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Comparison of the in vitro translated polypeptides from maize shoot, pollen and germinated pollen mRNAs

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Abstract

We have compared the two-dimensional gel (2D gel) profiles of in vitro synthesised polypeptides from maize shoot with that of pollen, and the latter with that of germinated pollen. In the former comparison, extensive quantitative and qualitative differences in the polypeptides are apparent. We describe an increase in the abundance of the major cytoskeletal protein, α tubulin, in pollen compared to shoot. We also show that the electrophoretically separable α tubulin polypeptides in ungerminated pollen are not obviously post-translationally modified. In the comparison between the 2D gel profiles of the in vitro synthesised polypeptides from pollen and germinated pollen a number of notable differences are apparent, the possible origins of which are described.

Key words: Pollen; Germinated pollen; In vitro translation; α Tubulin; Maize

1. Introduction

Microsporogenesis begins when the spore mother cells undergo a reductive division to form four haploid daughter cells. In maize, this meiotic division is accompanied by a rapid expansion in the size of the anthers. Within the locules of the anthers, the daughter cells mature into pollen grains. Just prior to anthesis and the shedding of pollen, two divisions occur in rapid succession to give rise to the vegetative nucleus and the two sperm cells. The two sperm cells are transported within the pollen tube through the silks to the embryo sac in the ovule where fertilisation occurs [1].

It has been shown that transcription of specific genes occurs in the haploid microspores after meiosis and during their subsequent development into pollen grains (reviewed in [2]). Renaturation kinetics reveals that pollen contains fewer, but more abundant, different sequences compared to the different sequences in shoot [3,4]. Heterologous renaturation analyses of pollen cDNA with shoot mRNA and the reciprocal studies reveal that at least 65% of the mRNAs in pollen are similar to those found in shoot [4]. By differentially screening pollen libraries with pollen and vegetative tissue cDNAs, it has been estimated that about 10% of the total sequences expressed in maize pollen might be pollen specific [5].

Several classes of pollen expressed genes have been studied, in particular, (i) genes for specific isozymes: although the majority of the isozymes expressed in the male gametophyte are also expressed in the sporophyte, certain enzymes in pollen are encoded by genes different from those in sporophytic tissues [2,6,7]; (ii) genes for specific cytoskeletal protein isoforms: different members of the tubulin and actin multigene families have been shown to be expressed differentially in pollen development [8,9,10]; (iii) genes expressed after microspore mitosis: generally isolated by differential or subtractive screening methods this class has been called the 'late' pollen genes [2,11,12,13]; some of these genes have homology to known proteins (reviewed in [14,15]).

Pollen contains a store of presynthesized RNAs and proteins [16]. The dependence of germination and early pollen tube growth on protein synthesis on presynthesized mRNAs varies with the plant species. It has been reported that new mRNAs are synthesised after the pollen tube is formed from most bicellular pollen grains [17,18]. So far, there are no reports which show that new genes are specifically transcribed during pollen germination and tube growth.

In this paper, we compare the in vitro translated polypeptides from maize shoot, pollen, and germinated pollen mRNAs. We note a vast increase in the abundance of α tubulins in pollen compared to shoot and reveal that the four α tubulin isotypes detected in the pollen grain are not obviously post-translationally modified. We show that there are subtle differences between the 2D gel constellations of in vitro translated polypeptides prepared from pollen and germinated pollen mRNAs.

2. Materials and methods

2.1. Plant material

Maize seeds (*Zea mays* inbred line A188) were germinated on filter paper in a moist chamber at 25°C in the dark. Shoot material was collected from 1-week-old etiolated seedlings. Leaf tissue was obtained from greenhouse grown plants with six leaves. Cob tissue and pollen were obtained from greenhouse or field grown plants. Endosperm tissue was obtained by dissection of kernels 12 days post-pollination. Pollen

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was germinated for 45 min in vitro on solid media containing 17% (w/v) glucose, 0.3 g/l $CaCl_2$ H_2O, 0.1 g/l H_2BO_3, pH 6.4 (using 1 M KOH), and 1% agarose. Germinated pollen was harvested only if germination exceeded 95%.

