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PHYTATE METABOLISM IN *PETUNIA* POLLEN

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Key Word Index—*Petunia hybrida*; Solanaceae; pollen; anther, metabolism; phytic acid; *myo*-inositol; phospholipids; phosphatidylinositol; pectic polysaccharide.

Abstract—Phytic acid has been detected in the anthers of young flower buds of *Petunia hybrida*, the amount increasing slowly as the flower develops until anther dehydration, when there was a more rapid increase in phytic acid content. In mature pollen, the phytic acid content was found to be 2.0% by weight, of which 90% was water soluble, while free *myo*-inositol was a relatively low 0.06% by weight. Breakdown of phytic acid was initiated soon after pollen germination began, and its degradation products, *myo*-inositol and inorganic phosphate, were rapidly mobilized for phospholipid and pectin biosynthesis. Both are in high demand during pollen tube elongation. Utilization of *myo*-[2-^{3}H]inositol for phospholipid biosynthesis was about five times that for pectin synthesis during the first few hours of pollen germination. The label in the phospholipid was identified as the *myo*-inositol moiety of phosphatidylinositol, while the pectin material contained predominantly labelled arabinose, with smaller amounts of label in galacturonic acid, glucose and xylose. A chase experiment showed that the *myo*-inositol moiety of phosphatidylinositol was subject to a relatively rapid turnover, while the label in pectin was not. Labelling germinating pollen with [^{32}P]orthophosphate gave label in phosphatidic acid, phosphatidylinositol, phosphatidylethanolamine and phosphatidylcholine of the phospholipids. Phosphatidylinositol contained 30% of this label initially, a proportion which declined to 10% over longer periods of germination.

INTRODUCTION

Phytic acid has recently been identified as a major constituent of the pollen of many plant species [1, 2]. When pollen from *Petunia hybrida* germinates, phytic acid is observed to break down and *in vitro* experiments with crude extracts suggest that *myo*-inositol and inorganic phosphate is produced [3]. The enzyme(s) responsible for this breakdown, phytase, can be found in ungerminated pollen, while further activity develops after germination begins, apparently as a result of translation of a stable mRNA already present in ungerminated pollen [3]. Accumulation of phytic acid is a characteristic of reproductive tissue, and the finding of this compound in pollen and more recently in stigma and styles of *P. hybrida* as well [4] emphasizes this fact. It has long been known to accumulate in seeds [5] where it is often concentrated in discrete regions [6]. Phytic acid is also known to break down to *myo*-inositol and inorganic phosphate during seed germination [6–9] but there are some differences from the degradation in pollen. In seed there is a much longer lag period before phytic acid is degraded [6] and this can be attributed to the necessity for the mRNA for phytase to be produced and then translated before the seed begins to break down phytic acid [10]. Another difference lies in the fact that in some species producing short pollen tubes, the pollen may have little or no phytic acid [1], while the seed of that species can have considerable phytate, as in wheat or barley [5]. Additionally phytic acid metabolism in pollen is partially controlled by the incompatibility genes [4]. In view of these differences, we have explored further the metabolism of phytic acid in *Petunia* pollen, and report here on the accumulation of phytic acid in anthers and the fate of the phytic acid breakdown product *myo*-inositol during subsequent germination of *Petunia* pollen.

RESULTS

Phytic acid accumulation in developing anthers

Paper electrophoretic determination of phytic acid [1] in extracts of developing anthers showed that phytate accumulates over a relatively long period in this tissue (Table 1). Moreover, the presumed intermediates at the

Table 1. Phytic acid content of developing anthers

Flower bud length (mm)	Fr wt (mg) (50 anthers)	Dry wt (mg) (50 anthers)	Phytic acid (% by dry wt)
10–14	172	35.2	0.01
15–19	220	40.0	0.02
20–24	275	47.5	0.025
25–29	281	46.5	0.03
30–39	310	47.5	0.05
35–39	311	53 0	0.20
40–44	284	52.5	0.40
45–49	241	50.0	0.80

