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In vitro protein synthesis in isolated microspores of *Zea mays* at several stages of development

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Summary. A new procedure has been used providing large and homogenous populations of pollen from maize at different stages of their development. In order to label proteins synthesized during the course of microsporogenesis, a method has been developed that allows an efficient uptake of amino acids in the microspores. Results are presented showing that during pollen development three specific steps are involved: an early period active in protein synthesis, followed by a rest period when starch is accumulated, and a third period preceding the sorting out of mature pollen grains and during which protein synthesis starts again at a relatively low level. New polypeptides, some of which are very basic, appear at the time of starch deposition and accumulate up to the mature stage.

Key words: Microsporogenesis – Pollen – Maize – Protein synthesis

Introduction

Pollen development is one subject of investigation in plants that has rapidly progressed in recent years (for review, see Raghavan 1987; Mascarenhas et al. 1984; Mascarenhas 1989). A number of experiments have been carried out concerning DNA and RNA synthesis during the course of microsporogenesis (Mascarenhas and Mermelstein 1981; Mascarenhas et al. 1984; Willing et al. 1984; Stinson et al. 1985; Mascarenhas 1989). In contrast, less information is available at the protein level. Among the results, the activity of some enzymes that are determined after meiosis has been studied in relation to the microspore developmental stage. This is the case of multimeric enzymes such as alcohol dehydrogenase, catalase, and beta galactosidase (Frova et al. 1987; Singh et al. 1985). The synthesis of some enzymes during microsporogenesis has also been studied (for a review, see Mascarenhas 1989). In more general studies, Zarsky et al. (1985) have shown that during *N. tabacum* microsporogenesis, both soluble and insoluble proteins exhibit an inital rapid rise followed by a decrease of accumulation during pollen maturation. More recently, Vergne and Dumas (1988) and Delvallée and Dumas (1988), using one-dimensional gel electrophoresis, have detected a set of new proteins appearing at the time of the second mitotic division, and have observed changes in relation to the stage of pollen development.

We have succeeded in obtaining pure populations of *Zea mays* microspores at several stages of development and also in establishing an efficient system of in vitro labeling of the newly synthesized proteins. With these new techniques, we have analyzed through two-dimensional gel electrophoresis the accumulation and the synthesis of proteins at the early, mid-, and late stages of development of microspores. Results presented here show at the protein level that pollen development is a stepwise process and that each of the steps can be characterized by a specific pattern of protein synthesis.

Materials and methods

Plant culture and pollen isolation

Corn plants (*Mo* 17 variety) were grown in a culture room at 26 °C during the day and 20 °C at night. Relative humidity was 60% with an illumination of 5,000 candles for 15 h/day. Tassels were collected from plants prior to their emergence to determine early stages of pollen development, and or after emergence to determine middle or later stages of development. Spikelets were

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dissected from the collected tassels and homogenized using a Polytron tissue grinder. The pollen and debris obtained were filtered successively through 100-, 50-, and 25-μm nylon sieves. Pollen samples retained on the sieves were rapidly resuspended in water, then centrifugated at low speed (4,000 *g*) to avoid pollen damage. Sedimented material was deposited on a 30%–100% percoll gradient and centrifugated at 4,000 *g* for 15 min to eliminate sporophytic debris. By using such a procedure, homogenous and pure pollen populations corresponding to defined stages of development can be obtained. Number of grains was determined using a hematocytometer. The final volume of pelleted pollens was adjusted to get an approximately equal number of pollen grains in samples, taking into account the volume of microspores (Table 1). Viability of mature pollen was tested by incubation on a germination medium according to Cook and Walden (1965). Collected material was used immediately or frozen in liquid nitrogen and stored at −80 °C until required for experiments.

Protein labeling in isolated microspores

Immediately after their extraction from spikelets, samples of microspores at different stages of development were prepared, each containing approximately the same number of grains. They were incubated for 2 h in a medium containing 1 m*M* H_3BO_3, 20 m*M* $Ca(NO_3)_2$, 4 m*M* $MgSo_4$, 0.6 m*M* KNO_3, 6% sucrose, 14 m*M* creatine phosphate, 1 m*M* ATP, 0.4 m*M* GTP, and 40 μ*M* each amino acids, except methionine, which was added as ^{35}S-Met (Amersham, 47.1 TBq/mmol) at a concentration of 37 kBq/μl of medium. Incubation was performed in the dark at room temperature with vigorous shaking.

