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# Messenger RNas: their utilization and degradation during pollen germination and tube growth

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ABSTRACT

During pollen germination and tube growth at least 230 new proteins are synthesized, as determined by $^{35}$S-methionine labeling and two dimensional gel electrophoretic analysis of the labeled proteins. The same number and pattern of protein spots is seen whether or not actinomycin D is included in the medium, indicating that the mRNAs present in the ungerminated pollen grain and those newly synthesized code for the same proteins. The genetic program during at least the latter part of pollen maturation prior to anthesis and that during pollen germination and tube growth thus appears to be similar if not identical. During the first hour of pollen tube growth about 50% of the protein synthesis that occurs utilizes previously synthesized mRNAs. The remaining 50% occurs on newly made mRNAs. The ungerminated mature pollen grain contains 196 pg of RNA and approximately $6 \times 10^6$ molecules of poly(A)$^+$ RNA, i.e. mRNAs. The rate of protein synthesis corrected for internal pool changes in the labeled amino acid used ($^3$H-leucine) is highest during the first 15 min of pollen tube growth. The rate decreases rapidly thereafter for the next 45 min. Concurrent with the reduction in rate of protein synthesis there is a reduction in the poly(A) content of the pollen RNA and in the amount of poly(A) per pollen grain. The total RNA per pollen grain, however, appears not to change during this period.

## INTRODUCTION

The ungerminated pollen grain at anthesis contains a store of stable messenger RNAs (mRNAs) that are translated early during germination. The evidence for this has been obtained indirectly, based on the effects of inhibitors of RNA and protein synthesis on germination and tube growth, and also on the rapidity of polysome formation and protein synthesis during germination [see review in Mascarenhas, 1975; Mascarenhas, Bell, 1969]. In addition recently, direct evidence for the presence of mRNAs in the ungerminated pollen grain of *Tradescantia* has been obtained (Frankis, Mascarenhas, 1980). Poly(A)$^+$

RNAs from the ungerminated pollen grain have been isolated and translated in a cell-free system into polypeptides several of which show similarity to proteins made during germination (Frankis, Mascarenhas, 1980).

During germination new RNA is synthesized (Mascarenhas, Bell, 1970). In *Tradescantia* the newly synthesized RNA is not ribosomal (Mascarenhas, Bell, 1970) or transfer RNA (Mascarenhas, Goralnick, 1971). These two species of RNA are present in the mature pollen and were synthesized prior to anther dehiscence. The ribosomal and transfer RNA genes are inactive during pollen germination and tube growth (Mascarenhas, Bell, 1970; Peddada, Mascarenhas, 1975). The RNAs that are synthesized during pollen germnation are thus mRNAs or mRNA precursors (Mascarenhas et al., 1974). When the proteins synthesized during pollen germination and tube growth in *Tradescantia* are labelled and analyzed by single dimension SDS-polyacrylamide gel electrophoresis about 20 new protein bands are seen (Mascarenhas et al., 1974). These same bands are synthesized whether or not actinomycin D is present in the growth medium, indicating that the presynthesized stored mRNAs and the newly made mRNAs code for the same proteins (Mascarenhas et al., 1974).

The rate of protein synthesis is highest during the first hour of pollen germination and tube growth in *Tradescantia;* thereafter it decreasess rapidly (Mascarenhas et al., 1974).

The purpose of this work was (1) to study in greater detail the proteins synthesized on pre-existing and newly made mRNAs during germination and (2) to determine the nature of the changes in mRNAs that might account for the decrease in the rate of protein synthesis during germination and tube growth.

## MATERIALS AND METHODS

Pollen of *Tradescantia paludosa* was collected, stored, and grown in shaking cultures at 25°C as previously described (Mascarenhas et al., 1974). For labeling pollen proteins with $^{35}$S-methionine, 10 mg of pollen was grown for one hour with or without 30 μg/ml of actinomycin D in 1 ml of medium with casamino acids omitted, and containing 100 μCi L-[$^{35}$S]-methionine (New England Nuclear, Sp. Act. 564.70 Ci/mmole). The pelleted pollen tubes were homogenized in a Dounce homogenizer with 0.5 ml of lysis buffer (O'Farrell, 1975). The homogenate was centrifuged for 15 min at full speed in a Clay Adams Dynac desk top centrifuge. To the supernatant additional urea was added to saturate the solution. The extract was either stored at —20°C or used immediately for further analysis. To determine the

