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PHYTIC ACID IN PETUNIA HYBRIDA POLLEN IS HYDROLYSED DURING GERMINATION BY A PHYTASE

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SUMMARY

Phytic acid, which makes up a large proportion of the phosphorus content of *Petunia* pollen, undergoes only a very slow rate of degradation during the first two hours of germination. After two hours an active phytase (E.C.3.1.3.8) can be detected in pollen extracts and rapid degradation of phytic acid begins in the germinating pollen. Both the rapid degradation of the phytic acid and appearance of phytase activity can be prevented by cycloheximide, but not by cordycepin or actinomycin-D. It is concluded that a pre-existing, long-lived m-RNA in mature, ungerminated pollen is used during germination for the synthesis of phytase, which then catalyses rapid degradation of phytic acid. The product of this reaction is myoinositol, which is needed for pollen tube wall polysaccharide synthesis. The phytase synthesized during pollen germination has maximum activity at pH 5, does not require metal ions and is inhibited by fluoride and inorganic phosphate. The degradation of phytic acid and pollen tube elongation are retarded by inclusion in the medium of concentrations of phosphate inhibitory to phytase.

1. INTRODUCTION

Phytic acid has recently been shown to be present in significant quantities in pollen from plants with style lengths greater than about 5 mm, while pollen from Compositae and grasses with very short styles have little or no phytic acid (JACKSON et al. 1982). It was deduced that phytic acid is needed as a reserve for myoinositol, which is in great demand for uronic acid formation during pollen tube wall synthesis. While there is known to be a small amount of free myoinositol in the pollen from lily, for example (MAITI et al. 1978) it is not enough for tube wall synthesis; nor is there enough myoinositol-1-phosphate synthetase activity in this pollen to convert phosphorylated glucose to myoinositol (LOEWUS & LOEWUS 1980).

Because it now seems likely, in view of the relatively high content of phytic acid in pollen developing long tubes, that phytic acid supplies the necessary myoinositol during the germination of most pollen, we report here on factors

Abbreviations: pCMB = parachloromercuribenzoate;
EDTA = ethylene diamine tetraacetic acid.

controlling phytic acid breakdown in germinating pollen. We have chosen to investigate *Petunia hybrida* pollen because of its high initial phytic acid content (JACKSON et al. 1982).

2. MATERIALS AND METHODS

Pollen: Pollen was collected from *Petunia hybrida* L. clones W166H, W166K and T_2U grown in glasshouse conditions. The pollen was stored at $-5\,^{\circ}C$.
Pollen germination: Frozen pollen was hydrated in a desiccator over water for 30 min at 25 °C and then shaken in a conical flask with sucrose-boric acid medium at 25 °C (35 mg pollen in 5 ml 10% sucrose-0.01% H_3BO_3, pH 6.0). Under these conditions maximum pollen tube elongation (approximately 600 μm) was achieved in 12 h (JACKSON & LINSKENS 1979). The pH of the medium did not change significantly during this time.
Phytic acid determination: Stored pollen or germinated pollen collected by centrifugation, was extracted with 0.02 M Na_4EDTA solution and phytic acid determined by paper electrophoresis as described previously (JACKSON et al. 1982).
Phytase assay: Phytase was determined as described by PEERS (1953). Occasionally the amount of inorganic phosphate liberated was compared to phytic acid degradation by paper electrophoresis of enzyme digests as described above for phytic acid determination.

3. RESULTS

3.1. Phytic acid content of Petunia hybrida pollen

Desiccated pollen from three clones of *Petunia hybrida* (W166K, W166H and T_2U) gave values of 2.0, 1.2 and 1.0% by weight phytic acid, respectively. All three clones were cultivated under similar glasshouse conditions, so it is considered that the differences in phytic acid content are largely genetic in origin. Similar differences have been found recently between clones of *Pinus radiata* (JACKSON & LINSKENS 1982). Clone W166K was used in a previous study of phytic acid in pollen, where we reported a figure (2.1% by weight) close to that obtained here (JACKSON et al. 1982). Clone W166H was used for the present studies, due to the availability of larger amounts of pollen from that clone.