Protein extraction and electrophoresis

Labeled or unlabeled pollen grains were ground in liquid Nitrogen in a Spex 6700 Freezer Mill (Bioblock, France). The powder obtained was solubilized in a phenolic extraction buffer according to Hurkman et al. (1986). After centrifugation at 20,000 *g*, proteins contained in the phenolic phase were precipitated by 0.1 *M* ammonium acetate in methanol at −20 °C overnight. After centrifugation at 100,000 *g*, the pellets were dissolved in a 9.5 *M* urea lysis buffer according to O'Farrell (1975). For other experiments, samples were dissolved in a SDS extraction buffer and proteins were precipitated by acetone according to Hurkman et al. (1986).

As the urea lysis buffer is required to dissolve proteins before their separation by the isoelectric focusing technique (IEF) according to O'Farrell (1975), protein content of each sample could not be determined by the usual methods. Each sample loaded on top of first-dimension gel corresponds to an equal number of pollen grains. Proteins were separated as indicated by O'Farrell (1975) with IEF in the first dimension and SDS-PAGE (7.5%–15% acrylamide) in the second dimension. Biolytes 3–10 (Biorad) were used as carrier ampholytes for the first-dimension run at 400 V for 16 h or at 400 V for 5 h, according to the NEPHGE (non-equilibrium pH gradient gel electrophoresis) developed by O'Farrell et al. (1977), in order to resolve very basic polypeptides. This system, used with carefully controlled experimental conditions (carrier ampholyte, time, and temperature), provided good reproducibility and resolution. Gels were stained with Coomassie Blue and were exposed for autoradiography to Kodak X-O mat ARS films or to Fuji X-ray films with intensifying screen at −70 °C for 2 or 3 weeks.

Results

Stages of pollen development

Several stages are discernible during microsporogenesis, from the formation of tetrads at the end of meiosis up to mature pollen. These stages are outlined in Fig. 1. Microspores resulting from tetrad (T) disruption present a central nucleus and a thin unpigmented wall (CN stage). Then spores increase in size and develop a large vacuole

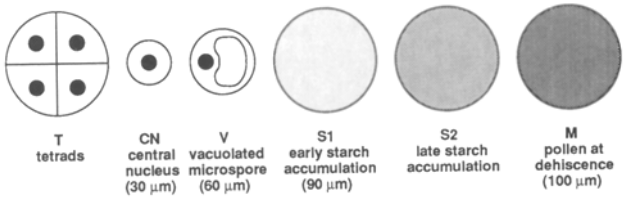

Fig. 1. Scheme showing different steps of microsporogenesis, from meiosis to mature pollen grain. The letters are used to design microspores at the different stages of development

Table 1. Incorporation of ^{35}S-methionine into proteins of microspores at different stages of their development. Samples containing an approximately equal number of microspores were incubated for 2 h in the labeling medium

Stage of pollen development	V	S_2	M
Morphological and cytological characteristics	Vacuolated bi- or tricell microspores Mitosis	Tricell microspores Starch accumulated Vol. of vacuole reduced	Dehydrated dehiscent Mature pollen
Pollen size, in μm	60	90	90–100
Volume of pelleted pollen with equal number of grains in the culture medium	250	700	700
cpm of ^{35}S-Met incorporated into proteins[a, b]	2,000	1,150	2,050

[a] The radioactivity reported corresponds to the number of cpm per microliter of urea containing lysis buffer, as indicated in 'Materials and methods'

[b] Each number corresponds to an average of 10 to 15 measurements

(V stage). At this time, the exine wall becomes yellow in color. Mitosis I and II take place and microspores start to accumulate starch. The S_1 stage corresponds to the beginning of the starch synthesis and the decrease of the vacuole in the tricellular pollen grain. The S_2 stage corresponds to microspores where starch is accumulated. At the mature stage (M), the pollen grain is full of starch and is released from the anther (dehiscence). Viability of mature pollen has been tested by fertilizing the female flower. For all experiments reported here, V, S_2, and M stages were used. Characteristics of these stages are shortly outlined in Table 1.

Protein labeling

In order to achieve an adequate labeling of the proteins synthesized during the course of microsporogenesis, classical methods of in vitro incubation in the presence of a marker had to be modified, taking into account that the exine wall behaves as a very efficient barrier between the spore and its environment. Release of corn pollen cells from the exine wall, according to the procedure of Baldi et al. (1987) used for *Lilium,* proved to be unsuccessful. The best results of labeling were obtained when incubation of pollen grain was performed in a calcium-enriched medium developed for this purpose. In order to verify that this method does not alter protein synthesis, control experiments were done using in vivo conditions. Tassels were soaked in the ^{35}S-Met-containing medium for 4–6 h. In this case the labeling was very low but results obtained were very similar to those using in vitro incubation of isolated spores (not shown). Therefore, in vitro conditions do not disturb the normal protein synthesis.