amount of $^{35}$S-methionine incorporated into protein, to 25 µl of each extract, 2 ml of 5% trichloroacetic acid (TCA) was added, heated to 95°C for 5 min, cooled in ice, filtered through Millipore HA 0.45 µm filters, dried and counted in a scintillation spectrometer with 5 ml of Liquifluor (New England Nuclear). For the two dimensional analysis of $^{35}$S-proteins, 30 µl of each extract in lysis buffer was analyzed according to the procedure of O'Farrell (1975). The first dimension was by isoelectric focusing in the pH range 4-7. The second dimension was in 8 to 12.75% gradient acrylamide slab gels in presence of sodium dodecyl sulfate (SDS). The gels were dried onto Whatman 3MM filter paper (Maizel, 1971) and then exposed against Kodak XR-2, X-ray film at —20° C.

To study the rate of protein synthesis at different times during germination and pollen tube growth, 5 mg of pollen was grown in 1 ml of growth medium (Mascarenhas et al., 1974) minus casamino acids in each of several 25 ml Erlenmeyer flasks. At 15 min intervals from 0-60 min after addition of pollen to the medium, 5 µCi of L[3,4-$^{3}$H]-leucine (Amersham Searle; 48 Ci/mmole) was added and the pollen grown for 15 min longer. To terminate the experiment the pollen was transferred with excess ice cold medium to a centrifuge tube and centrifuged at 4° C in the Clay Adams Dynac for 10 min at full speed. To the pellet 3 ml of growth medium was added, the pollen gently resuspended and pelleted again. The pellet was washed once more with 3 ml of growth medium. The pollen pellet was suspended in 1 ml of 1% SDS and immediately boiled for 2 min to destory all proteolytic activity. To each sample, 75 µl of 100% TCA was added, boiled for 5 min, cooled in ice and centrifuged for 10 min at full speed in the Dynac. The supernatant was saved and the pellets washed twice with 3 ml of 5% TCA. The supernatants were pooled in a 10 ml volumetric flask and the volume made to 10 ml. To determine TCA soluble cpm, 50 µl of the pooled supernatant was added to a Whatman GF/C glass fibre filter disc, dried and counted in Liquifluor. The pellet which contained the TCA insoluble material was resuspended in 1.5 ml of 5% TCA, filtered through a Whatman GF/C glass fibre filter and washed 10 times with 2 ml each time of cold 5% TCA. The filter was dried and counted. To determine the absolute rate of protein synthesis taking into consideration the internal pools of $^{3}$H-leucine, the TCA insoluble cpm were expressed per equal number (20,000) of TCA soluble cpm.

For the isolation of RNA for determining the changes in poly(A) content during germination, 20 mg pollen was grown in medium for 0, 15, 30, 45 and 60 min. The pollen was pelleted and homogenized with 2 ml of homogenization buffer (0.2 M NaCl, 0.005 M $MgCl_2$, 0.01 M Tris (pH 7.4)) and 2 ml of phenol:$CHCl_3$:isoamyl alcohol (50:48:2)

and the RNA extracted as described (Frankis, Mascarenhas, 1980). RNA concentrations were determined by assuming 1 mg of RNA/ml had an $A_{260nm}$ of 20.

The poly(A) content of the RNA was detrmined by hybridization with $^3$H-poly (U) as described (Gillespie et al., 1972; Wilt, 1973). A 100 µl hybridization reaction contained (final amounts or concentrations) 50% formamide 3 X SSC (0.45 M NaCl, 0.045 M Na Citrate), 0.01 M Tris (ph 7.2), 0.11 µg 5-$^3$H-polyuridylate (361 µCi/µmole P; Miles Laboratories, Elkhart, Indiana), plus pollen RNA or poly(A) standards. After incubation at 36° C for 18 hr, 2 ml of 0.01 M Tris (ph 7.2), 0.1 M $MgCl_2$, 0.5 M NaCl was added, followed by 5 µg/ml of boiled pancreatic RNase (Sigma Chemical Co., St. Louis, Missouri) and 20 µg/ml of electrophoretically pure DNase 1 (Sigma). After incubation for 2 hr at 30° C, the RNase resistant hybrids were precipitated at 4°C with 2.5% TCA (Williams, Klett, 1978) after adding 0.02 $A_{260nm}$ units of carrier poly (A) and 20 µg of yeast RNA. Within 5 min of precipitation, samples were filtered on Millipore HA 0.45 µm filters, washed with cold 2.5% TCA, dried and counted in 5 ml scintillation fluid. The poly (A) content of the samples was determined from a standard curve constructed with known amounts of poly(A).