The development shown took place over a period of six to seven days, the earliest stage being at the microspore stage, and the latest about one day before anthesis. Both fresh and dry weight are shown to indicate the onset of dessication at the later stages of development

various lower levels of phosphorylation *en route* to the biosynthesis of the hexaphosphorylated phytic acid (*myo*-inositol monophosphate, diphosphate, etc., all of which should be seen during paper electrophoresis) were not observed at any stage. The greatest increase in phytic acid took place after dessication of anthers had begun at about 35 mm flower bud length. Highest phytate levels were obtained just before anthesis, anthers then achieving 0.8% phytic acid by weight. Mature pollen, as described previously [1], contained 2.0% by weight phytic acid. At least 90% of the phytate in mature pollen was water-soluble, while further analysis of the mature pollen showed that the free *myo*-inositol content was low (0.06% by weight) compared to phytic acid. Feeding detached, developing flower buds with *myo*-[2-^{3}H]inositol gave only a small amount of labelled phytic acid in the anthers, some of the label also being recovered as free *myo*-inositol. Because much of the supplied label was directed to other parts of the flower, the amount of radioactivity in pollen phytic acid was thus too small to be used to trace the fate of its breakdown products during subsequent pollen germination. The presence of labelled free *myo*-inositol would also complicate the interpretation of such an experiment. Since cell-free extracts of pollen degraded added phytic acid to inorganic phosphate and *myo*-inositol (no phosphorylated intermediates were detected), and because no phosphorylated intermediates accumulated during phytate breakdown in pollen germinating *in vitro* [3], we considered it a reasonable alternative to study the utilization of added labelled *myo*-inositol and inorganic phosphate in attempting to determine the fate of phytate breakdown products during pollen germination. A similar approach has been used for seeds [6].

myo-*Inositol uptake and incorporation by germinating pollen*

After a slight lag the rate of *myo*-[2-^{3}H]inositol uptake into the 'alcohol-soluble, non-lipid cell contents' of germinating pollen increased up until about 90 minutes, after which the uptake slowed down and reached a plateau by 2.5 hours (Fig. 1). The decrease of radioactivity in the medium followed a complementary pattern, except that it continued to decrease throughout the incubation period of four hours.

The tritium label taken up by germinating pollen was incorporated into a lipid fraction and into alcohol-insoluble material (Fig. 2a). Incorporation of label into the lipid fraction started promptly at the onset of germination and followed a hyperbolic course for the first 45 minutes. Then the incorporation became linear for at least four hours. A lag period of about 30 minutes was observed for the incorporation of tritium label into alcohol-insoluble material. After four hours a linear incorporation rate was reached which was one-fifth of the value for the lipid fraction.

Incorporation of inorganic phosphate into phospholipids

In contrast to the incorporation of *myo*-[2-^{3}H]inositol into lipids, which began without delay, labelling with [^{32}P]orthophosphate had a lag period of 30 minutes (Fig. 2b). After a sharp increase up to two hours the incorporation rate slowed down until the end of the incubation period.

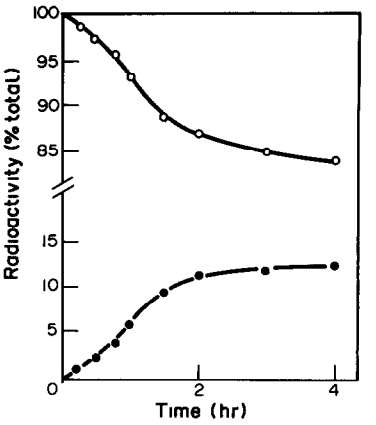

Fig. 1. Time course of incorporation of tritium from *myo*-[2-^{3}H]inositol into alcohol-soluble cell contents by germinating pollen. Results are expressed as percent of total radioactivity, remaining in the medium (○) and in alcohol-soluble cell contents (●).

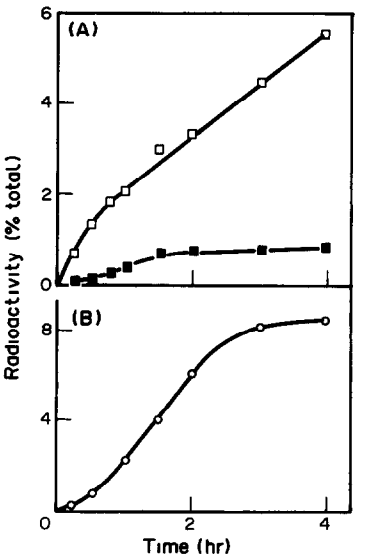

Fig. 2. Incorporation of labelled substrates into the lipid fraction and alcohol-insoluble (pectic polysaccharide) material in germinating pollen. (A) Incorporation of tritium from *myo*-[2-^{3}H]inositol into lipid (□) and alcohol-insoluble (pectic polysaccharide) material (■) and (B) incorporation of [^{32}P]orthophosphate into lipid.

