Origins and Genetic Nonvariability of the Proteins Which Diffuse from Maize Pollen

by Elizabeth K. Porter*

The major function of pollen is to deliver the sperm nuclei to the embryo sac. It does this by germinating and producing a pollen tube and thus provides a relatively simple developmental system for study. Mutants for many pollen functions are accessible, as it is a haploid cell. Mature pollen was fractionated into diffusible proteins, soluble proteins, and proteins insolubly associated with membrane or wall; these protein fractions have been quantified and cataloged by native and SDS polyacrylamide gel electrophoresis. Diffusible proteins are localized in the pollen grain wall whereas soluble proteins are cytoplasmic. The roles of haploid and diploid genomes in specifying these proteins is discussed. Pollen from maximally divergent maize lines was examined for quantitative and qualitative variation in the diffusible proteins. A surprising conservation was found for these proteins indicating some functional role which is, at present, unknown. Initial experiments on the incorporation of ^{35}S-methionine into germinating pollen indicate that major representatives of the diffusible proteins are made within the pollen grain itself. They are presumably included in the pollen wall during development and diffuse out through the pore region. Studies with pollen mRNA and experiments on incorporation of ^{35}S-methionine into developing anthers are underway and will identify the origin of these proteins. A knowledge of the basic developmental biology of maize pollen is a prerequisite to its judicious use as a monitor of environmental mutagens.

Introduction

Pollen represents the male gametophytic stage in the life cycle of flowering plants in which the haploid genome is expressed. It is adapted to function in germination and pollen tube growth for the transport and delivery of the sperm nuclei. The growth of the pollen tube also involves synthesis and assembly of membrane and wall components. Pollen thus presents a relatively simple and interesting developmental system with the advantage that, being haploid, mutants in many aspects of pollen development and metabolism may be recovered directly.

Pollen Wall Proteins

The first report showing the presence of proteins in the pollen grain wall came from light microscopic

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observations of pollen after staining for protein and enzymes (1). Proteins were localized in both the outer layer of the wall—the exine—and the inner layer—the intine. Since then, electron microscopic observations have shown protein to be present in the pollen walls of more than 70 flowering plant species (2). Wall proteins are released rapidly on moistening of the pollen grain (3) and show considerable heterogeneity. The proteins released include a number of enzymes (4-6), and failure of pollen to germinate after short-term washing (7) suggests that such rapidly diffusing enzymes may function in normal pollen germination. The diffusible protein fraction from a number of different pollen samples is also involved in inter-and intraspecific incompatibility responses (8-14).

The development of the pollen grain wall has been studied in a wide range of species (15, 16) and the origin of the wall proteins has been a subject of speculation. There are three possible sources of origin for the pattern information and molecular components of pollen. One of these is the anther

tissue surrounding the developing pollen grain—in particular, the closely associated tapetal cells, which are known to act as "nurse cells" in contributing material, including protein, to the outside of the pollen grain (5, 11, 17, 18). An alternative source of information, which is also sporophytic in nature, is the maternal cytoplasmic determinants remaining in the developing grain from the primary meiocyte. The final possible site of control lies in the gametophytic genome of the pollen grain vegetative nucleus; it is known that protein inclusions of the intine are derived from the microspore cytoplasm as it develops (16, 19). It is generally accepted that exine proteins are sporophytic in nature, being contributed by the tapetum, while intine proteins are gametophytic in origin (20). This supposition has been supported by data on the localization of proteins associated with either sporophytic or gametophytic incompatibility systems (11, 21–23).

This report presents data on some qualitative and quantitative aspects of maize pollen proteins.

Maize Pollen Proteins

Maize pollen has been separated into diffusible proteins, soluble proteins, and those that remain insolubly associated with wall and membrane fragments. Pollen was place in chilled 0.1M sodium phosphate buffer (pH 7.5) for various times up to 90 min and then pelleted at 20,000g for 5 min at 5°C. The supernatant comprised the diffusible protein fraction, and the soluble proteins were extracted by grinding the pelleted pollen in a glass homogenizer until > 90% grain breakage has occurred. The homogenate was spun at 20,000g for 60 min at 5°C to yield a soluble protein fraction in the supernatant and an insoluble protein fraction in the pellet. After diffusion, examination of pollen by phase contrast light microscopy and scanning electron microscopy indicated that the grains remained intact; there was a slight swelling in the pore region but no bursting of grains.