The uptake of ^{35}S-Met in pollen grains during incubation was poor, so that ^{35}S-Met labeling of proteins appears at a low level for all stages of development (Table 1). However, a slight decrease of the incorporated ^{35}S-Met during the late S phase followed by an increase in the M stage was repeatedly observed in all assays (15 independent experiments). These differences correspond with a decrease in protein synthesis at the time of starch accumulation, as verified by two-dimensional gel electrophoresis analysis.

Separation of proteins by 2D-electrophoresis at three stages of pollen development

Two-dimensional electrophoresis of polypeptides isolated from microspores at three distinct developmental stages was carried out. Representative gels and their corresponding autoradiography are shown in Fig. 2. Repeated analysis produced the same basic pattern with only slight variations.

The comparison of the three stages revealed qualitative and quantitative differences in both accumulation and synthesis of polypeptides. As an example, more than 15 polypeptides (some are circled in Fig. 2, 1 A) present in the early stage (V) disappeared as soon as starch became synthesized (Fig. 2, 2 A). At this latter stage (S_2), more than ten polypeptides were new (some are boxed in Fig. 2, 2 A). Quantitatively, polypeptides accumulated during the S_2 stage, as shown by the intensity of staining of many spots (see spots b,c,d,i, and o in Fig. 2, 2 A), which increased from the V to the S_2 stage. In contrast, from mid-(S_2) to mature (*M*) stages (2 A and 3 A in Fig. 2), little qualitative differences were revealed; only a few number of spots have disappeared at the mature stage (spots d, e, l, and o). The weaker intensity of protein staining in the *M* stage relative to the S_2 stage could reveal a lower amount of proteins accumulated at the end of the microspore development.

Labeling experiments give information on protein synthesis. Autoradiographs (Fig. 2, 1 B–3 B) corresponding to the Coomassie Blue-stained gels provided interesting results. Synthesis of polypeptides was very active at the early stage (Fig. 2, 1 B), decreased thereafter (Fig. 2, 2 B), and re-increased at the mature stage (Fig. 2, 3 B). As expected, polypeptides which are only detected by their radioactivity in the early stage (V) accumulated at the following stage (S_2) and became visible by Coomassie Blue staining (e.g. see spots a, d, and e). Some polypeptides are synthesized in a short period of time (spots e, d and l) during the V or S_2 stages. These polypeptides disappeared in the mature stage. In contrast, some polypeptides were continuously synthesized (spots a, b, c, i, k, m, and those included in brackets, Fig. 2). Very basic polypeptides are characteristic of the S_2 and *M* stages and their synthesis started at the time of starch deposition (compare 2 A and 1 A in Fig. 2).

Discussion

New methods have been developed providing a successful approach to the protein synthesis during the whole course of maize microsporogenesis. First, large and homogenous populations of microspores were isolated and separated according to their developmental stage. Second, isolated spores were incubated in a simple medium in the presence of a marker (^{35}S-Met) in order to label newly synthesized polypeptides.

Results reported here reveal important changes in protein synthesis during microsporogenesis. The first to occur is concomitant with the beginning of starch deposition in vegetative cells. It is characterized by a drastic decrease in the synthesis of a number of polypeptides relative to young spores and, at the same time, by the synthesis of new ones. In particular, very basic polypep-