2.2. Isolation and translation of RNA

Total RNA was prepared as described in Hussey et al. [19]. Poly(A)$^+$ RNA was purified from shoot, pollen and germinated pollen RNA by two cycles of Oligo-dt cellulose chromatography (Collaborative Research Incorporated, Bedford, M.A.). Poly(A)$^+$ RNA was translated in rabbit reticulocyte lysate incorporating [^{35}S]methionine as described in the Promega protocol (Promega Corporation, Madison, WI).

2.3. Two-dimensional gel electrophoresis

The in vitro translation products were prepared as samples for 2D gel electrophoresis as described by Burland et al. [20]. Two dimensional gel electrophoresis was performed according to the method of O'Farrell [21]; in the isoelectric focussing gels, the ampholines (Pharmacia LKB, Sweden) were used in a ratio of 3:2 of pH 3.5–10:pH 5–7. The maize pollen total protein sample was prepared using the method described in Hussey et al. [8]. Fluorography was performed using EN^{3}HANCE (New England Nuclear).

The 2D gels in Fig. 1 were loaded with equal counts and were aligned using fortuitous internal marker proteins. These marker proteins are indicated by the '+' and 'O' signs on the gels. The markers are reticulocyte lysate proteins that have produced a 'negative' imprint on the fluorograms.

2.4. Immunoblotting

Immunoblotting was performed essentially as described by Towbin et al. [22]. Only the tubulin area (defined in Hussey et al. [8]) of the 2D gel immunoblot is shown in Fig. 2b. This blot was probed (as described in Hussey et al. [8]) sequentially with the anti α tubulin monoclonal antibody, B-5–1–2, and the anti β tubulin monoclonal antibody, 2–10-B6. The two monoclonal antibodies were raised against sea urchin tubulin and were a gift from Dr. G. Piperno, Mount Sinai School of Medicine, New York.

2.5. Hybridisation selection

α tubulin mRNA was purified from maize (inbred line A188) pollen poly(A)$^+$ RNA using the hybridisation selection procedure of Ricciardi et al. [23]. 10 μg of a full-length α tubulin cDNA clone in Bluescribe M13+ [24] was linearised with *Eco*RI, immobilised on a 1 cm^2 nitrocellulose filter, and hybridised to 10 μg maize pollen poly(A)$^+$ RNA. The α tubulin selected RNA was translated in rabbit reticulocyte lysate.

2.6. Northern blotting

Maize tissue total RNAs were fractionated on a formaldehyde agarose gel and transferred to nylon membrane (Nytran, Schleicher and Schuell) using 20× SSC as transfer solution [25]. The Northern blot was probed with a full length α tubulin cDNA which was radiolabelled by primer extension [19]. Filters were prehybridised and hybridised at 42°C in 50% formamide, 5× SSC, 50 mM phosphate buffer pH 6.5, 1% (w/v) SDS, 5× Denhardts, 100 μg/ml calf thymus DNA and 50 μg/ml poly A. Prehybridisation and hybridisation were for 3 h and 24 h, respectively. The filter was washed at 60°C in four changes of 0.1 × SSC/0.1% (w/v) SDS. Each lane on the Northern blot in Fig. 3 was loaded with 10 μg of total RNA as quantified by A_{260} spectrophotometric assays supplemented by comparison of ribosomal RNAs on an ethidium bromide stained agarose gel.