For the calculation of quantities of RNA species per pollen grain, the value of 88,100 pollen grains per mg pollen was used. This value was obtained by counting pollen both with a Coulter counter and in a Sedgwick-Rafter counting chamber.

## RESULTS

Single dimension SDS-polyacrylamide gel electrophoresis of proteins synthesized during the first hour of pollen tube growth in the presence and absence of actinomycin D seem to indicate that the presynthesized mRNAs and the newly made mRNAs code for the same proteins (Mascarenhas et al., 1974). Since the resolution of single dimension gels is relatively poor, pollen proteins synthesized in the presence and absence of actinomycin D were labeled to high radioactivity with $^{35}$S--methionine during the first hour of germination and tube growth and analyzed by two dimensional gel electrophoresis (O'Farrell, 1975). The autoradiogram of the gel of proteins synthesized in the absence of actinomycin D is shown in Fig. 1. On this autoradiogram 230 radioactive spots. i.e. polypeptides can be resolved. The autoradiogram of proteins synthesized in the presence of actinomycin D (Fig. 2) also shows 230 spots. A careful comparison of the two autoradiograms shows that there are no qualitative differences in the pattern of protein spots seen in the two gels.

When the incorporation of $^{35}$S-methionine into TCA insoluble material was studied it was found that about 50% fewer counts were in-

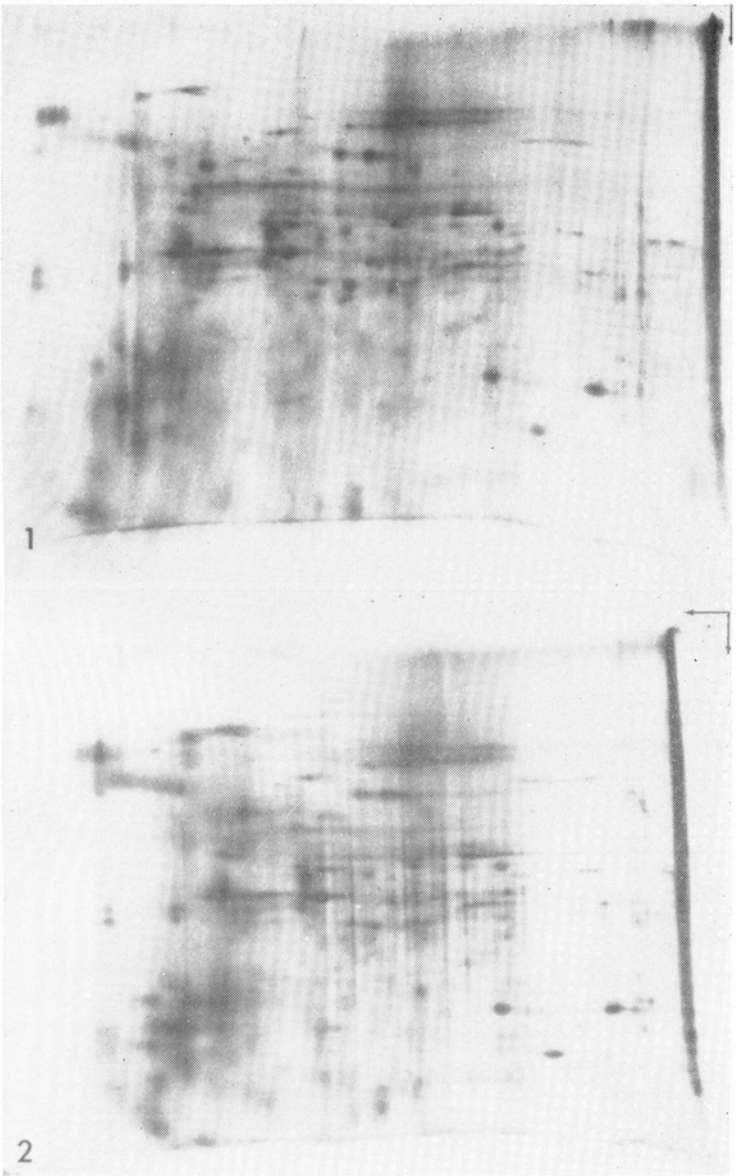


Fig. 1 and 2. Autoradiograms of $^{35}S$-labeled proteins from pollen grown for 1 hr without (Fig. 1) and with actinomycin D (Fig. 2) analyzed by two dimensional gel electrophoresis.

