Theor Appl Genet (1983) 67:11–16

Wall-bound proteins of pollen tubes after self- and cross-pollination in *Lilium longiflorum*

Yi-qin Li and H. F. Linskens

Department of Botany, Section Molecular Developmental Biology, University, NL-6525 ED Nijmegen, The Netherlands

Received June 17, 1983
Communicated by P. L. Pfahler

Summary. Wall-bound proteins of *Lilium longiflorum* pollen tubes grown in vivo constitute 20–27% of the dry matter. Twenty-two–twenty-six percent of these proteins are NaCl soluble. Wall-bound proteins of in vivo pollen tubes are present in amounts 5–7 times that found in tubes grown in vitro. The protein pattern of wall-bound proteins is different between in vitro and in vivo grown pollen tubes. There are two kinds of pollen tube wall proteins: loosely bound and tightly bound. The latter are NaCl insoluble, contain hydroxyproline and are assumed to be covalently bound. No significant differences have been found in the amount of wall-bound proteins present between pollen tubes resulting after self-pollination and those resulting from cross-pollination. However, some band differences between self- and cross pollen tubes have been observed after gel electrophoresis. It can be supposed that some wall-bound proteins of pollen tubes are associated with the incompatibility reaction.

Key words: Wall bound proteins – Pollen tubes – Incompatibility – Lily – *Lilium longiflorum*

Introduction

Formation and elongation of pollen tubes play a decisive role in fertilization (Linskens 1975a, b, 1981, 1983). After pollination, in many species only pollen tubes from a cross pollination can reach the ovary and lead to the fusion of male and female cells. Because of gene control, self incompatibility is expressed in *Lilium longiflorum* by a restriction of the incompatible pollen tube length to half that of the compatible pollen tubes in the time the latter travels through the whole length of style. The exudate secreted in lily by the stigma and stylar canal can be utilized by pollen tubes. Kroh et al. (1970) found incorporation of label into pollen tube walls from ^{14}C-myo-inositol labelled lily pistils. When pistils were labelled with ^{14}C-glucose, Labarca and Loewus (1972, 1973) found that a portion of the pistil exudate contributed to the synthetic requirements of the developing pollen tubes and that the label was incorporated into polysaccharides in pollen tube walls after self- and cross-pollination. Campbell and Ascher (1975) reported that incorporation of radioactivity from ^{3}H-uridine labelled styles into nucleic acids of lily pollen tubes is different between different cultivars. Differences in metabolism and synthesis of nucleic acids in their turn may possibly influence wall formation as well as cell wall composition of pollen tubes as indicated already by electronmicroscopy (Mühlethaler and Linskens 1956).

In the study of *Neurospora,* Manocha and Ross Colvin (1967) found that protein exists throughout the cell wall as a part of a 3-dimensional network. They suggested that the wall proteins may serve as a conduit for transport of macromolecular substances across the wall. In the sporophytic self incompatibility system, pollen grain wall proteins are involved in the recognition reaction between pollen grains and stigma (Knox and Heslop-Harrison 1970; Knox et al. 1972; Yang and Tsao 1981). However, basic knowledge about proteins in walls of in vivo pollen tubes is still not available. Pollen tubes do have tip growth, and during the incompatibility reaction, wall formation at the pollen tube tip is disturbed (Linskens and Kroh 1967; Shirvanna et al. 1982). Pollen tube wall proteins may thus possibly play a role in this process.

The purpose of this research was to study the wall proteins of in vivo *L. longiflorum* pollen tubes after self- and cross pollination. Several basic aspects of pollen tube wall proteins, such as composition and content of

the proteins, nature of the proteins and partial separation of the proteins, were investigated. Investigations of wall proteins of in vitro pollen tubes have been conducted earlier (Li et al. 1983). The differences in the wall-bound proteins between in vivo and in vitro, and between compatible and incompatible pollen tubes, were compared.