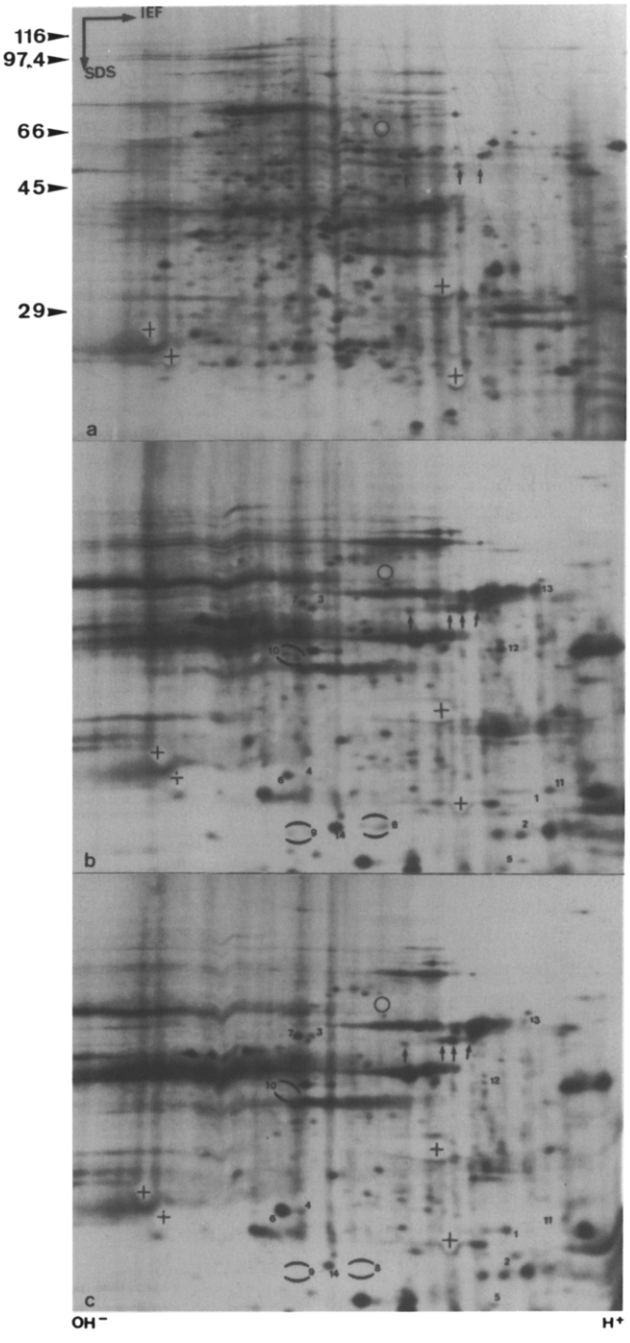

Fig. 1. 2D gel fluorograms of in vitro translated polypeptides from maize shoot (a), pollen (b), and germinated pollen (c), mRNAs. The arrows denote the α tubulin polypeptides. The numbers indicate the positions of the polypeptides that differ between the pollen and germinated pollen 2D gel profiles. The '+' and 'O' signs point out the internal marker proteins which produce a negative imprint on the fluorograms. These marker proteins were used to align the 2D gels.

3. Results

3.1. Comparison of shoot and pollen in vitro translated polypeptides

Poly(A)$^+$ RNAs from shoot, pollen and germinated pollen were translated in vitro in rabbit reticulocyte lysate and the polypeptide products fractionated on 2D gels. Fig. 1 shows the resulting 2D gel fluorograms of the in vitro synthesized polypeptides from shoot (Fig. 1a), pollen (Fig. 1b) and germinated pollen (Fig. 1c). Comparison of the 2D gel profiles of in vitro synthesied polypeptides from shoot mRNA with those from pollen and germinated pollen mRNA reveals that the former is

