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Localization of Phosphorus and Cation Reserves in *Lilium longiflorum* Pollen[1]

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ABSTRACT

Transmission electron microscopy of pollen from *Lilium longiflorum* Thunb. reveals electron-dense inclusions in storage body organelles ubiquitous in the cytosol. In ungerminated pollen, these inclusions are rounded in appearance and appressed to the inner surface of the smooth membrane of the storage body. During pollen germination, these inclusions become less rounded, smaller, and enclosed in storage bodies that have developed crenated membranes. Energy dispersive x-ray analysis reveals high levels of P, Mg, K, and Ca in the inclusions relative to other regions of the cytosol in which elemental signals can be obtained. The elemental composition and the degradation of inclusions during germination are offered as evidence for storage of phytin in these structures which are thus analogous to phytin storage globoids of seed tissues.

Energy dispersive x-ray analysis (EDX)[2] has been used extensively as a method of determining the elemental composition of globoid inclusions of protein bodies in seed tissues (14–16). Characteristically, the EDX spectra of seed globoids show large P and cation (most notably Mg, Ca, K) signals relative to those signals generated from surrounding cytosolic regions (13–18). The elemental composition of globoids is indicative of the presence of phytin which is composed of salts of *myo*-inositol hexa*kis*phosphate (14, 15).

In seeds, phytin may account for 60 to 90% of the total phosphorus content and it is present in amounts ranging from 1 to 3% of the dry weight (3, 7, 15). The sequestration of Pi, *myo*-inositol phosphates, free *myo*-inositol and essential cations into phytin bodies provides an important stable molecular reserve for use during germination (14).

Phytin has recently been found to occur in pollen grains (9). Electron dense particles similar in appearance to seed phytin globoids have been seen in thin sections of mature pollen (4, 10, 21, 24) and their dissolution during germination (6) parallels the fate of seed phytin particles. We have used EDX analysis to determine if the electron dense particles in *Lilium longiflorum* pollen have chemical characteristics comparable to those reported for seed globoids. Preliminary reports have appeared (1, 22).

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[2] Abbreviations: EDX, energy dispersive x-ray; TEM, Transmission electron microscopy; SEM, scanning electron microscopy.

MATERIALS AND METHODS

Plant Material. Pollen from *Lilium longiflorum* Thunb., cv Nellie White harvested from commercial bulb fields during 1982 and 1985 seasons was used in this study. Anthers gathered during the final bud stage preceding anthesis were dried at room temperature and the pollen recovered by passage through a No. 10 brass screen. Pollen was stored in plastic containers at −20°C until used. Electron microscope studies were performed on ungerminated pollen and on pollen that had been germinated for 2 h at 30°C as described by Dickinson (5).

Fixation and Embedding. For TEM studies, tissue was fixed at 4°C in 2.5% glutaraldehyde and 2% paraformaldehyde in 25 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.2), for 5 h and then postfixed in 1% OsO_4 in 10 mM sodium cacodylate buffer for 2 h. After fixation, the tissue was dehydrated through an acetone series, infiltrated with Spurr's resin, and polymerized. Tissues were sectioned on a Reichert OM-U2 microtome equipped with a glass knife, mounted on formvar-coated copper grids and stained with 2% uranyl acetate and Reynold's lead citrate. For EDX analysis, tissues were prepared similarly but the OsO_4 postfixation was deleted, sections were thicker (about 200 nm), and no stain was applied (13).

TEM and EDX. Analyses were performed on a Hitachi H600-2 transmission electron microscope equipped with a H6010A scanning unit and a KEVEX x-ray detector. TEM was performed at a 50 kV accelerating voltage. EDX analyses were carried out at an accelerating voltage of 75 kV in the scanning-TEM mode. X-ray spectra were taken for 120 s (at 30–40% dead time) and elemental peak data were analyzed with a UNISPEC 7000 system (KEVEX). This system allowed complete background subtraction and elimination of artifacts such that only peaks of elements of interest were analyzed. Peak data were obtained by counting total x-ray signals within windows that centered on K_α emission peaks of each element and included the complete peak area but without overlap from other elemental peak areas. K_β signals were not significantly above background and were not analyzed.