The protein fractions were subjected to polyacrylamide gel electrophoresis on both nondenaturing (native) and denaturing (SDS) gels. Figure 1 shows the diffusible proteins from a maize pollen sample which had been in buffer for 90 min. The protein banding pattern, shown by Coomassie Blue staining, is simple and clear in both gel systems. In the native gel there is some retention of material at the gel origin, but the main feature is the three fast moving bands that comprise a large part of the total protein. The SDS gel separates the diffusate into 17 major bands ranging in size from 80,000 to less that 12,400 daltons. These gel patterns are both reproducible.
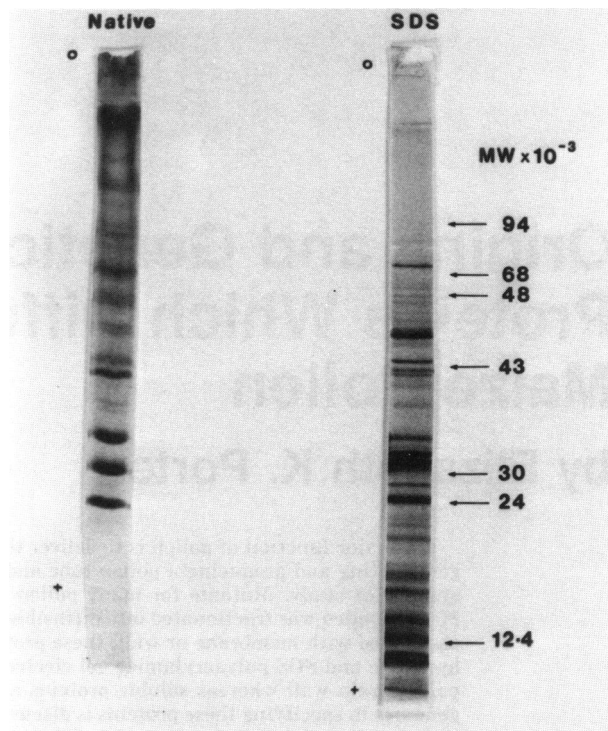

FIGURE 1. Native and SDS polyacrylamide gels of maize pollen diffusate. Maize pollen (200 mg) from a hybrid between Std F and Std S inbreds was allowed to diffuse in PO_4 buffer for 90 min as described in the text. Native acrylamide slab gels contained 10% (w/v) acrylamide, 0.1% (w/v) ·N,N methylaminebisacrylamide (Bis), 0.375M Tris-HCl pH 8.9. The stacking gel contained 5% (w/v) acrylamide, 0.07% (w/v) Bis, and 0.125M Tris-HCl pH 6.8. Gels were polymerized by bringing the gel solution to 0.5% (w/v) ammonium persulfate and 0.5% (w/v) N,N,N^1,N^1, tetramethylethylenediamine. Gels were run at 4°C with 10mM Tris, 77mM glycine electrode buffer at 10 mA for 1 hr then 25 mA for 2 hr. The discontinuous SDS gel system of Laemlli was employed with 5% (w/v) acrylamide in stacking gels, 15% (w/v) in separating gel. Gels were run at 30 mA for 4.5 hr. All slab gels were run in an apparatus similar to that described by Ames (25). Gels were fixed and stained for protein with Coomassie Blue R-250 (26). Molecular weight markers on six gels were as follows: rabbit phosphorylase a (Sigma 94,000 d), bovine serum albumin (Sigma 68,000 d), bacterial a-amylase (Sigma type II-A 48,000 d), chicken ovalbumin (Sigma 43,000 d), carbonic anhydrase (Worthington 30,000 d), trypsin (Worthington, TPCK-treated 24,000 d), horse heart cytochrome C (Sigma type IV 12,400 d).