TLC of the ^{32}P-labelled lipids showed four radioactive compounds, which comigrated with phosphatidic acid, phosphatidylinositol, phosphatidylethanolamine and phosphatidlycholine respectively in four solvent systems (A–D). Labelled phosphatidylinositol-4-monophosphate, phosphatidylinositol-4,5-biphosphate, phosphatidylglycerol and phosphatidylserine were not detected. Incorporation of ^{32}P into the four radioactive phospholipids did not follow the same patterns.

In the initial stages of germination (up to 15 min) both

Table 2. Time course of [^{32}P]orthophosphate incorporation into phospholipids during pollen germination

Time (min)	Radioactivity (dpm/35 mg of pollen) PC	PI	PE	PA	% Phospholipid ^{32}P in PI
15	820	794	540	564	29.2
30	3220	1486	14734	1606	13.5
60	10064	3508	18116	2776	10.2
90	17604	4904	28176	4224	8.9
120	29392	7268	39016	7836	8.7
180	41868	11524	40748	15740	10.5
240	66872	16440	54250	22986	10.2

phosphatidylinositol and phosphatidylcholine each contained about 30% of the radioactivity (Table 2). As germination proceeded phosphatidylcholine and phosphatidylethanolamine became the most highly-labelled phospholipids, while phosphatidylinositol contained only 10 per cent of the radioactivity.

Characterization of tritiated phospholipid

The only ^{32}P-labelled lipid component which was labelled with *myo*-[2-^{3}H]inositol as well, was identified as phosphatidylinositol. Thus, after TLC in solvent D no tritium was observed in positions corresponding with phosphatidylinositol-4-monophosphate and phosphatidylinositol-4,5-biphosphate. Further, mild hydrolysis of the tritiated lipid component in 0.01 M HCl (20 min, 100°) [11] gave only 16 percent of the tritium label in the aqueous phase after shaking with methanol–water–chloroform mixture used for pollen extraction. With 2 M HCl (50% methanol, 2 hr, 100°) 90–100% of the label was found in the aqueous layer. Paper chromatography of this hydrolysate in solvent E gave two radioactive peaks, one remaining near the origin (R_f 0.06), the other comigrating with authentic *myo*-inositol (R_f 0.35). The latter contained 30–50% of the label. After treatment of the hydrolysate with alkaline phosphatase, *myo*-inositol was then the only labelled compound on the paper chromatogram. It cocrystallized with authentic *myo*-inositol, with virtually no loss of specific activity. The relative resistance of phosphatidylinositol to acid hydrolysis necessitating the higher acid concentration described here has been observed previously [12].

As calculated from the amount of label from *myo*-[2-^{3}H]inositol appearing in phosphatidylinositol, the biosynthesis of this phospholipid in germinating *Petunia* pollen is of the same order (on a weight basis) as that reported for the very active Castor bean endosperm by Sexton and Moore [13], and much higher than that earlier described for cauliflower [12]. This is consistent with the suggestion by Hoeberichts and Linskens [14] that *P. hybrida* has 'fatty' and not 'starchy' pollen.

Characterization of labelled alcohol-insoluble material

Hydrolysis of alcohol-insoluble material with 2 M trifluoracetic acid dissolved virtually all tritiated compounds (Table 3). Eighty percent of the solubilized radioactive compounds were neutral sugars, which were not retained by the anion exchange resin. Paper chromatography in solvent F showed arabinose as the major labelled compound. Xylose and glucose were observed as minor components. Radioactivity was also found at the origin of the chromatogram, indicating the presence of oligomeric sugars, due to incomplete hydrolysis. Approximately one fifth of the radioactivity in the hydrolysates was retained by the exchange resin and released by elution with 4.0 N formic acid. Paper chromatography of these acidic sugars in solvent G showed about 4% of the label from alcohol-insoluble material at the origin, while more than 10% was galacturonic acid. We concluded that the bulk of the labelled alcohol-insoluble material was pectic cell wall polysaccharide. A similar label distribution was found for the pectic cell wall polysaccharides of Lily pollen germinated in *myo*-[2-^{3}H]inositol-containing medium [15, 16] and of *Petunia* pollen incubated with extracts of *myo*-[2-^{3}H]inositol-labelled styles [17], and is consistent with the composition of such polysaccharides [18].