Material and methods

Plant material

Flower buds from two cross compatible cultivars of *Lilium longiflorum*, cv. 'Arai No. 5' (self-incompatible) and 'Mount Everest' (pseudo-self-incompatible) were detached from plants 1 day before anthesis and placed in jars with water at room temperature. Before anthesis the anthers were removed from flowers to be cross-pollinated.

Incubation and recovery of pollen tubes

Flowers of *L. longiflorum* have approximately an 100 mm long style with a hollow stylar canal, through which the pollen tubes travel down to the ovary. Because these pollen tubes can be easily lifted out of the canal after bisecting the style, a large number of compatible and incompatible pollen tubes can be obtained after pollination and incubation of detached lily pistils. Twenty-four hours after anthesis, the flowers were stripped of petals and stamens. Each pistil was then self- or cross-pollinated, i.e. 'Arai 5' × 'Arai 5' (A × A), 'Arai 5' × 'Mount Everest' (A × M), 'Mont Everest' × 'Mount Everest' (M × M) and 'Mount Everest' × 'Arai 5' (M × A), with fresh pollen, placed in a moisture chamber and incubated at 25 °C for 72 h in the dark.

The stigma, characterized by a curved, 3-lobed papillate surface above an expanded stylar portion, was excised with three incisions with a sharp blade, so as to leave as much of the style, including the expanded portion, as possible. A shallow ringing incision was then made on the style below the expanded portion, shallow enough so as to not cut through pollen tubes, and the expanded portion then broken off. The pollen tube mass could then be pulled free of the expanded portion. Using a needle-point forceps, the style was then longitudinally sliced open along one side and held open with another forceps. The free pollen tube mass at the stigma end was then clustered together and held in a forceps, and the pollen tube mass lifted free of the length of the style, plunged into liquid nitrogen and stored at −80 °C.

Preparation of pollen tube walls

Pollen tubes were ground in liquid nitrogen and subsequently homogenized in 0.05 mol l^{-1} phosphate buffer, pH 6, with a French Press (AMINCO J4-3398A) at 1.24×10^8 N m^{-2}. Pollen tube walls were sedimented by centrifugation at 0 °C 5,000 × g for 10 min and the pellet was successively washed at 0 °C in the following steps, each followed by centrifugation at 1,000 × g:

0.05 mol l^{-1} phosphate buffer, pH 8 (3×); distilled water (6×); 1% Triton X-100 (1×); distilled water (1×); 1% Triton X-100 (1×); distilled water (4×).

To remove nuclear material, the pellet was resuspended in 0.02 mol l^{-1} Tris HCl buffer (pH 7.6). RNase, which was preheated at 70 °C for 2 min, and DNase were added to the suspension and made up to a final concentration of 50 μg ml^{-1}.

The enzymatic degradation was performed at 0 °C for 30 min with gentle stirring. After centrifugation, the pellet was washed 9 times with distilled water. A portion of the wall material was lyophilized and weighed.

Analysis of the walls and wall proteins

Elementary analysis of the tube walls, amino acid analysis, saline extraction of the wall-bound proteins, SDS (sodium dodecyl sulfate) gel electrophoresis and staining of the proteins were performed as earlier described (Li et al. 1983), except that the silver stain employed was according to Eschenbruch and Bürk (1982).

Isolation of cytoplasmic proteins from pollen tubes

Pollen tubes were ground in liquid nitrogen and homogenized in 0.5 mol l^{-1} NaCl at 0 °C. After centrifugation at 160,000 × g for 30 min in a Airfuge (Beckman), the supernatant was mixed with the same volume of cold acetone and the proteins precipitated at −5 °C.

Results

Production of pollen tubes

After self- or cross-pollination, pistils of 'Arai 5' or 'Mount Everest' were incubated at 25 °C. Cross-compatible pollinations after an incubation of 3 days produced a 43–76% greater weight of pollen tubes than self-incompatible pollinations. The dry weight of pollen tube walls collected from self- and cross-pollinated 'Arai 5' and 'Mount Everest' pistils were about the same, being only about 0.7% of the fresh pollen tubes weight. For example, from 1,397 cross-pollinated 'Arai 5' pistils, only 200 mg dry weight of pollen tube wall material was recovered (Table 1).