Figure 2 shows a time course of diffusion taken over periods of 0–5, 5–10, 10–30, and 30–90 min. The pollen was pelleted between each period of diffusion and extracted further in fresh buffer; the diffusates were separated on a SDS gel. The proteins in the two bands marked by arrows were very rapidly emitted and decrease in the longer diffusates, while the other bands were represented in the same proportions after the first 5 min of extraction. It has been shown that exine bound proteins are
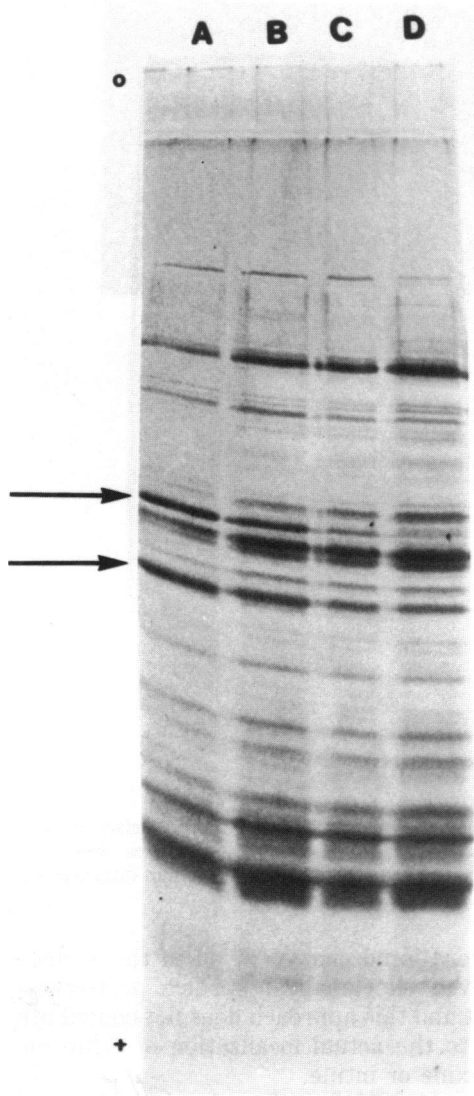
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FIGURE 2. Time course for diffusion of proteins from maize pollen; proteins were separated on a SDS gel. *(A)* 0–5 min; *(B)* 5–10 min; *(C)* 10–30 min; *(D)* 30–90 min. Arrows mark protein bands which are eluted rapidly and decrease with time.
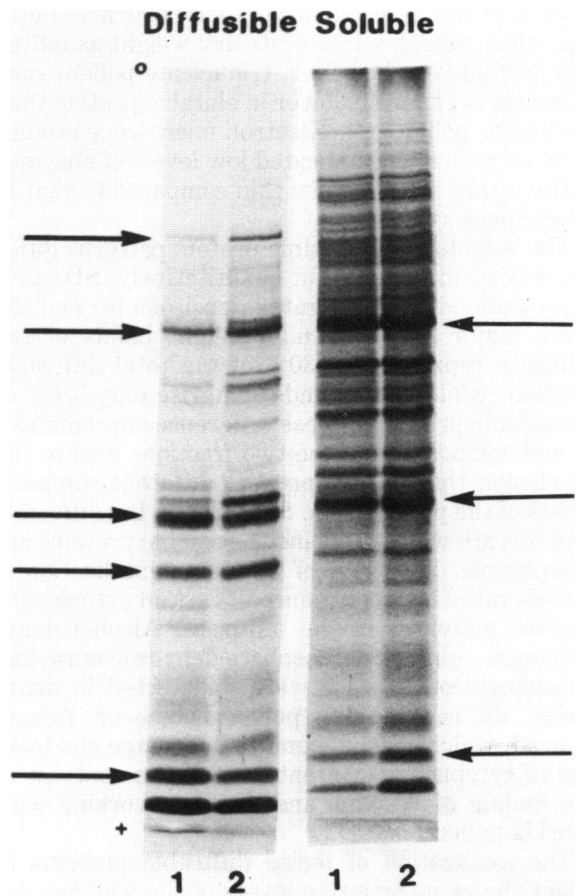

FIGURE 3. Comparison of diffusible and soluble pollen protein fractions from two individual maize plants separated on a SDS gel. Arrows mark bands which react positively with periodate-Schiff reagent indicating the presence of carbohydrate moieties (*43*).

generally released very rapidly upon moistening of pollen, whereas intine bound proteins diffuse out more slowly through the pore regions (*21,27*).