Desiccated pollen (used previously in pollen studies and for the values given above), consistently gave higher apparent values for phytic acid content than did pollen which was first hydrated at 100% humidity after weighing and then allowed to imbibe solution from the germination medium before extraction. These differences were considered to be due to the absorption by pollen of large amounts of moisture in the manner described by GILISSEN (1977), leading to dilution of the extract.

For comparison, the phytic acid content of *Petunia hybrida* W166H (S_2S_3) seed was determined to be 2.0% by weight.

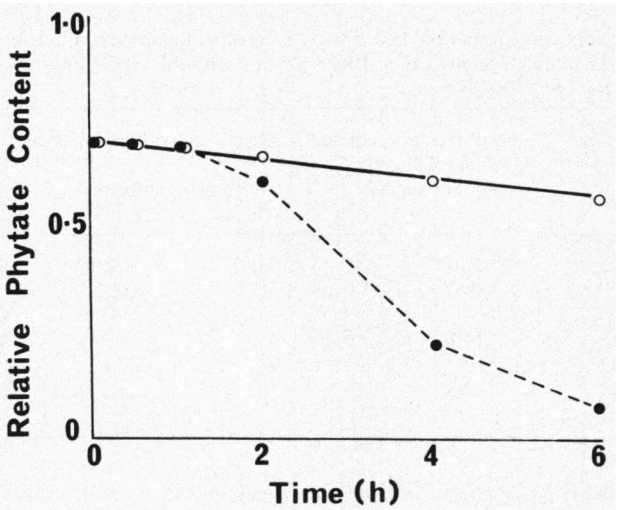

Fig 1. Phytic acid content of germinating W166H pollen and effect of protein synthesis inhibitor, cycloheximide. The medium contained 10% sucrose – 0.01% boric acid (---- ● ----) or 10% sucrose – 0.01% boric acid – 100 μg/ml cycloheximide (——— ○ ———). No phytic acid was found in the medium during germination.

3.2. Changes in phytic acid content during pollen germination

Over the first hour of germination there was little change in the phytic acid content of pollen. However, after two hours germination, degradation began to occur more rapidly, until by 6 hours there was little phytic acid remaining (*fig. 1*). Because it is likely that phytic acid is needed for pollen tube wall synthesis, it may be significant that under the *in vitro* germination conditions used, pollen tube length increases only slowly until about 4 to 6 hours, when it accelerates to a maximum rate of increase (JACKSON & LINSKENS 1979). This therefore corresponds to the time when myoinositol has just become available in large amounts. Examination of the medium during and after these incubations showed that the phytic acid lost from the pollen did not appear in the medium.

3.3. Effect of protein and RNA synthesis inhibitors on phytic acid content during germination

When 100 μg/ml cycloheximide was included in the germination medium, the phytic acid content of the germinating pollen did not show the rapid decrease seen in its absence after two hours germination. Instead, the very low rate of degradation (0.000 35 μM/h/mg pollen at 25°C, see *fig. 1*) apparent in the first hour of germination is continued. The concentration of cycloheximide used here is sufficient to inhibit protein synthesis in pollen (HOEKSTRA 1979) and was observed to inhibit tube growth as well, as described earlier by SONDHEIMER & LINSKENS (1974) for *Petunia hybrida* pollen. On the other hand the RNA synthesis inhibitors actinomycin-D and cordycepin were without effect on the degradation of phytic acid in germinating pollen (*table 1*). These results suggest that

Table 1. Phytic acid content and phytase activity of germinating pollen. Phytase assay mixtures contained 0.05 M sodium acetate, pH 5, 1 mM phytate, 10 mM $MgCl_2$ and pollen extract, and were incubated for 2 h at 55°C.