Turnover of the myo-*inositol moiety of phosphatidylinositol (chase experiment)*

To investigate whether the *myo*-inositol moiety of phosphatidylinositol was subject to turnover, a chase experiment was carried out. Germinating pollen was incubated for two hours with 50 μM *myo*-[2-^{3}H]inositol,

Table 3. Composition of alcohol-insoluble material formed after 4 hours incubation in 50 μM *myo*-[2-^{3}H]inositol

Radioactivity (% total radioactivity in alcohol-insoluble material)					
TFA soluble material	99	Neutral sugars	80	Origin	8
				Galactose	0
				Glucose	4
				Arabinose	66
				Xylose	2
				Rhamnose	0
		Acidic sugars	19	Origin	4
				Galacturonic acid	11
				Glucuronic acid	0
				Other	4
TFA insoluble material	1				

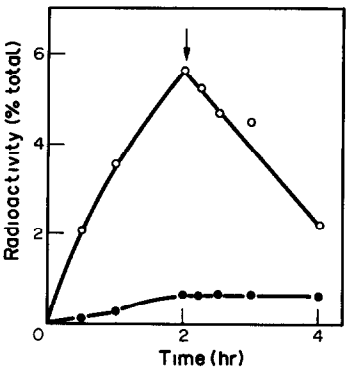

Fig. 3. Effect of radioisotope dilution on the radioactivity in phosphatidylinositol (○) and alcohol-insoluble (pectic polysaccharide) material (●). After 2 hr of germination (arrow) 73 kBq of *myo*-[2-^{3}H]inositol was diluted from 50 μm to 20 mM by the addition of unlabelled *myo*-inositol.

resulting in the incorporation of tritium into phosphatidylinositol and pectic polysaccharides (Fig. 3). A four hundred fold radioisotope dilution, achieved by addition of unlabelled *myo*-inositol after two hours of germination, chased the tritium label out of phosphatidylinositol. The decrease in radioactivity took place at a linear rate. Radioactivity in alcohol-insoluble material (containing pectic polysaccharides) remained at the same level as attained by two hours, when unlabelled *myo*-inositol was added.

DISCUSSION

The present investigation shows that phytic acid, a compound not generally found in vegetative tissue [2–5], begins to accumulate at a very early stage in the development of the reproductive (flower) bud of *P. hybrida*. Since it is also found in all the later stages of development of reproductive tissue such as mature pollen and stigma [4] and in seeds [5], phytic acid accumulation can now indeed be seen as a characteristic of reproductive tissue. Investigations into the biochemical aspects of differentiation from vegetative to reproductive growth should take account of this finding. The acceleration in phytic acid accumulation described here during the later dessication stage of anther development can only partly be explained by a fresh weight decrease. In seeds, changes in protein synthesis are known to occur during an equivalent dessication stage; however, the change in phytate seen here is one of increased accumulation and not a change in direction of metabolism as described for protein synthesis in developing seeds [19].

There is a change in direction of phytate metabolism however, during pollen germination, when a net degradation of the compound occurs soon after germination begins [2]. We conclude from our present studies that the *myo*-inositol moiety released during this degradation is utilized for phosphatidylinositol and pectic polysaccharide biosynthesis, both of which are needed in large amounts for pollen tube assembly as pollen tube elongation gets under way. The phosphate moieties released from phytate may also be utilised for phospholipid biosynthesis. We find that substantially more of the *myo*-inositol label is used for the synthesis of the phospholipid component phosphatidylinositol than for pectic polysaccharide. *myo*-Inositol had previously only been shown to be a precursor of pectic cell wall polysaccharides in germinating *Lilium* pollen [15, 16]; the phospholipid fractions (containing most of the label in *Petunia* pollen at least) were not investigated in *Lilium*.

For *Lilium* it was possible to germinate the pollen in a medium free of metabolizable sugar, using pentaerythritol. *P. hybrida* pollen shows very poor germination in this medium, so the present experiments were carried out in the usual sucrose medium where germination is extremely good (> 80% germination). It is difficult therefore to compare our results for pectic-substance labelling with the *Lilium* experiments. Roggen and Stanley [20] also studied incorporation of labelled *myo*-inositol into pollen and found that the highest proportion of label appeared near the tube tip of pear pollen germinating *in vitro*. As with *Lilium* pollen, these results were also interpreted in terms of utilization of labelled *myo*-inositol for pectin biosynthesis only. It is possible that the high density of label found near the pear pollen tube tip was due at least in part to labelled phospholipid taking part in fusion of Golgi vesicles with the plasma membrane in elongating pollen tubes [21].

In view of our earlier observation that the pattern of phytic acid degradation can be altered by the incompatibility genes [4], it remains to be determined how the relative utilization of *myo*-inositol for phospholipid or pectic polysaccharide biosynthesis can be affected by these genes. It is not clear either how the turnover in *myo*-inositol labelling of phosphatidylinositol described herein affects the observed ratio of labelling of pectin and phospholipid. However, the finding that one of the enzymes thought to be involved in the so-called 'PI response' of animal tissues has been found in higher plants [22], could make it of interest to investigate the possibility that the turnover observed here in germinating pollen can also be related to a 'PI response' to some biological functions (e.g. incompatibility reactions, or transport across membranes) as it is for certain other functions in animal tissue [23, 24].