The horizontal arrow indicates the direction of isoelectric focusing and the vertical arrow the direction of SDS-electrophoresis.

corporated in pollen tubes grown with actinomycin D as compared to the control. This would indicate that within the first hour of pollen tube growth, about 50% of the protein synthesis occurs on previously existing mRNAs and the remaining 50% on newly synthesized mRNAs.

RNA was isolated from ungerminated pollen grains, its poly(A) content determined by $^3$H-poly(U) hybridization and the amount of RNA per pollen grain and the number of poly(A)$^+$ RNA molecules per pollen grain calculated. On an average each pollen grain contains 196 picograms ($10^{-12}$ g) of RNA and 0.340 pg of poly(A). From the number average size of the poly(A) segments, the percentage of RNA which exists as poly(A) and the amount of RNA per pollen grain, the number of poly(A) molecules (mRNAs) can be calculated. Based on an average poly(A) segment of 100 nucleotides, the ungerminated pollen grain of *Tradescantia* contains approximately $6 \cdot 2 \times 10^6$ molecules of poly(A)$^+$ RNA.

Earlier work with *Tradescantia* pollen had shown that the rate of protein synthesis was highest during the first hour of pollen tube growth, decreasing thereafter. In these studies (Mascarenhas et al., 1974), however, no corrections were made for the changes in specific activity of the internal pools of the radioactive amino acids used. In

Table 1

Uptake of $^3$H-leucine and its incorporation into TCA insoluble material at different periods during pollen germination and tube growth

| Period of label min. | Total TCA insoluble cpm in 5 mg pollen | Total TCA soluble cpm $\times 10^{-2}$ in 5 mg pollen |
|---|---|---|
| 0-15 | 4,785 | 3,168 |
| 15-30 | 4,152 | 8,582 |
| 30-45 | 4,145 | 9,898 |
| 45-60 | 2,931 | 10,468 |

Table 2

Changes in the amount of poly (A) per pollen grain at different periods during pollen germination and tube growth

| Time min. | pg poly (A) per pollen grain |
|---|---|
| 0 | 0.379 |
| 15 | 0.333 |
| 30 | 0.195 |
| 45 | 0.198 |
| 60 | 0.169 |

order to study the absolute rates of protein synthesis during the first hour of pollen growth, pollen was pulse labeled for 15 min intervals from time 0-60 min after addition to the growth medium. The TCA soluble and insoluble cpm were determined (Table 1). The incorporation of $^3$H-leucine into TCA insoluble material was normalized by expressing the results based on an equal number of TCA soluble cpm. The data in Fig. 3 show that the rate of protein synthesis is highest during the first 15 min of pollen tube growth. The rate decreases drastically during the next 15 min of pollen tube growth and continues to decrease although at a slower rate for the next 30 min.

To attempt to determine the reasons for the rapid drop in rate of protein synthesis, the percentage poly(A) content of RNA at dif-

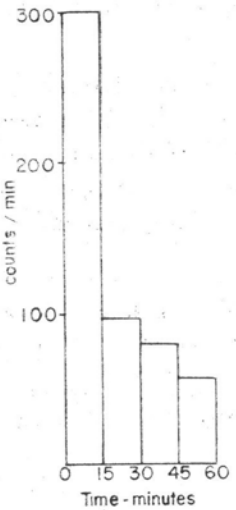


Fig. 3. The change in rate of protein synthesis during the first hour of pollen tube growth as studied by pulse labeling with $^3$H-leucine. The rates have been normalized to take into account changes in the internal pools of $^3$H-leucine.

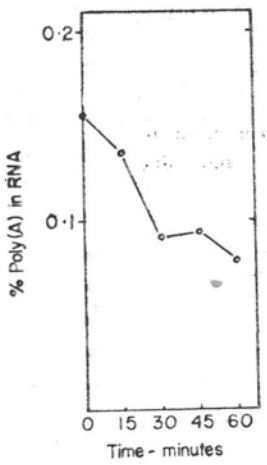


Fig. 4. Changes in the poly(A) content of pollen RNA with time after addition of pollen to a growth medium

ferent times after start of germination was determined (Fig. 4). The % poly(A) content of RNA decreases continuously with increasing time and seems to parallel the decrease in rate of protein synthesis. There is a similar reduction in the poly(A) content per pollen grain with time (Table 2). The total amount of RNA, however, appears to be constant during the first hour and averages about 196 pg/grain or pollen tube.