Additions to medium	Phytic acid content of pollen after 6 h % by weight	Phytase activity μmole/h/mg		
		Pollen germinated for: 0 h	1 h	2 h
Nil	0.07	0.002	0.003	0.013
Cycloheximide 100 μg/ml	0.57		0.002	0.003
				0.002
Cordycepin 150 μg/ml	0.10			0.014
Actinomycin-D 250 μg/ml	0.15			0.012

Table 2. Properties of pollen phytase activity. Assay conditions were as described in *table 1*, except that phytase was extracted from pollen germinated for 2 h.

Addition to assay mixture	Phytase activity μmole/h/mg
Nil	0.013
50 mM $MgCl_2$	0.010
2 mM pCMB	0.013
10 mM NaF	0.007
3 mM KH_2PO_4	0.006
10 mM KH_2PO_4	0.000

Table 3. Effect of inorganic phosphate on phytic acid breakdown and tube elongation in germinating pollen. Phosphate was added as the potassium salt, pH 6.0. Pollen tube length was determined from 200 measurements in each case; a standard deviation is added below.

Inorganic phosphate concentration in medium mM	Phytic acid content of pollen at 6 h % by weight	Length of pollen tube at 12 h μm
0	0.15	610 ± 196
3	0.30	441 ± 132
10	0.45	30 ± 5

there is a long-lived m-RNA already present in *Petunia* pollen, which is translated into a protein during the early stages of germination, this protein effecting phytic acid hydrolysis. Such a protein could be phytase.

3.4. Appearance of phytase activity in germinating pollen

Examination of pollen extracts for phytase activity showed that a small amount of phytase is present in ungerminated pollen, an amount that does not change

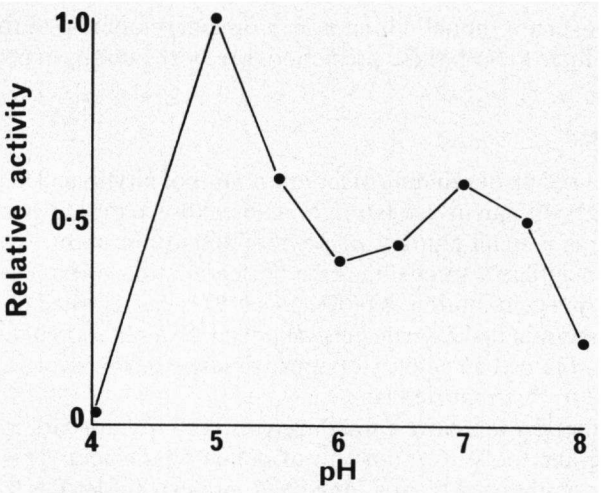

Fig. 2. Effect of pH on phytase activity from pollen germinated for 2 h. From pH 4–6.5, 0.05 M sodium acetate was used as buffer, from pH 7–8, 0.05 M Tris-acetate.

significantly until about 2 hours. By this time there has been a six-fold increase in phytase activity, which is not prevented by the RNA synthesis inhibitors cordycepin and actinomycin-D. It is, however, prevented by the protein synthesis inhibitor cycloheximide (*table 1*), which is consistent with the enzyme being translated from a pre-existing m-RNA. The appearance of the enzyme activity is consistent with the observed changes in phytic acid content during germination. The amount of phytase measured in ungerminated pollen is not enough to degrade the known amount of phytic acid in pollen in 6 hours (0.002 μM/h/mg at 25 °C). The results of PEERS (1953) are used to allow for differences in rates at the different temperatures. The activity observed at 2 hours is, however, sufficient to degrade all the phytic acid in pollen in a few hours.