EXPERIMENTAL

Pollen. Pollen was collected from *Petunia hybrida* L. clone W166H, grown under greenhouse conditions, and stored at −20°.

Chemicals. All chemicals were reagent grade. Standard phospholipids and alkaline phosphatase from *E. coli* were obtained from Sigma Chemical Co. *myo*-[2-^{3}H]Inositol (12.2 kBq/nmole) and [^{32}P]orthophosphate (carrier free) were purchased from the Radiochemical Centre, Amersham.

Pollen germination and fractionation Pollen (35 mg per 25 ml-Erlenmeyer flask) was hydrated for 60 min according to Gilissen [25]. Germination was carried out in 5 ml 10% sucrose, containing 0.01% boric acid, at 25° in a shaking water bath. *myo*-[2-^{3}H]Inositol (73 kBq, final concentration 50 μM), and, if indicated, [^{32}P]orthophosphate (800 kBq, final concentration 110 μM) was added as radioactive substrate. Radioisotope dilution of *myo*-[2-^{3}H]inositol from 50 μM to 20 mM was achieved by the addition of 100 μl 1 M *myo*-inositol

Germinated pollen was collected after different incubation periods by centrifugation (1 min, 1200 *g*) and washed (× 2) with germination medium lacking radioactive substrate. The super-

natant was combined with the two washes. After homogenization of the pollen in 2.0 ml MeOH, 1.8 ml H_2O and 4.0 ml $CHCl_3$ were added and the suspension was thoroughly mixed. Phase separation was performed by centrifugation (5 min, 1200 *g*). After removal of the organic phase, 4.0 ml $CHCl_3$ and 0.2 ml conc HCl were added to the remaining aq. and interphases, followed by phase separation as described above. The two organic phases were combined and will be referred to as 'lipid fraction'.

The insoluble interphase material was washed (× 4) with 2.0 ml 80% EtOH. The aq. phase was combined with the EtOH washes and referred to as the 'alcohol-soluble, non-lipid cell contents', the remaining insoluble material as 'alcohol insoluble material'. Hydrolysis of this insoluble material with 2 M TFA and fractionation of the hydrolysate on Dowex-1 (formate form) anion exchange resin was carried out according to Chen and Loewus [16].

Chromatography. TLC was carried out on Kieselgel 60 plates (Merck no. 5721) in the following solvents: A, $CHCl_3$–MeOH– H_2O (62:25:4); B, $CHCl_3$–MeOH–HOAc–H_2O (25:14:2:4); C, $CHCl_3$–MeOH–HOAc (60:25:8) and D, $CHCl_3$–MeOH–3.3 M NH_3 (43:38:12). PC was performed on Whatmann 3 MM paper in solvents; E, EtOAc–C_5H_5N–H_2O (10:6:5); F, EtOAc–C_5H_5N–H_2O (8:2:1) or G, EtOAc–C_5H_5N–HOAc–H_2O (5:5:1:3).

Determination of myo-*inositol in pollen. P. hybrida* pollen (200 mg) was homogenized in 2 ml MeOH. After addition of 4.0 ml $CHCl_3$ and 1.8 ml H_2O, followed by phase separation as described above, *myo*-inositol was recovered in the aq. phase. *myo*-Inositol remaining in the interphase material was extracted with 4 ml H_2O (three such extractions carried out). The combined extracts and aq. phase were applied to cation (Dowex-50, H^+ form) and anion (Dowex-1, formate form) exchange resins and eluted with H_2O.

After reduction in vol. under red. pres. an aliquot of the eluate was subjected to descending PC in solvent E (above) for 90 hr together with *myo*-inositol standards (10 μg to 100 μg). *myo*-Inositol, which migrated 8 cm, was visualized by passing the paper through methanolic solns of $AgNO_3$ and KOH (diluted tenfold from that described by Trevelyon *et al.* [26]). The amount of *myo*-inositol in pollen extracts was thus estimated by reference to the colour developed in the standards.

Radioactive measurements. Aliquots from aq. and lipophilic samples were adjusted to 0.5 ml with H_2O and MeOH, respectively. Insoluble samples were suspended in 0.5 ml H_2O. All radioactivity measurements were carried out with 5.0 ml Tritosol [27] as a scintillation cocktail in a Philips PW 4540 liquid scintillation analyser.

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