A minimum of 9 x-ray scans were taken on each of 8 ungerminated pollen grains at a magnification sufficient to scan completely the single electron-dense inclusions (×300,000). Following background subtraction, peak areas of elements of interest were obtained, and the elemental composition of each structure that had been analyzed was calculated as percentages of total peak area. A total of 17 x-ray scans were taken in areas of cytoplasm not containing electron-dense inclusions (but in areas where electron density was high enough to detect elemental peaks above background noise levels) on 8 ungerminated pollen grains in order to determine baseline elemental composition values for cytoplasm. Thirteen x-ray scans were taken of electron-dense inclusions within germinated pollen to determine changes in elemental composition after germination. Scans of the resin bed were taken in an area free of tissues to determine background

levels and artifactual peaks to be subtracted from data obtained on tissues.

RESULTS

TEM. Micrographs of thin sections from ungerminated and germinated (2 h) pollen are presented in Figure 1. Ungerminated pollen grains are densely packed with lipid bodies, mitochondria, and numerous membrane-bound storage bodies (Fig. 1A), the latter closely resembling protein bodies as found in seed tissues (14–16, 20). The storage bodies range in size from 0.2 to 1 μm in diameter and, in counts taken from low magnification micrographs, average about 8/10 μm^2 in sections from ungerminated pollen (data not shown). Forty-four percent of these storage bodies had electron dense inclusions within the electron transparent matrix of the organelle. These inclusions were typically rounded in shape and tightly appressed to the inner surface. Only about 5% of the storage bodies contained evidence of more than one inclusion (Fig. 1B). The varying sizes and shapes of storage bodies, as well as the varying size and occurrence of inclusions, are indications of the amorphous structure of the storage bodies, and the relatively small volume occupied by inclusions. The inclusions are apparently small enough and eccentrically located within the storage body, such that they are not always within the plane of section through a body. A separate limiting membrane does not exist around the inclusion (Fig. 1, B–D).

After germination, the limiting membranes of these storage bodies develop a crenated shape that is not seen in sections from ungerminated pollen grains which have undergone the same procedures of fixation, dehydration, and embedding. Inclusions lose their rounded appearance and also appear to be reduced in size in all sections examined, presumably due to loss of material rather than due to tangential sectioning of inclusions. Loss of material is also indicated by a decrease in the number of storage bodies with inclusions in germinated pollen (Fig. 1, E–G).

EDX. Elements found in the electron-dense inclusions by EDX analysis include Mg, P, S, K, and Ca as well as Si, Cl, and Cu