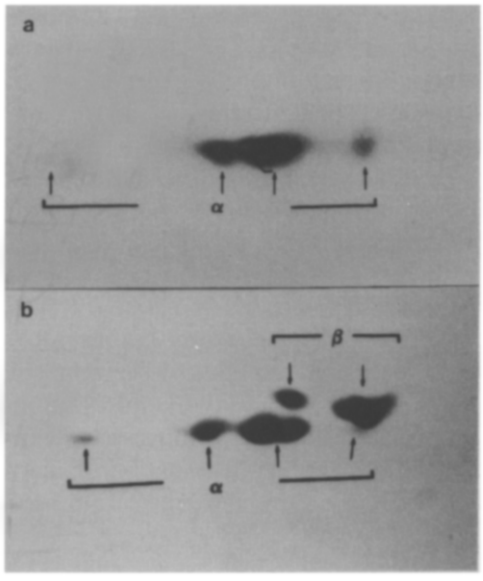

Fig. 2. Tubulin areas of, (a) a 2D gel fluorogram of in vitro translated, hybridisation selected α tubulin mRNA and, (b) a 2D gel immunoblot probed sequentially with the anti α tubulin monoclonal antibody, B-5-1-2, and the anti β tubulin monoclonal antibody, 2-10-B6.

qualitatively far more complex. Also, there are a number of significant quantitative differences in similar polypeptides in pollen and germinated pollen compared to shoot. In particular, we have identified a marked increase in the abundance of the major microtubule protein, α tubulin, in the two pollen developmental stages compared to shoot.

In order to identify the coordinates of the α tubulin polypeptides on the 2D gel fluorograms shown in Fig. 1, we have hybridisation selected total α tubulin mRNA from pollen poly(A)$^+$ RNA, translated the selected RNA in vitro and fractionated the resulting α tubulin polypeptides on a 2D gel. The result shown in Fig. 2a shows a constellation of four spots representing at least four α tubulin polypeptides; one spot is elongated and may be composed of more than one polypeptide. This constellation of polypeptides can be seen at the same coordinates on the 2D gel fluorograms of the pollen and the germinated pollen in vitro synthesized polypeptides; indicated by the arrows in Fig. 1b,c, respectively. A very similar constellation of spots can be seen at similar coordinates on the shoot 2D gel fluorogram; there are three, rather than four, spots depicted by arrows in Fig. 1a, which is consistent with a differential expression of α tubulin isotypes between maize seedling and pollen tissues observed by Joyce et al. [26]. In comparing the relative abundance of these polypeptides within the whole 2D gel profiles of the shoot or pollen in vitro synthesised polypeptides it is apparent that the α tubulins show a marked increase in abundance in pollen compared to shoot (compare Fig. 1a with Figs. 1b and 1c). This increase in the relative abundance of the α tubulin in vitro translated polypeptides in pollen is complemented by the Northern analysis shown in Fig. 3. Fig. 3 is a Northern blot of various maize tissue RNAs probed with a maize α tubulin coding sequence probe. The data shows that there is an increased abundance of total α tubulin transcript in pollen and germinated pollen compared to the other tissues examined.

The electrophoretic mobility of the in vitro synthesised α tubulin polypeptides identified by hybridisation selection (Fig. 2a) would appear to be very similar to the electrophoretic mobility of the in vivo synthesized α tubulin isotypes that were detected by immunoblotting a 2D gel of a maize pollen total protein extract (Fig. 2b). These data imply that heterogeneity of α tubulins in the pollen grain does not appear to arise as a result of post-translational modification.

3.2. *Comparison of pollen and germinated pollen* in vitro *translated polypetides*

A comparison of the 2D gel profiles of in vitro synthesized polypeptides from pollen (Fig. 1b) and germinated pollen mRNAs (Fig. 1c) identifies a number of significant differences. There are qualitative differences. Polypeptides 1 to 5 (see Fig. 1c) are present only in the germinated pollen 2D gel profile, whereas polypeptide clusters marked 8, 9 and 10 and polypeptide 11 (see Fig. 1b) are found only in the pollen 2D gel profile. These differences reflect qualitative differences in the translatable mRNAs between the two stages in pollen development, mature pollen and germinated pollen. Also clearly evident are several quantitative differences. That is, polypeptides 6 and 7 (see Fig. 1b,c) appear to increase in abundance in germinated pollen whilst, in contrast, polypeptides 12, 13 and 14 (see Figs. 1b and 1c) appear to be more abundant in pollen. Other qualitative and quantitative differences are also apparent but those described above are the most reproducible and the most striking. These data do suggest that there are changes in the type