3.5. Properties of the pollen phytase

The pH/activity curve for the activity extracted from pollen germinated for 2 hours shows maximum activity at pH 5 (*fig. 2*). However, activity is seen over quite a wide range of pH, with a second but lower peak of activity at pH 7. The pollen phytase does not need $MgCl_2$ for activity like many acid phosphates (COSGROVE 1980) and is in fact inhibited by high concentrations of $MgCl_2$ (*table 2*). The chelating agent EDTA, and the sulphydryl binding compound pCMB, were without effect on phytase activity. The pH 5 activity is inhibited by fluoride and inorganic phosphate (*table 2*), again as one would expect for an acid phosphatase (SHAW 1966).

The inhibition of extracted *Petunia* pollen phytase *in vitro* by inorganic phosphate predicts that phosphate should inhibit phytic acid breakdown when included with pollen in the liquid culture medium. Furthermore, if the breakdown product from phytic acid (myoinositol) is essential for tube development, then

inorganic phosphate should inhibit pollen tube development at the same time. As shown in *table 3,* both these predictions are borne out by experiment.

4. DISCUSSION

We have followed up our finding of large amounts of phytic acid in pollen (JACKSON et al. 1982) with an investigation of its breakdown during germination. Although there is a small amount of phytase activity in mature, ungerminated pollen, it is insufficient to give the rate of degradation of phytic acid actually measured during germination. BREDEMEIJER (1971) has reported phytase activity in mature, ungerminated *Petunia hybrida* pollen. Examination of his results suggests that he obtained an activity of approximately 0.004 μmole/h/mg at 55°C, which is close to that reported here.

After 2 hours germination, enough phytase activity appears in germinating pollen to degrade the known amount of pollen phytic acid. This new activity is apparently synthesized from a long-lived, pre-existing m-RNA, since its appearance is prevented by cycloheximide and not by cordycepin or actinomycin-D. It has long been known from studies with RNA and protein synthesis inhibitors on pollen germination and tube growth that the ungerminated pollen grain at anthesis contains a store of stable m-RNA (MASCARENHAS 1975). This includes pollen from *Petunia hybrida* (SONDHEIMER & LINSKENS 1974). The poly(A)RNA from ungerminated *Tradescantia* pollen has been extracted, translated in cell free systems and shown to code for similar proteins as are synthesized during pollen germination (FRANKIS & MASCARENHAS 1980). It seems likely then that one at least of these proteins in *Petunia hybrida* pollen is the enzyme phytase. We have therefore established for the first time for pollen germination that a substrate (phytic acid) is degraded during germination by an enzyme (phytase) which is synthesized from pre-existing stable m-RNA, and we have mapped the time course of these events. In addition, since little or no phytic acid is found in vegetative tissue (JACKSON & LINSKENS 1982), these events are likely to be more specific to pollen and perhaps other reproductive tissue. So far only one enzyme (an esterase) has been shown to be unique to pollen and not expressed at all in vegetative tissue (TANSKLEY et al. 1981).

The properties of the phytase appearing in germinating pollen are not unlike those of phytase prepared from various seeds (COSGROVE 1980). The existence of two pH optima in phytase preparations has been noted before in lettuce seed (MAYER 1958) and cotton seed (FONTAINE et al. 1946) and like the situation in wheat brand (LIM & TATE 1973) could be due to the presence of two phytase enzymes. BREDEMEIJER (1971) obtained two peaks of phytase activity on sephadex G-200, when looking at extracts of ungerminated *Petunia hybrida* pollen, again suggesting two enzymes.

Another property of pollen phytase, that of inhibition by inorganic phosphate, was exploited to show the importance of phytase activity to the supply of raw materials (myoinositol) for tube wall growth. This finding, together with others described here, provide further support for the hypothesis generated from

our earlier studies (JACKSON et al. 1982) that the phytic acid found in pollen is essential for tube elongation, and thereby for successful fertilization. The myoinositol derived from phytic acid can be converted via the myoinositol oxidation pathway (ROSENFIELD et al. 1978) to uronic acid and pentose units of pollen tube wall polysaccharides.

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