Protein content in tube walls from self- and cross pollination

Protein content in the pollen tube walls was indirectly estimated from total nitrogen content (Table 1) and from the amount of 17 amino acids recovered after hydrolysis of the walls (Table 2). The amount of protein estimated from the latter was some 5–7% lower than that from the former. The total wall-bound protein of pollen tubes was very high and amounted to 20–27% of the dry weight of the walls (Table 1). The content of total protein from pollen tube walls grown in styles of 'Arai 5' was about 6–7% higher than that in 'Mount Everest'. No significant difference was found between self- and cross-pollinated samples (Table 1). In all 4 pollination combinations, the concentration of 1 mol · l^{-1} NaCl soluble wall proteins was more or less the same (Table 1) – 22–26% of the total wall bound proteins.

Amino acid composition

A portion of the tube walls isolated from self or cross pollinated 'Arai 5' pistils and the 1 mol · l^{-1} NaCl

Table 1. Production of pollen tube walls and content of wall-bound proteins after self- and cross-pollination

Pollination[a]	No. of pistils	Pollen tubes (fresh wt)		Wall content of tubes[b]	Protein content in walls		
		Total (g)	(mg pistil^{-1})	(dry wt μg mg^{-1})	Total[c] (μg mg^{-1})	NaCl soluble fraction[d] (μg mg^{-1})	(6)/(5)
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
M×A	433	9.7	22.4	7.2	201.5	43.6	21.6%
M×M	703	8.9	12.7	7.2	198.3	45.8	23.1%
A×M	1397	27.4	19.6	7.4	256.2	65.6	25.6%
A×A	2037	28.0	13.7	7.0	273.0	60.1	22.0%

[a] *M Lilium longiflorum* 'Mount Everest'; *A Lilium longiflorum* 'Arai No 5' [b] dry weight of walls per mg fresh weight of tubes [c] total protein content: calculated from total N content multiplied by a factor of 6.5, μg per mg dry weight of walls; [d] protein content in 1.0 mol l^{-1} NaCl extract: calculated from the content of 17 amino acids after proteins were hydrolyzed, μg per mg dry weight of walls

Table 2. Total amino acid composition (%) in tube walls and in their NaCl soluble fraction after self- and cross-pollination

Amino acid	in wall		NaCl soluble fraction			
	A×A	A×M	A×A	A×M	M×M	M×A
Asp	10.0	9.5	9.6	9.5	9.9	9.3
Thr	7.6	6.3	9.0	8.5	8.2	9.5
Ser	9.6	8.7	9.9	9.7	9.0	10.3
Glu	5.8	5.5	6.1	6.1	6.8	5.6
Pro	5.1	5.4	4.7	4.9	5.9	4.2
Gly	9.7	9.1	9.4	9.4	9.3	8.9
Ala	8.7	8.8	8.3	8.1	8.0	8.1
Val	7.9	8.3	8.7	7.9	7.6	9.8
Met	0.5	0.5	0.5	0.5	0.5	0.4
Ileu	3.8	4.6	4.7	5.3	5.4	4.8
Leu	8.2	8.9	7.7	8.2	8.6	7.7
Tyr	3.1	3.7	3.4	4.4	4.5	3.3
Phe	5.6	6.3	4.6	5.2	4.5	4.5
Lys	6.3	6.3	6.1	5.6	5.5	6.4
His	2.4	2.4	2.4	2.3	2.3	2.8
Arg	4.8	4.7	4.8	4.4	4.0	4.3
Hyp	1.0	0.9	tra[a]	tra[a]	tra[a]	tra[a]
Total	100.1	99.9	99.9	100.0	100.0	99.9

[a] *tra* trace. In NaCl extracts, only trace amounts of hydroxyproline were found (less than 0.1%)

soluble proteins extracted from pollen tubes of self or cross pollinated 'Arai 5' or 'Mount Everest' pistils were hydrolyzed in 6 mol · l^{-1} HCl and the 17 common amino acids were analyzed (Table 2).

