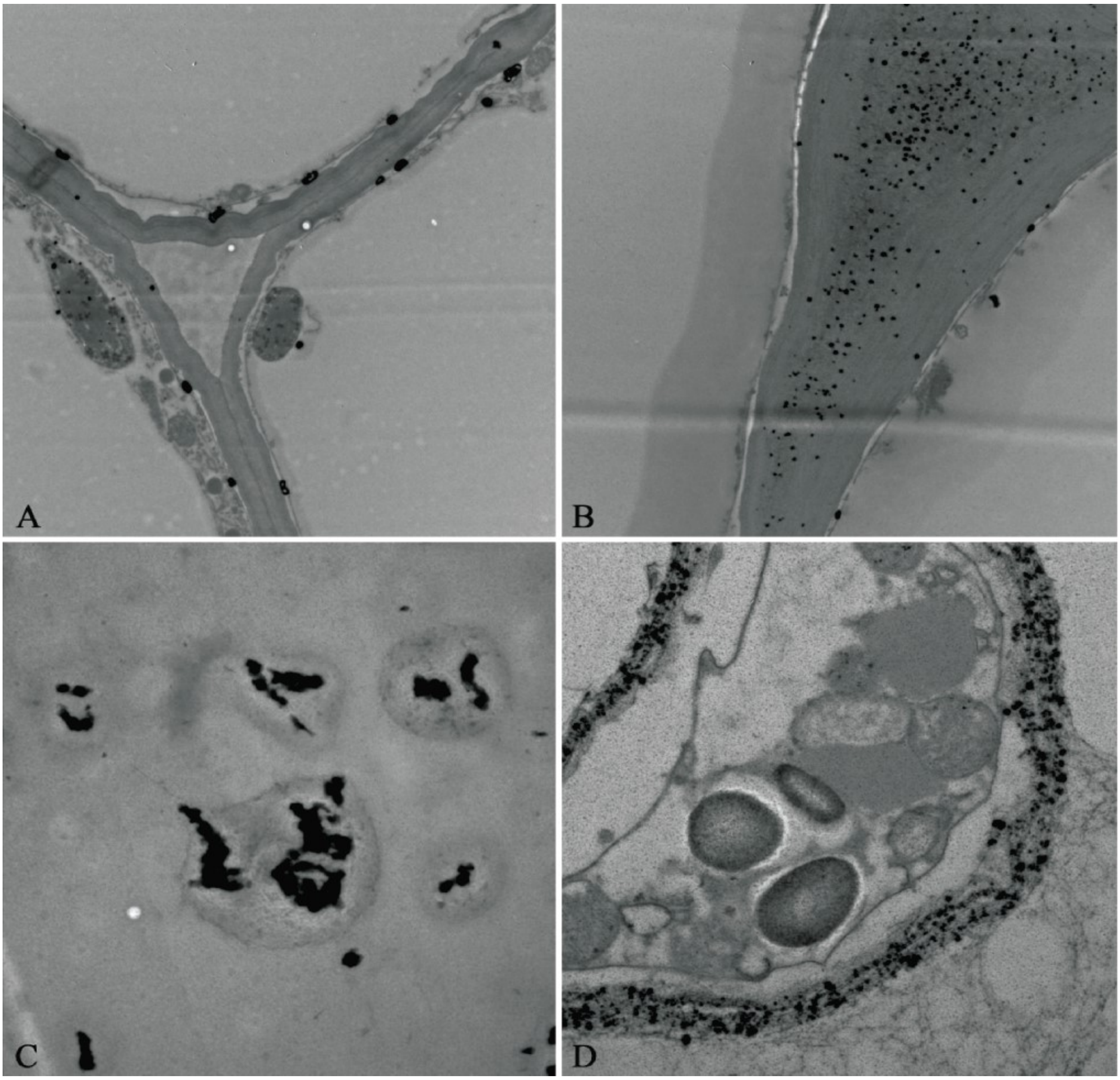

Fig. 3. Existence form and distribution of calcium precipitates. A, scattered granular precipitates; B, scattered fine granular precipitates; C, scattered flocculent precipitates; D, dense flocculent precipitates.

Discussion

Exudate in the stylar canal and its relationship with cross compatibility: In the current study it was observed that in lily, the exudate in the stylar canal of the female parent increased obviously after pollination, signalling the existence of certain relationships between cross compatibility and variation in the exudate. Our results showed that compatible pollens could activate an intense secretion of exudate in the stylar canal, and that the amount of exudate accumulated obviously as time progressed. If the female parent was pollinated with incompatible pollen, a small amount of exudate was observed in the top segment of the style, while no exudate was produced in the middle and basal segments. It is known that when a compatible pollen grain lands on the stigma, it germinates and forms a pollen tube (Arzani & Javady, 2002; Rodriguezenriquez *et al*., 2013). Therefore, it is speculated that the exudate has the effect of promoting pollen tube growth. This is in agreement with the results of previous studies in *L. longiflorum* (Ascher & Drewlow, 1971) which found that stylar exudate significantly increased the length of the pollen tube. Accumulation of exudate was previously found to significantly promote the growth of the pollen tube (Ichimura & Yamamoto, 1992, 1991). It was also reported that the inability of incompatible pollen to grow in *Lilium* may be attributed to insufficient exudate secretion (Amaki & Higuchi, 1991).