In Figure 3, diffusible and soluble protein extracts from two individual maize plants are shown after separation on a SDS gel. The insoluble protein fraction was treated with SDS and subjected to electrophoresis, but suffered high interference from the presence of large amounts of starch and polysaccharides, and so it is not shown. The bands marked with arrows were positive for periodate-Schiff's stain indicating that these proteins are probably glycosylated. Glycoproteins have also been reported to be present in the pollen diffusates from a number of other species (*11, 13, 14, 20*). The soluble protein fraction is much more complex than the diffusible protein fraction, with many high molecular weight components. Many of these are probably enzymes; at least 40 enzyme activities have been reported in pollen grains of higher plants (*28*). The two most rapidly emitted protein bands of the diffusate are almost absent from the soluble fraction.

The diffusates were low in protein containing only 10–15% of the total soluble protein which itself is only 4–6% of pollen dry weight, although values for maize pollen protein of up to 28% dry weight

have been reported (29). The maize diffusate is low in protein compared to other species such as Petunia, which releases 18% of its dry weight as diffusible protein (30); however, trinucleate pollen, such as maize, is generally lower in elutable protein than binucleate pollen (31). Electron microscope studies of maize pollen also indicated low levels of enzymes in the intine, with is very thin compared to that in other species (32).

The soluble and diffusible protein patterns differ not only qualitatively, but quantitatively. SDS gels were scanned on a calibrated densitometer and the three major low molecular weight bands of the diffusate represent 28–30% of the total diffusible fraction, while those bands comprise only 5–6% of the soluble proteins. These differences in complexity and composition of the two fractions lead to the conclusion that they represent different compartments of the pollen grain. Specifically, the diffusible proteins are well bound and the soluble proteins are cytoplasmic. The idea of compartmentalization is corroborated by the absence of certain cytoplasmic enzyme activities in the diffusate. Alcohol dehydrogenase, glucose-6-phosphate dehydrogenase and phosphoglucoisomerase were undetected in maize pollen diffusates until pollen had been freeze-thawed, which caused membrane damage and leakage of cytoplasmic contents. This is in contrast to the finding of Weeden and Gottlieb working with Clarkia pollen (33).

The localization of maize diffusible proteins in either the exine or intine layers of the wall has yet to be established. The surface of a maize pollen grain can be seen in Figure 4. The area around the single pore is the only place at which the intine is exposed (Fig. 4a). Higher magnification of the surface (ca. 60,000 ×) before and after diffusion for 90 min (Fig. 4b, 4c) shows no significant change in the pollen surface patterns. Similar findings were reported for pine pollen, whereas pear pollen showed marked changes after diffusion (7). I have made some calculations of the surface area of the pollen grain that would be occupied by the total amount of diffusible proteins. The average size of a maize pollen grain is 80 μm diameter; 1 mg of pollen represents approximately 2000 grains and produces approximately 2 μg diffusible protein. Assuming that the protein are globular and that the average size and shape is similar to a typical, medium-sized proteins such as hemoglobin (34), the number of protein molecules eluted from a single grain would cover only 0.2% of the surface area. The diffusate does contain other substances such as pigments, but it is likely that those small amounts of material washing off the outside of the grain would not be seen on a scanning electron micrograph. It can be
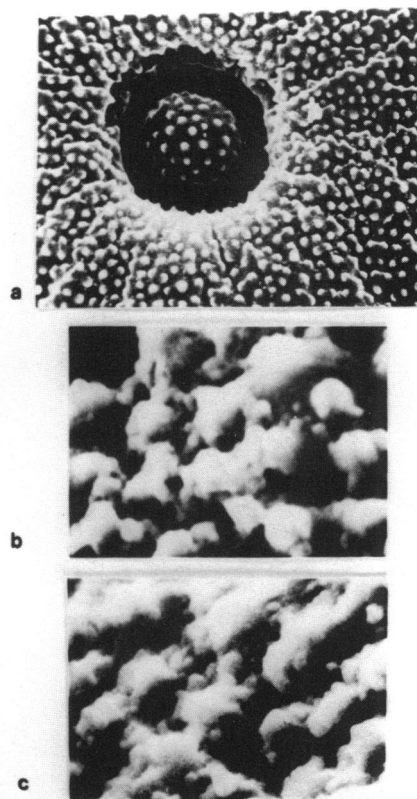

FIGURE 4. Scanning electron micrographs of the surface of maize pollen grains: (a) surface showing pore region, ca. 6000×; (b) surface, ca. 60,000×; (c) surface, after 90 min diffusion, ca. 60,000×.

seen that there is no change at all in the surface structure, even the smallest surface projections remain intact and this approach does not contribute any further to the actual localization of diffusible proteins in exine or intine.