In all six different samples, i.e. between self- and cross pollination and between NaCl soluble and insoluble wall proteins, no substantial difference was found in percent compositions of the 17 amino acids with the exception of hydroxyproline. Only trace amounts of this amino acid was present in the NaCl soluble fraction. In contrast, about 1% of total wall protein was present as hydroxyproline (Table 2). This might be an indication that most hydroxyproline-containing proteins are covalently bound to pollen tube walls.

Gel pattern of wall protein

SDS gel electrophoresis of NaCl soluble wall bound proteins of pollen tubes grown under self or cross pollination gave a large number of similar protein patterns.

The molecular weight of the majority of the proteins that stained with Coomassie blue was between 33 k and 91 k (Fig. 1 a). In addition to many similarities between the four pollinations, some different protein bands were observed in the samples. For example, there was a protein band of 86 k in sample A×A that was barely present in M×A. This difference is likely not to due to the pollen tubes being from different cultivars because in sample M×M the 86 k protein was also present although this band was not as densely stained as in A×A. In the 20 k to 23 k protein, each sample had its own unique band. They are 21 k in M×A, 20 k in M×M, 23 k in A×M and 21.5 k in A×A (Fig. 1 a). These differences could be seen even more clearly in the gel stained by silver (Fig. 1 b). Moreover, in both M×A and A×A two clear bands were found between the molecular weights of 33 k and 34.5 k, but only one in the samples from M×M or A×M. This might reflect a cultivar difference. Because silver stain is more sensitive to low molecular proteins than Coomassie blue, many protein bands, the molecular weight of which were lower than 33 k, become visible. In the 20–39 k range for 'Arai' pollen tubes and in the 22–25 k range for 'Mount Everest' pollen tubes, more bands were