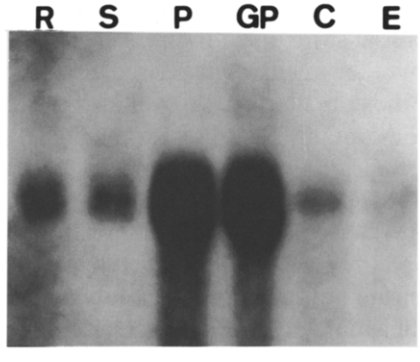

Fig. 3. Northern blot containing 10 μg of total RNA from maize root, R, shoot, S, pollen, P, germinated pollen, GP, cob, C, and endosperm, E. The Northern blot was probed with a radiolabelled full length maize α tubulin cDNA clone.

and abundance of translatable mRNAs in pollen and germinated pollen. It must be noted however that these diferences are subtle when compared to the quantity of similarly translated polypeptides in the pollen and germinated pollen 2D gel profiles.

There does not appear to be a difference in the quality or quantity of in vitro translated α tubulin polypeptides (compare Fig. 1b,c) or in the abundance of α tubulin transcript between pollen and germinated pollen (see Fig. 3).

4. Discussion

The mRNAs from shoot, pollen and germinated pollen have been translated in vitro and fractionated on 2D gels. A comparison of the resulting 2D gel fluorograms has allowed us to define and characterise the differences in the translatable mRNA populations in the three tissues examined.

These data show that there is a vast increase in the quantity of translatable total α tubulin mRNA in mature pollen tissue compared to shoot. It has been reported that the transript for another cytoskeletal protein, actin, increases in abundance post-meiotically in maize [5]. In all plants examined to date, the α and β tubulins and actin are encoded by large gene families, certain members of which show preferential or specific expression in pollen. The transcripts for the maize α tubulin genes, *tua5* and *tua6*, and the maize β tubulin genes, *tub3* and *tub4*, are most abundant in pollen [9,26,27] and the *Nicotiana tabacum* actin gene, Tac25, is expressed specifically in pollen [10]. The maize *tub3* transript shows a marked increase in abundance in post-meiotic anthers in pollen development [9]. Tubulin and actin are the major components of two of the three fibrous cytoskeletal elements in eukaryotic cells, and their increase in abundance in pollen emphasises the important role played by cytoskeleton based functions in pollen development [28].

These data also show that all the α tubulin polypeptides represented by translatable RNAs can be detected in ungerminated pollen thereby showing that the pollen grain contains a store of synthesized α tubulin RNAs. A comparison of the in vitro synthesised α tubulin polypeptides with those made in vivo would indicate that the pollen grain contains a similar store of presynthesised α tubulin polypeptides. In addition, the fact that the constellations of the in vitro and in vivo synthesised α tubulin polypeptides are very similar would indicate that the electrophoretically separable α tubulins in ungerminated pollen are not subject to the α tubulin post-translational modification, acetylation. Acetylation of α tubulin results in a characteristic mobility shift on 2D gels [29]. An antibody that recognises acetylated α tubulin, 6–11B-1 [30], has been used successfully to identify specific sets of microtubules in *Nicotiana tabacum* pollen tubes [31]. It must also be noted that no qualitative changes in the in vitro translated α tubulins can be detected between the maize germinated and ungerminated pollen.

We have shown that there are qualitative as well as quantitative differences between the different translatable mRNAs in the pollen grain and in germinated pollen. There are two possible origins of the in vitro translated polypeptides specific to the germinated pollen, (i) in the pollen grain, there are presynthesised mRNA precursors which are modified to produce functional RNAs in germinated pollen or, (ii) the new translatable mRNAs represent genes specifically expressed in germinated pollen. With regards to those translatable mRNAs which are present in pollen but absent from germinated pollen, it is conceivable that specific degradation of these RNAs occurs and /or that the specific genes are turned off.

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