The development of the exine of maize pollen has been studied (35, 36), and none of the electron microscopic studies of this or other grass exines indicate a massive transfer of tapetal material to the exine (37). However, the complexity of proteins held in the intine is remarkable; a least 30 components have been separated from the intine of Rye pollen (30, 37). The Gramineae have imcompatibility mechanisms of the gametophytic type. It is likely, therefore, that most of the proteins seen in maize pollen diffusates are intine borne; the most probable candidates for exine borne proteins are those in the two rapidly diffusing bands seen in SDS gels. To determine whether these diffusible proteins are gametophytic or sporophytic in origin, a study of diffusates from a number of maize races was carried out to look for variants of the diffusible protein pattern.

Genetic Variability of Maize Pollen Proteins

Pollen diffusates from 40 races of corn were separated on native and SDS gels. The races chosen covered the widest possible genetic background from USA corn-belt corn, through the South American races, to the close relative of maize, teosinte.* The seeds for the South American races were supplied by the USDA Seed Storage Lab and the Maize Genetics Cooperative, University of Illinois.

The results of this survey of pollen diffusates show surprising conservation among the proteins. There were no electrophoretic variants of the proteins separated on native gels. The SDS gels also showed great conservation of the protein pattern; there were a few minor quantitative variations but no bands missing or new bands appearing in any of the races. The conservation applies not only to protein structure, but also to post-translational modifications such as glycosylation. The conservation of pollen diffusible proteins is in sharp contrast to the pattern of the anaerobically induced proteins in other tissues from these maize races, which show considerable variation (39).

The fact that the proteins are so conserved indicates that they have a functional role to play although none has, as yet, been assigned. The possible involvement of diffusible proteins in germination and growth of pollen tubes down the silks will be studied using diffusates from maize plants carrying gametophytic factors (40). If these proteins function in pollen/silk interaction, we may expect to find a class of male sterile mutants which bear normal pollen that lacks only certain diffusible proteins. Such pollen would be unable to effect fertilization, and mutants of this type may thus have gone largely undetected.
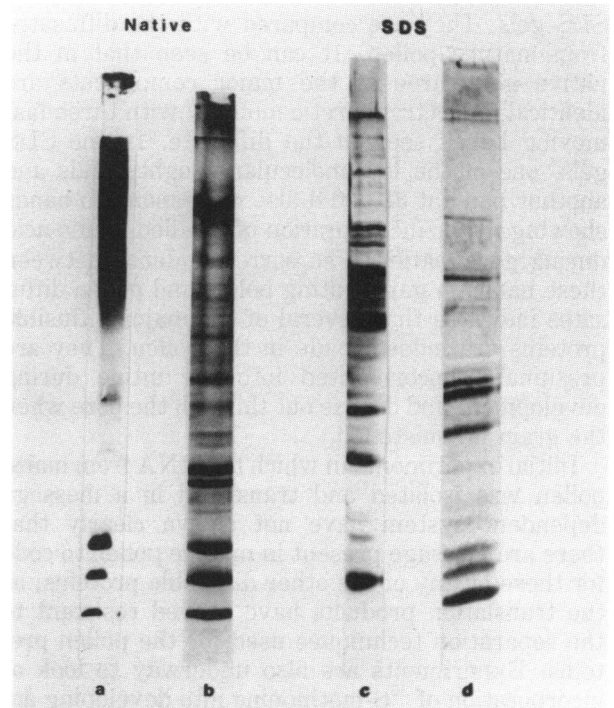
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* Races of corn used for study of pollen diffusates. Races of the USA: Tama Flint, Gaspe Flint, Papago Flour, Ohio Yellow, Yellow Dent Reid, Missouri Cob. Races of Mexico, Central America and West Indies: (a) Mexico-Tabloncillo, Harinoso de Ocho, Chapolote, Jala, Nal-Tel, Tuxpeno, Cacahuacintle, Pepitilla; (b) Guatemala-Imbricado blanco, Oloton; (c) West Indies-St. Croix Long ear. Races of South America: (a) Lowland Northern South America-Yucatan blanco, Guirna segregations, Cariaco amarillo, Negrito, Costeno blanco, Comun blanco, Puya blanco; (b) Amazon Basin-Pirinco composite, Moradoandaluz, Enano; (c) Lowland Southern South America-Caingang mangueirinha, Lenha white soft corn, Cristal, Cateto paulista, Sabuyo grosso, Canaria de Ocho, Paulista Dent; (d) Andean Complex-Pollo amarillo; (e) Central Andean-Blanco San Geronimo, Chulpi. Unknown origin: Red popcorn, South American Popcorn, Black Mexican Sweet (38).