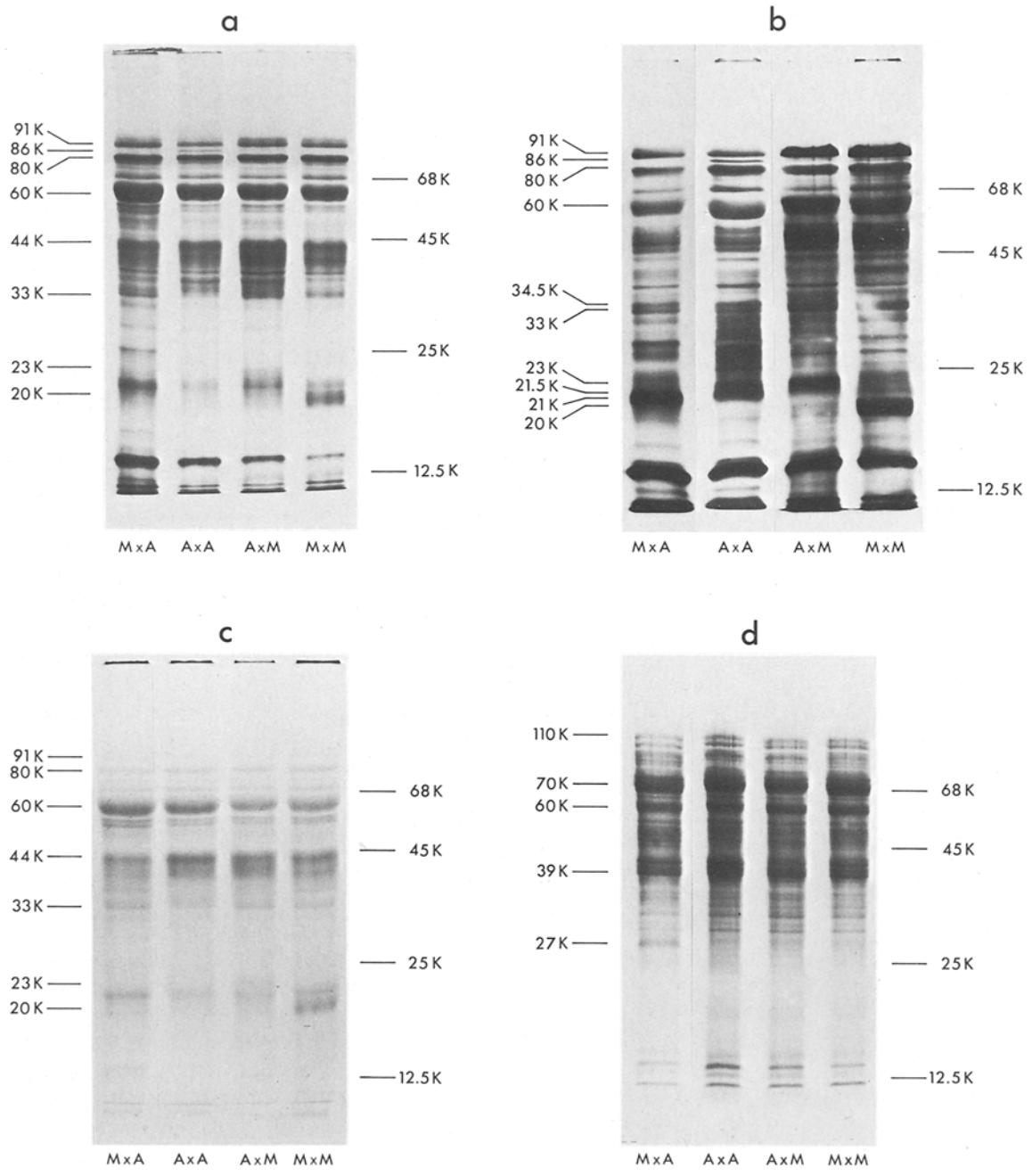

Fig. 1 a–d. Gel electrophoresis of wall-bound and cytoplasmic proteins of lily pollen tubes after self- and cross-pollination. Electrophoresis was performed on an 11% SDS polyacrylamide gel slab. Cytochrome C, chymotrypsinogen A, hen egg albumin and bovine serum albumin (Combithek calibration proteins, Boehringer Mannheim GmbH; Biochemica) were used as molecular weight markers. Wall-bound proteins: **a** coomassie blue stain; **b** silver stain; **c** Periodic acid Schiff's reagent stain. Cytoplasmic protein: **d** coomassie blue stain.

found in self-pollinated samples than in cross-pollinated ones (Fig. 1 b). Fig. 1 c shows that the most of the wall proteins with high molecular weight visualized by Coomassie blue staining were stained by the Periodic Acid Schiff's reagent (Dubray and Bezard 1982).

Only in the 20–30 k range were different proteins bands found, corresponding to those shown in Fig. 1 a, b.

Figure 1 d shows the protein pattern of the cytoplasm of the pollen tubes from the four different pollinations. Except that the 27 k protein band was more

densely stained in the sample of M×A, they are very similar to each other and are quite different from that of NaCl soluble wall-bound proteins. The molecular weight of predominant proteins was between 39 k and 110 k. A comparison of the protein pattern shows that, except for the 60 k protein, the main bands recovered from the sample of pollen tube walls, for instance 91 k, 80 k and 44 k proteins, were not densely stained. In contrast, the 70 k main band is not present in the gel of wall-bound proteins (compare Fig. 1 a, d).

Discussion

Lily pollen tubes grown in vivo are quite different from in vitro tubes, although the technique of in vitro germination of lily pollen grains has been much used (Dickinson 1968). Both cross and self lily pollen tubes are much longer than those grown in vitro, at least five times longer in the case of self tubes. The wall composition of the pollen tubes grown in detached pistils is also quite different from that of pollen tubes incubated in vitro. Wall-bound proteins of in vivo pollen tubes constitutes 20–27% of the total wall material – about five to seven times that found in the walls of pollen tubes germinated in vitro (Li et al. 1983). Since there might be other nitrogen containing components, e.g. amino sugars, in the pollen tube walls in addition to proteins, the protein content value estimated in this study from total elementary nitrogen present in walls might be too high. However, amino acid analysis confirms that the greater part of the nitrogen comes from proteins. The amount of wall-bound proteins calculated from the presence of the 17 amino acids is still about four to six times greater in the walls of pollen tubes grown in vivo than in vitro. The amount of wall-bound proteins in cultured hypha (Harris and Faber 1973), in suspension cultured sycamore cells (Talmadge et al. 1973) and in in vitro *Petunia* pollen tubes (Engels 1974) was found to be greater than that in in vitro lily pollen tubes but they are all much lower than that of in vivo grown *Lilium* pollen tubes. The percent content of NaCl soluble wall-bound proteins is slightly higher in pollen tubes grown in vivo than those grown in vitro.