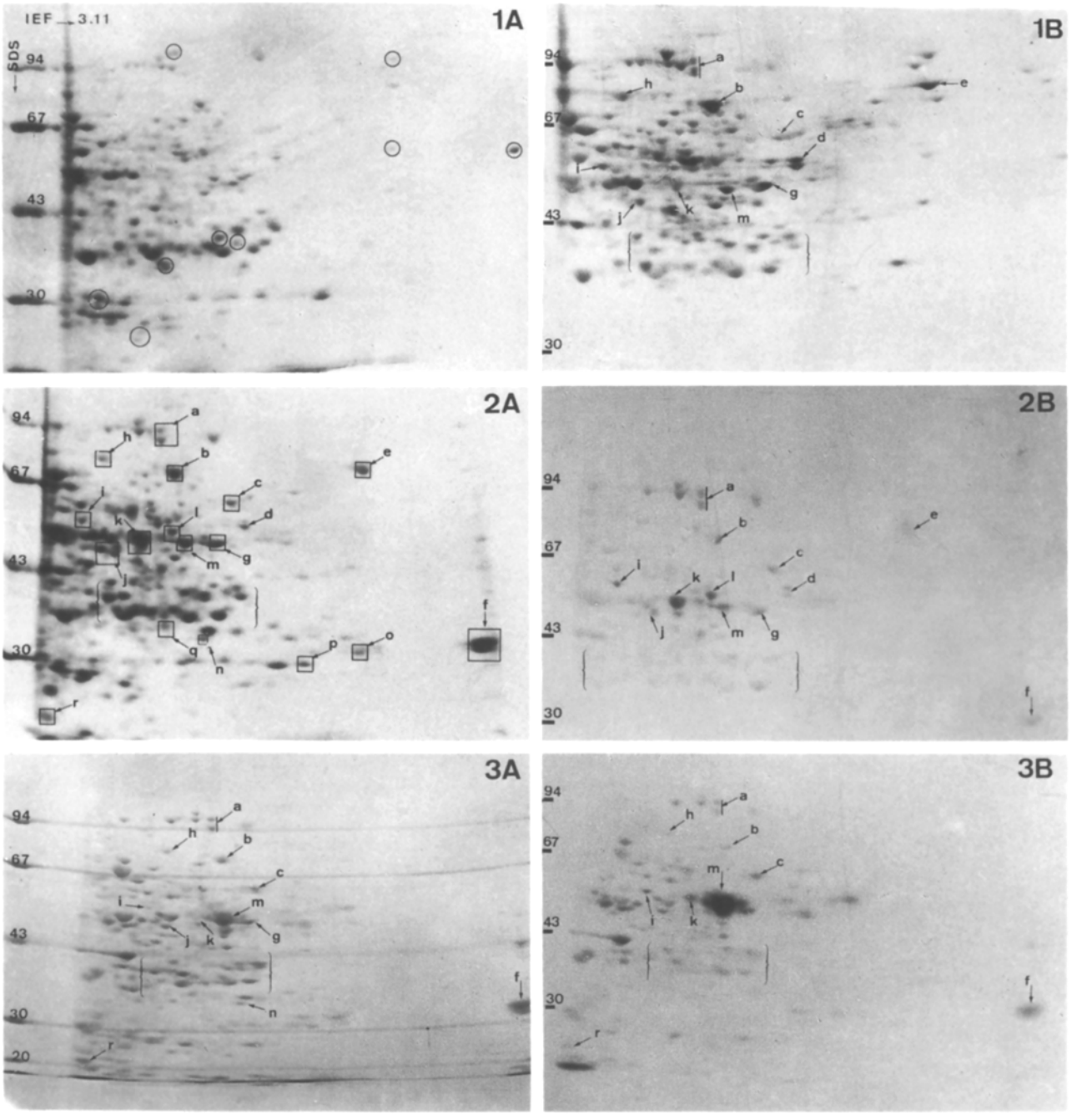

Fig. 2. Two-dimensional electrophoresis of proteins isolated from microspores at three different stages of development. *1*: V stage; *2*: S2 stage; *3*: M stage. The 2D-gels, after their Coomassie-Blue staining, are shown in the *left hand* side (*A*) and autoradiograms of the same gels are shown in the *right hand* side (*B*). Molecular weight markers are indicated in kDa. A number of spots, taken as example, are pointed out by *arrows* and *letters*. Some others are indicated as follows: (O), polypeptides present at the vacuolated stage (V) disappearing later; (□), polypeptides newly appearing, or whose synthesis drastically increases at the S_2 stage relative to the V stage; in brackets, a polypeptide group present during all the steps of the development but whose synthesis varies greatly according to the stage; after a high level during the V stage followed by a drastic decrease at the S_2 stage, the synthetic activity for this polypeptide group resumes at the late M stage

tides appear as soon as starch becomes cytologically detected. This change in the pattern of protein synthesis could be correlated with the cytological stage where microspores become incompetent for androgenesis (M. Becker, personal communication). It can be speculated that at this stage totipotency of cells is lost. The few polypeptides that are newly synthesized could reflect the first sign of irreversible differentiation.

Another change occurs at the end of pollen development just before anthesis. At this time, polypeptide synthesis seems to be reactivated and most of the polypeptides synthesized when starch deposition begins can also be detected. This late activity may be correlated with the ability of the pollen grain to germinate.

The results presented here indicate clearly that our attention has to be focused on what happens at the stage when the drastic change in the polypeptide synthesis occurs. In particular, it would be worthwhile to characterize the basic polypeptides synthesized at this stage of development.

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