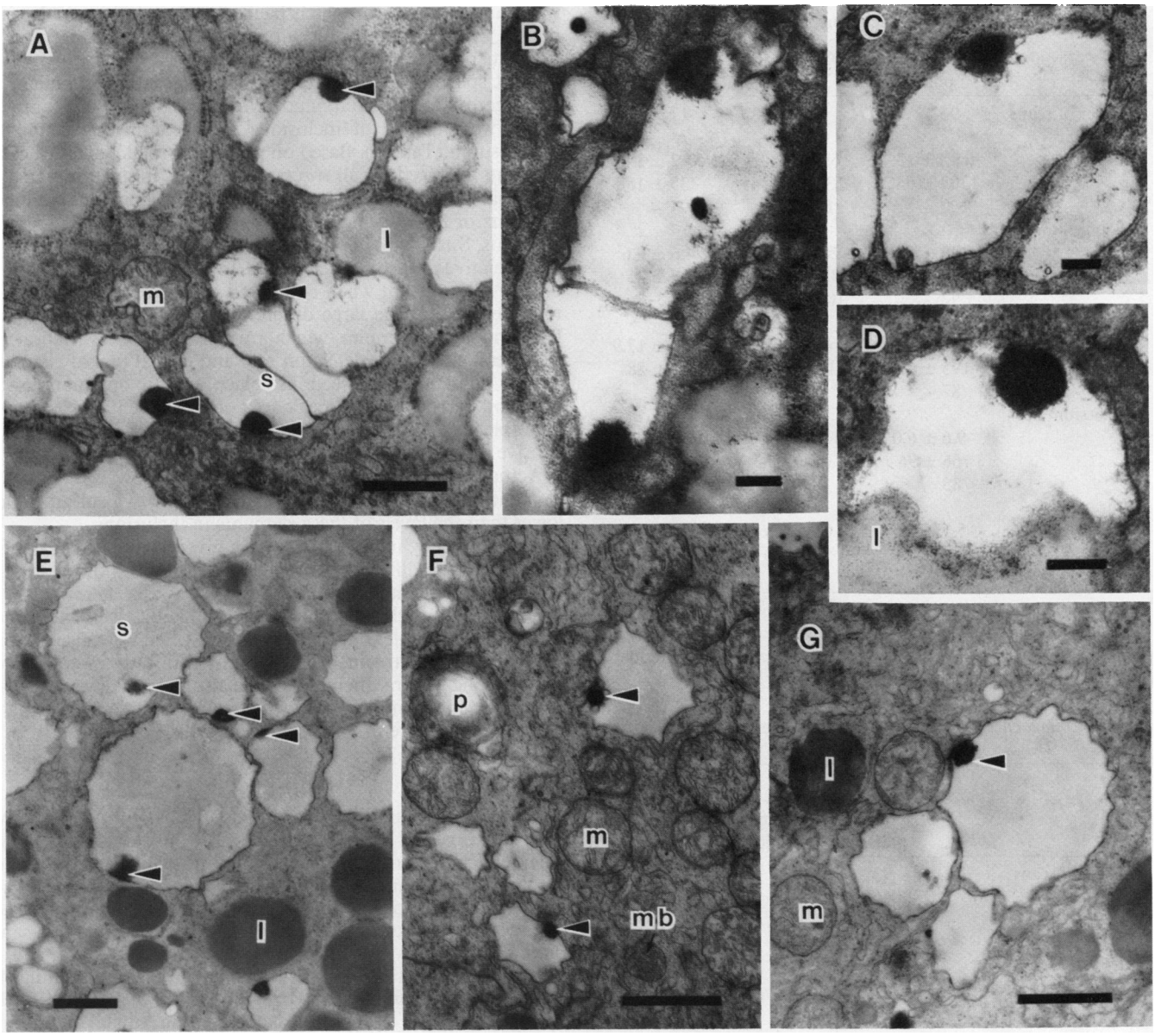

FIG. 1. TEM micrographs of ungerminated (A–D) and 2 h germinated (E–G) sections from lily pollen. Magnification/bar symbol: A, ×25,000/0.5 μm; B, ×34,000/0.2 μm; C, ×29,000/0.2 μm; D, ×45,000/0.2 μm; E, ×9,000/1 μm; F, ×15,000/1 μm; G, ×14,000/1 μm. Letter symbols: l, lipid body; m, mitochondrion; mb, microbody; p, plastid; s, storage body. Pointer indicates electron-dense inclusion.