Although the molecular weights of the predominant proteins extracted from walls of both in vitro and in vivo pollen tubes are larger than 33 k in SDS polyacrylamide gels, the protein patterns are different. For instance, the main band is a 60 k protein in the gels containing extracts from in vivo pollen tubes but is 82 k in similar extracts from in vitro pollen tubes. These results might be correlated with those of an electron-microscopically study of *Petunia* pollen tubes (Kroh 1967): in vitro and in vivo grown tubes did not have a different cytoplasmic structure, but had a different wall structure.

Very little is known about the nature of in vivo pollen tube wall proteins. Two kinds of wall proteins were found in *lily* tubes: loosely bound and tightly bound ones. The latter are NaCl insoluble and assumed to be covalently bound to the walls. Most of the wall-bound proteins are not rich in hydroxyproline; only 1% of the total amount of amino acids recovered from wall proteins was hydroxyproline. Furthermore, only trace amounts of hydroxyproline was found in NaCl soluble proteins, which is much lower than that found in total walls. This indicates that the hydroxyproline-containing, perhaps hydroxyproline-rich (Dashek et al. 1971), proteins are tightly bound to the wall structure. Most of the NaCl soluble proteins are positive to PAS staining and are therefore supposed to be glycoproteins. These characters are similar to those shown by the wall proteins found in lily pollen tubes grown in vitro (Li et al. 1983). Self- or cross-pollination does not change the relative production of pollen tube walls. In the samples M×A, M×M, A×M and A×A, the wall content constitute about 0.7% of the total pollen tube. All four samples resemble each other in the amino acid composition of the wall-bound proteins.

After SDS polyacrylamide gel electrophoresis, wall proteins extracted from M×A, M×M, A×M and A×A pollen tubes show similar but different patterns. One of the differences found in the protein patterns may be based on the genotype of the cultivars used. As Fig. 1 b shows, bands 33 k and 34.5 k are present only in 'Arai 5' pollen tubes. A difference was found between self-incompatible and compatible pollen tube walls. The 86 k protein band being predominant in incompatible pollen tubes from both A×A and M×M self-pollination but not in compatible ones from A×M and M×A (cross pollinations). It is therefore reasonable to assume that some of the pollen tube wall proteins are involved in the incompatibility reactions. Supporting evidence also comes from morphological studies on pollen tubes (Linskens and Kroh 1967; Shivanna et al. 1982) which indicates that wall formation is influenced by incompatibility.

In addition to the difference in the content and pattern of wall-bound proteins between in vitro and in vivo pollen tubes, it was also found that pollen tubes from both 'Arai 5' and 'Mount Everest' contained more wall-bound proteins when they grew in the style of 'Arai 5' than in 'Mount Everest'. Taking this into account, it strongly suggests that the medium, e.g. pistil exudate, also plays an important role in the formation of pollen tube walls.

Acknowledgements. We are grateful to M. Kroh, T. H. Tsao and R. Campbell for the critical reading of the manuscript.