Calcium distribution in the stigma: The stigma and style are the necessary pathways of transmitting male gametes (Erbar, 2003). Earlier studies have shown that calcium is a requirement for pollen germination and tube growth (Brewbaker & Kwack, 1963). Using X-ray microanalysis, Bednarska (1991) found that the germinating pollen of

Primula officinalis and *Ruscus aculeatus* absorbed calcium from the stigma. Using potassium pyroantimonate precipitation, it was also found that loosely bound calcium in sunflower was abundant on the receptive surface of the stigma, indicating that large amounts of calcium on the stigma benefits pollen germination (Zhang *et al*., 1995). Large amounts of calcium precipitates were observed on the stigmatic surface of *Brassicaoleracea* after pollination (Elleman & Dickinson, 1999). A recent study of *Arabidopsis* revealed that there are three stages of Ca^{2+} uptake/increase. The first stage is at pollen grain attachment, soon after pollen hydration. The second is after pollen protrusion and tube initiation, and the third stage is during pollen penetration of the stigmatic surface (Iwano *et al*., 2004). This study showed that the accumulation speed of calcium in the receptive stigma varied in different cross combinations. It was also hypothesized that the rapid accumulation and high concentration of calcium in the stigma could negatively impact pollen tube growth. This phenomena was previously observed in *Tradescantia* (Picton & Steer, 1983), in which growth of the pollen tube was stimulated when calcium ion concentration was raised from 10^{-8} to 10^{-5} M. However, a marked decline in the growth rate of the pollen tube was observed when the level of calcium ions was further increased. The optimal concentration of calcium plays an important role in the growth of the pollen tube, and the threshold varies in different species or genera.

Calcium distribution in the style: The lilaceous style is hollow and the canal cells are the intra-epidermal cells of the stylar canal, and are proven to be the ‘transfer cells’ in the style (Rosen & Thomas, 1970). Vascular bundles form part of the transport system in vascular plants. The vascular bundle in the style connects the stigma and receptacle, which conducts the transport of water and dissolved mineral substances. It is the main pathway for the vertical transportation of calcium ions and also constitutes the distribution centre of the transverse section. The amount of calcium in the canal cells and vascular bundle of the style is greater than in the adjacent parenchyma cells. Few antimonate precipitates localize within the cells of the parenchymatous tissue (Ge *et al*., 2007).

In the canal cells and vascular bundles of the style of compatible crosses, calcium presented an obvious transfer from the top to the base, and a rising gradient of Ca^{2+} was ultimately formed. Conversely, no calcium gradient was observed in the canal cells of incompatible crosses. This study showed that high cross compatibility was related to the transfer of Ca^{2+} from the top to the base in the style and the ultimate high calcium concentration in the basal segment. A previous study confirmed the presence of a calcium gradient in the pistils of some flowering plants, including *Gladiolus gandavensis* (Day *et al*., 1971). Mascarenhas & Machlis (1964) observed the chemotropic response of pollen tubes to calcium in *Antirrhinum*. It is hypothesized that the growth of the pollen tube towards the embryo sac is induced by sufficient calcium. In some flowering plants, however, it was reported that calcium gradients were absent in the style (Glenk *et al*., 1971; Mascarenhas, 1975). This is probably due to the differences in calcium distribution in the style not being very obvious, and therefore difficult to detect using currently available techniques. Another possibility is that the gradient distribution of calcium is related to plant species. The length of the style varies in different species. For species that have short styles, the pollen tubes can reach the ovary easily in the absence of a calcium gradient. But for species with long styles, the calcium gradient appears to play an important role in the growth of pollen tubes. In the style of tobacco, a significant calcium increase was observed in the basal segment near the ovary at 22 h after pollination (Ge *et al*., 2009). Zhao *et al.* (2004) discovered that pollination in lilies induced an apparent increase in calcium within the intracellular matrix of the transmitting tissue. Observation of a much wider variety of plants exhibiting different floral biology is required in order to better understand the role of calcium in the stigma and style of flowering plants.

Acknowledgment

We are thankful for the foundation from CAS "Light of West China" Program (2014), the supportfrom National Natural Science Foundation of China (NO. 31660582)and the foundation from ‘State Key Laboratory Breeding Base——Key Laboratory of Qinghai Province for Plateau Crop Germplasm Innovation and Utilization’ (2013-07). We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

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(Received for publiation 12 December 2016)