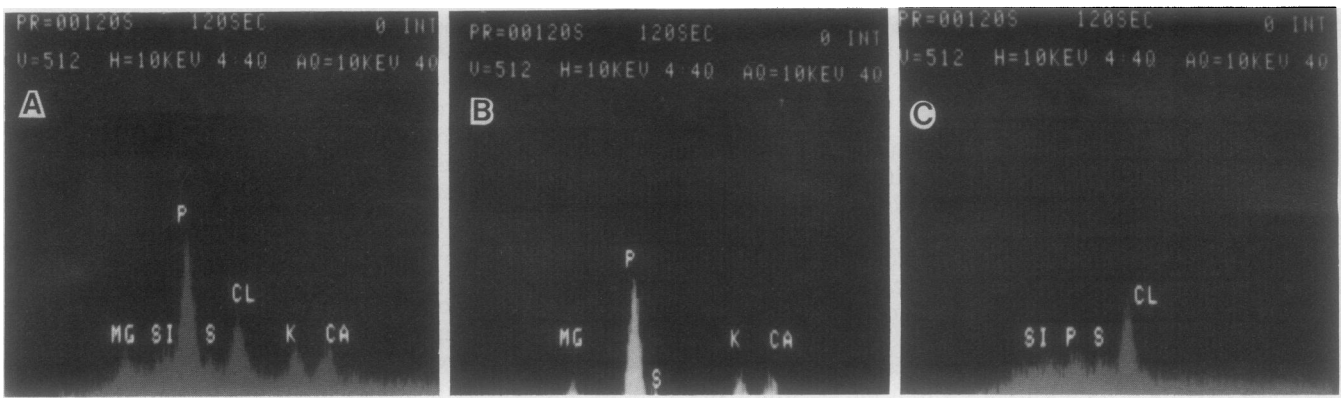

FIG. 2.. EDX spectra of ungerminated lily pollen. A, Spectrum of an electron-dense inclusion at low energy scale (0–5 Kev). Elements detected and K_α energy emission values in kev: Mg, 1.25; Si, 1.74; P, 2.02; S, 2.31; Cl, 2.62; K, 3.31; Ca, 3.69; Cu, 8.05 (off scale). B, Spectrum in A after subtraction of background and elemental peaks for Si, Cl, and Cu. C, Spectrum of cytosolic region under the same conditions of analysis used in A.

Table I. *EDX Peak Area Data from L. longiflorum Pollen*

	Inclusions		Cytosol
	Ungerminated pollen	Germinated pollen	
Number of scans	93	13	17
Phosphorus			
a	65.8 ± 13.1	56.4 ± 12.9	54.2 ± 24.7
b	1302 ± 641	2307 ± 1741	235 ± 168
c	100	100	94
Calcium			
a	12.9 ± 5.8	26.3 ± 6.0	4.6 ± 8.6
b	254 ± 132	1082 ± 723	19 ± 35
c	92	100	29
Magnesium			
a	11.6 ± 7.8	4.1 ± 3.4	12.4 ± 17.9
b	195 ± 95	165 ± 188	38 ± 58
c	87	92	41
Potassium			
a	9.6 ± 6.0	13.2 ± 14.1	5.3 ± 9.3
b	166 ± 96	386 ± 363	20 ± 39
c	85	85	29
Sulfur			
a	5.1 ± 4.0	0.3 ± 0.6	23.7 ± 15.1
b	59 ± 35	13 ± 23	118 ± 116
c	69	31	76

a, Averge (±SD) peak area values (as per cent of total peak area) for all scans containing that element. b, Raw data average (±SD) in x-ray counts of peak areas of each element for all scans. c, Frequency (percent) of peak signal appearance in scans.

Table II. *Coincidence of Cations with Phosphorus in EDX Analyses of Lily Pollen*

	Inclusions		Cytosol
	Ungerminated pollen	Germinated pollen	
Number of scans	93	13	17
P and (Mg + K + Ca)	66	10	2
P and (Mg + K)	7	0	3
P and (Mg + Ca)	8	2	2
P and (Ca + K)	7	1	0
P and (Mg or K or Ca)	5	0	3
P only	0	0	7

which are components of the embedding and mounting materials (Fig. 2A). Cu is not seen in Figure 2 since its high energy K_α peak is beyond the scale of the spectra shown here. A difference spectrum (Fig. 2B), in which background and elemental peaks due to embedding and mounting were subtracted, is used to integrate peak areas of elements of interest.

EDX spectra of inclusions (93 from ungerminated pollen, 13 from germinated pollen) and cytosolic regions (17 samples) which were devoid of inclusions show contrasting elemental composition (Table I). Based on percent of total peak area of all elements averaged for all spectra, inclusions in ungerminated or germinated pollen are high in P and contain significant but lower amounts of Mg, K, and Ca (Table I, row a). Relative to these elements, S is low. After germination, Ca is higher in the inclusions relative to the other elements. In the cytosol, apart from P, only S is significant when compared on the basis of percent of total peak area. The large differences in total elemental signal from individual samples, which are due in part to variations in thickness of sections, detection rate and partial loss of elements during preparation of sections, place a limit on quantitation based on percent of total peak area of all elements as presented in Table I (row a) but the data still allow for comparison of relative differences between samples.