References

Campbell RJ, Ascher PD (1975) Incorporation of radioactive label into nucleic acids of compatible and incompatible pollen tubes of *Lilium longiflorum* Thunb. Theor Appl Genet 46:143–148

Dashek WV, Harwood HI, Rosen WG (1971) The significance of a wall-bound, hydroxyproline-containing glycopeptide in lily pollen tube elongation. In: Heslop-Harrison J (ed) Pollen: development and physiology. Butterworth, London, pp 194–200

Dickinson DB (1968) Rapid starch synthesis associated with increased respiration in germinating lily pollen. Plant Physiol 43:1–8

Dubray G, Bezard G (1982) A highly sensitive periodic acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. Anal Biochem 119: 325–329

Engels FM (1974) Function of Golgi vesicles in relation to cell wall synthesis in germinating *Petunia* pollen. 4. Identification of cellulose in pollen tube walls and Golgi vesicles by X-ray diffraction. Acta Bot Neerl 23:81–89

Eschenbruch M, Bürk RR (1982) Experimentally improved reliability of ultrasensitive silver staining of protein in polyacrylamide gels. Anal Biochem 125:96–99

Harris JL, Faber WA (1973) Compositional studies on the cell walls of the synnema and vegetative hyphae of *Ceratocystis ulmi.* Can J Bot 51:1147–1153

Knox RB, Heslop-Harrison J (1970) Pollen-wall proteins: localization and enzymic activity. J Cell Sci 6:1–27

Knox RB, Willing RR, Ashford AA (1972) Role of pollen – wall proteins as recognition substances in interspecific incompatibility in poplars. Nature 237:381–383

Kroh M (1967) Fine structure of *Petunia* pollen germinated in vivo. Rev Palaeobotan Palynol 3:197–203

Kroh M, Miki-Hirosige H, Rosen W, Loewus F (1970) Incorporation of label into pollen tube walls from *myo*-inositol-labeled *Lilium longiflorum* pistils. Plant Physiol 45:92–194

Labarca C, Loewus F (1972) The nutritional role of pistil exudate in pollen tube wall formation in *Lilium longiflorum.* 1. Utilization of injected stigmatic exudate. Plant Physiol 50:7–14

Labarca C, Loewus F (1973) The nutritional role of pistil exudate in pollen tube wall formation in *Lilium longiflorum.* 2. Production and utilization of exudate from stigma and stylar canal. Plant Physiol 52:87–92

Linskens HF (1975 a) The physiological basis of incompatibility in angiosperms. Biol J Linnean Soc 7 (Suppl) 1:143–152

Linskens HF (1975 b) Incompatibility in *Petunia.* Proc R Soc London, Ser B 188:299–311

Linskens HF (1981) Barreras en la fertilización de las plantas superiores. Kurtziana (Cordoba, Argent) 14:7–20

Linskens HF (1983) Pollination processes: understanding fertilization and limits to hybridization. In: Meudt WJ (ed) Strategies of plant reproduction. Beltville Symposia in Agricultural Research 5. Allanheld-Osmond, Granada, pp 35–50

Linskens HF, Kroh M (1967) Incompatibilität der Phanerogamen. In: Ruhland W (ed) Encyclopedia of plant physiology, vol 18. Springer, Berlin Heidelberg New York, pp 506–530

Li Yi-qin, Croes AF, Linskens HF (1983) Cell wall proteins in pollen and roots of *Lilium longiflorum:* extraction and partial characterization. Planta 158:422–427

Manocha MS, Ross Colvin J (1967) Structure and composition of the cell wall of *Neurospora crassa.* J Bacteriol 94: 202–212

Mühlethaler K, Linskens HF (1956) Elektronenmikroskopische Aufnahmen von Pollenschläuchen. Experientia 12: 253–254

Shirvanna KR, Heslop-Harrison Y, Heslop-Harrison J (1982) The pollen-stigma interaction in the grasses. 3. Features of the self-incompatibility response. Acta Bot Neerl 31: 307–319

Talmadge KW, Keegstra K, Bauer WD, Albersheim P (1973) The structure of plant cell walls. 1. The macro-molecular components of the walls of suspension-cultures sycamore cells with a detailed analysis of the pectic polysaccharides. Plant Physiol 51:158–173

Yang ZH, Tsao TH (1981) Physicochemical properties of pollen wall proteins of *Cucurbita pepo* and *Luffa cylindrica* and their possible role in 'recognition' reaction. Acta Gen Sin 8:75–83