FIGURE 5. Comparison of pollen diffusible proteins and proteins incorporating ^{35}S-methionine during germination *in vitro*. Liquid media containing ^{35}S-methionine (Amersham) at 20 µCi/ml was used to germinate pollen (approximately 15 mg pollen/ml of media) as described in the text. The soluble proteins were separated on polyacrylamide gels and autoradiographs of a native (a) and SDS (c) gel are shown next to diffusates of mature pollen separated on the same gels and stained for protein (b, d).

Pollen Proteins Synthesized during Germination

Maize pollen will germinate in liquid media and produce pollen tubes of × 4–8 pollen grain diameter in 60 min. The liquid media is based on Cook and Walden's recipe for solid germination media (41) without addition of agar. The solution is sterilized by autoclaving and stored at 4°C; the pH is adjusted to 6.4 immediately prior to use and germination is carried out at 26–29°C. The pH and temperature range tolerated by maize pollen for good germination (80%) is very narrow.

Pollen has been germinated in this manner in presence of ^{35}S-methionine to trace protein synthesis. The germinated pollen was pelleted at $500g$ for 5 min at 5°C and washed three times in fresh media to remove excess radioactivity. The pelleted pollen was homogenized and the soluble fraction subjected to gel electrophoresis as described above.

Figure 5 shows the newly synthesized proteins of germinating pollen separated on both native and

SDS gels. They are compared with the diffusates from mature pollen. It can be seen that in the native gel, three of the major components are identical in electrophoretic mobility with three fast moving bands seen in the diffusate. In the SDS gels, one of the low molecular weight bands and another band at 32,000 d also correspond to bands showing major incorporation of labelled amino acid during germination. The correspondence between these bands in germinating pollen and pollen diffusates indicates that several of the major diffusible proteins are indeed made in the pollen. They are presumably incorporated into the intine during development and diffuse out through the pore when the grain in moistened.

Initial experiments in which the RNA from maize pollen was isolated and translated in a message dependent system have not shown clearly that there are message present in mature pollen to code for these or any of the other diffusible proteins, as the translation products have proved resistant to the separation techniques used for the pollen proteins. Experiments are also underway to look at incorporation of ^{35}S-methionine into developing anthers at various stages and preliminary results do not indicate any transfer of tapetal material to developing pollen. Moss and Heslop-Harrison carried out a cytochemical investigation of protein in developing maize anthers (*42*) and did not find any evidence of tapetal transfer from such "balance sheet" studies.

Conclusions

The diffusible protein fraction of maize pollen provides a relatively simple, reproducible and conserved pattern on polyacrylamide gels. The proteins are located in the external compartment of the pollen grain—the wall—and are readily accessible for study.

Major diffusible proteins are synthesized within germinating pollen grains, thus eliminating the tapetal cells as a source. However, we have not yet distinguished between "masked" maternal messages and new messages from the gametophytes as the source for this protein synthesis.

If pollen is to be used discerningly as a monitor of environmental mutagens and toxins, we will need to know much more about its basic developmental and molecular biology. At the very least, such studies should continue, in order to identify specific genes whose functions are essential for pollen structure and effective fertilization.

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