Actual peak integration values (Table I, row b) show that elemental levels of P, Ca, Mg, and K in the cytosol are much lower than the levels of these elements in inclusions.

The frequency in appearance of a given elemental peak also emphasizes the differences in elemental composition of storage body inclusions and surrounding cytosol (Table I, row c). P is detected in all inclusions that were sampled as well as 94% of the cytosolic samples but Ca, Mg, and K which were also present in 85 to 100% of the inclusions appeared in only 29 to 41% of the cytosolic spectra. Inclusions were also more likely to have all three cations accompanying P whereas cytosolic samples usually lacked one or more of these cationic signals (Table II).

DISCUSSION

Organelles containing electron-dense inclusions in pollen have been described by others (4, 10, 21, 14) but the correlation of chemical characteristics of such structures with those of phytin globoids as encountered in seeds has not been previously reported. Evidence of phytin in *Lilium longiflorum* pollen's electron dense inclusions includes: (a) EDX spectra of a large sampling of inclusions which show much higher P, Mg, Ca, and K signals relative to the general cytosolic regions, results similar to those obtained from seed phytin globoids (3, 8, 14–16, 19); (b) an ultrastructure of inclusions enclosed in single membrane storage bodies analogous to that of a seed protein body containing a large single globoid as seen in tissues with high Ca plus Mg (18); and (c) degradation of these inclusions during germination;

an indication of utilization of stored materials such as occurs in seed globoids (14). Moreover, SEM-EDX spectra of freeze-fractured pollen grains obtained at low magnifications (×5000) contain P and K peaks of similar magnitude, each about 40% of the total peak area in the spectra (data not given). The freeze-fracture procedure preserves solutes that might otherwise be lost in part, as appears to have occurred in TEM sections in which the K peak was only 10%.

Phytic acid is known to be present as a phosphorus reserve in *L. longiflorum* pollen (11, 22). EDX data and ultrastructural features implicate the inclusion-containing bodies of lily pollen as the storage organelle of phytin. Conclusive proof will require isolation of these putative storage bodies followed by biochemical analysis. as has been done for globoids of rice and wheat grain (11, 25).

The germination-induced degradation of lily pollen inclusions (Fig. 1, E–G) may be caused by the activity of one or more of the phytases present in this pollen. One constitutive form (6), optimally active at pH 5, is similar to wheat bran phytase (3) and the phytase associated with globoids from rice (26). Another constitutive phytase, optimally active at pH 8 and dependent on Ca ions for maximal activity, has been described recently (23). An induced form, optimally active at pH 6.5, reaches maximal activity 2 h post-germination (6). The increased Ca signals in EDX spectra of germinated pollen storage bodies suggests an involvement of the pH 8, Ca-activated form.

Sections from germinated pollen contain more Ca relative to other elements than ungerminated pollen in the inclusions (Table I). Bound Ca may account for this in part. It should be noted that the germination medium used here (5) contains 1.27 mM Ca $(NO_3)_2$ and 1 mM KNO_3. If medium-derived Ca contributes to that retained by inclusions in germinated pollen, one might also expect a correspondingly higher level of K relative to that found in ungerminated pollen (even with partial extraction) and this is not observed. The medium-derived Ca contribution to cytosol is also negligible based on EDX data (Table I).

Our future efforts will deal with isolation and biochemical characterization of the lily pollen storage bodies and the subcellular localization of the various phytases. A recent development in the preparation of pollen sporoplasts (protoplasts) (2, 12) has promise as a practical route to the isolation of intact storage bodies and globoids. With this approach, a study of the relationship between phytases and phytate breakdown at the subcellular level is possible.

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