## DISCUSSION

The two dimensional electrophoretic separations of pollen proteins synthesized in the presence and absence of actinomycin D (Fig. 1, 2) shows that at least 230 different proteins are synthesized during pollen germination and tube growth. This is likely to be an underestimate of the number of proteins synthesized, for if the gels were exposed longer, several additional proteins present in very small amounts would probably show up on the autoradiograms. On single dimension SDS gels only about 20 new proteins can be resolved (Mascarenhas et al., 1974). Since there are no detectable differences in the proteins made in the presence or absence of actinomycin D our previous conclusions (Mascarenhas et al., 1974) are confirmed that the presynthesized mRNAs present in the mature pollen grain and the mRNAs newly synthesized during pollen germination, code for the same proteins. In other words, the genetic program during at least the latter part of pollen maturation prior to anthesis and that during germination and tube growth appear to be the same.

Each mature *Tradescantia* pollen grain at the time of anthesis contains about $6 \times 10^6$ molecules of poly(A)$^+$ RNA, i.e. mRNA, and these mRNAs are used in the early protein synthesis that occurs during germination.

The rate of protein synthesis is high during the first 15 min of pollen germination and tube growth. Thereafter, the rate drops off rapidly. Concurrent with the reduction in rate of protein synthesis a similar drop is seen in the poly(A) content of the RNA (Fig. 4) and in the poly(A) content per pollen grain (Table 2). The total amount of RNA per pollen grain, however, appears to be constant during the first hour. This would appear to indicate that the poly(A) content of the RNA decreases without a concomitant destruction of the rest of the mRNA molecule. Since the quantity of mRNA is probably very small compared to the ribosomal and transfer RNAs, it is possible that the method used to determine the total RNA in the pollen is not sensitive enough to measure the loss of small amounts of mRNA. Further experiments are in progress to determine whether the drop in the rate of

protein synthesis during germination is because of the destruction of just the poly(A) portion of the mRNAs or because of the degradation of entire mRNA molecules.

Acknowledgments

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REFERENCES

Frankis R. C., Mascarenhas J. P., 1980. Messenger RNA in the ungerminated pollen grain: A direct demonstration of its presence. Ann. Bot. 45: 595-599.

Gillespie D., Marshall S., Gallo R. C., 1972. RNA of RNA tumor viruses contains poly(A). Nature New Biol. 236: 227-231.

Maizel J. V., 1971. Polyacrylamide gel electrophoresis of viral proteins. In "Methods in Virology". Ed., Maramorsch, R. and H. Koprowski. Vol. 5. Academic Press, N. Y. pp. 219-220.

Mascarenhas J. P., 1975. The biochemistry of angiosperm pollen development. Bot. Rev. 41: 259-314.

Mascarenhas J. P., Bell E., 1969. Protein synthesis during germination of pollen: studies on polyribosome formation. Biochim. Biophys. Acta 179: 199-203.

Mascarenhas J. P., Bell E., 1970. RNA synthesis during development of the male gametophyte of *Tradescantia*. Develop. Biol. 21: 475-490.

Mascarenhas J. P., Goralnick R. D., 1971. Synthesis of small molecular weight RNA in the pollen tube of *Tradescantia paludosa*. Biochim. Biophys. Acta 240: 56-61.

Mascarenhas J. P., Terenna B., Mascarenhas A. F., Rueckert L., 1974. Protein synthesis during germination and pollen tube growth in *Tradescantia*. In "Fertilization in Higher Plants". (Ed.) H. F. Linskens, North-Holland Publ. Co., Amsterdam, pp. 137-143.

O'Farrell P. H., 1975. High resolution, two dimensional electrophoresis of proteins. J. Biol. Chem. 250: 4007-4021.

Peddada L., Mascarenhas J. P., 1975. The synthesis of 5S ribosomal RNA during pollen development. Develop. Growth. Diff. 17: 1-8.

Williams D. L., Klett H., 1978. Measurement of polyadenylic acid by hybridization with polyuridylic acid: A source of error due to the lability of tritiated polyuridylic acid in trichloroacetic acid. Anal. Biochem. 91: 137-179.

Wilt F. H., 1973. Polyadenylation of maternal RNA of sea urchin eggs after fertilization. Proc. Nat. Acad. Sci. USA 70: 2345-2349.