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Localization of Phytic Acid in the Floral Structure of *Petunia hybrida* and Relation to the Incompatibility Genes

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Summary. In three clones of *Petunia hybrida* with different incompatibility genes, phytic acid is detected exclusively in pollen, stigma and style. These are all parts of the floral structure involved in the incompatibility reaction. Phytase activity was detected in these tissues as well as in the ovary. The level of phytic acid and phytase activity varied between clones with different S alleles. This difference was most evident in stigma and style. The pattern of phytic acid breakdown following pollination depends on whether pollen and style form a compatible or incompatible combination. Incompatible pollination results in a higher rate of degradation. Consideration is given to the relationship between breakdown of phytic acid to myo-inositol and cell wall thickening and plug formation, which occurs to a greater extent in the incompatible combination.

Key words: Self incompatibility – Phytic acid – *Petunia hybrida* – Pollen – Stigma – Style

Introduction

Gametophytic incompatibility involving control of pollen tube growth in the style, as seen for example in *Petunia hybrida*, is genetically directed by one multiallelic S locus (Linskens 1975 a). When the pollen is compatible with the style, tube growth continues down the style to the ovary and fertilization follows. Incompatibility between pollen and style leads to cessation of tube growth halfway down the style so that fertilization cannot be achieved. Factors relating to control of the elongation of the pollen tube are therefore worthy of investigation in seeking a mechanism for this type of incompatibility. Recently we have shown that pollen from species which need to develop tubes long enough to negotiate styles greater than about 4 mm, have significant quantities of phytic acid (Jackson et al. 1982). Furthermore, pollen from species showing strong gametophytic incompatibility of the single multi-allelic S locus type (*Petunia hybrida, Antirrhinum majus, Lilium henryi*) had an extraordinary high phytic acid content. These finding, together with the realization that little is known about the distribution of phytic acid within the flower other than its presence in pollen, prompted us to investigate the interaction of the S genes with both the localization of phytic acid and phytic acid metabolism.

Materials and Methods

Petunia hybrida clones W166K (self incompatibility alleles S_1S_2), W166H (S_2S_3) and T_2U (S_3S_3) were grown in temperature controlled glasshouses. The parts of the flower were excised on the day of anthesis and snap-frozen in liquid nitrogen prior to freeze-drying. The freeze dried components were stored at −15 °C before analysis. Phytic acid was determined as described previously (Jackson et al. 1982). Extracts for phytase determination were prepared by grinding frozen segments in a mortar precooled in liquid nitrogen, and allowing 0.05 M sodium acetate, pH 5 buffer to freeze over the powder. The frozen buffer and plant material were then powdered together, allowed to thaw while being ground, and centrifuged at 10,000×g for 30 min. The supernatant was poured off and adjusted to 90% saturation ammonium sulphate by the addition of solid ammonium sulphate. The precipitate was collected by centrifugation, and dissolved in 0.05 M sodium acetate, pH 5. Phytase activity was determined at a substrate concentration of 0.5 mM sodium phytate in 0.05 M sodium acetate, pH 5 and at 45 °C. The reaction was stopped by the addition of trichloroacetic acid to 5% (W/V) and the inorganic phosphate released determined after separation from the phytic acid substrate (Irving and Cosgrove 1970). In terms of P released as inorganic phosphate from phytic acid substrate, it was found that the phytase activity in W166K pollen extracts was 3 µg P/h/mg pollen. All phytase activity is given here relative to this value. Germination of pollen in vitro with addition of stylar tissue was carried out as described previously (Jackson and Linskens 1982 a).

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Table 1. Phytic acid distribution in the various parts of the flower and variation between clones W166K, W166H and T_2U. The corresponding incompatibility genes are shown in brackets

	W166K (S_1S_2)			W166H (S_2S_3)			T_2U (S_3S_3)		
	Phytic acid (% by wt)	P (μmol per mg)	Av. wt. (mg)	Phytic acid (% by wt)	P (μmol per mg)	Av. wt. (mg)	Phytic acid (% by wt)	P μmol per mg)	Av. wt. (mg)
Stigma	0.3	0.17	0.85	0.3	0.18	0.92	0.6	0.22	0.70
Upper style	0.02	0.14	0.53	0	0.14	0.50	0.05	0.16	0.41
Lower style	0	0.14	0.49	0	0.15	0.46	0.01	0.17	0.30
Ovary	0			0			0		
Pollen	2.0	0.52		1.8	0.3		1.0	0.42	
Filaments	0			0			0		
Petals & other	0			0			0		

Results

1 Distribution of Phytic Acid in the Floral Structure

Phytic acid was found to be localized in the pollen (anthers) and in the stigmas in all three clones tested, with pollen always having the highest concentration (Table 1). The filaments however contained no significant amounts of phytic acid. While in two of the three clones the style showed some phytic acid, the concentration was always lower than the stigma and increased towards the stigma end. No stylar phytic acid was detected in clone W166H (S_2S_3). Although there was some correlation between phytic acid and total phosphate in the styles, this was not the case for pollen from the three clones (Table 1).

While the anthers, stigmas and in some cases, the styles were sites of phytic acid accumulation, other portions of the flower showed no significant phytic acid, including the ovary. The latter after fertilization becomes the site of accumulation of phytic acid in the developing seed (Jackson and Linskens 1982a). In *Petunia hybrida,* excluding the reproductive organs of the plant, no other tissue tested (leaves, stem, apical meristem, and roots) contained significant accumulations of phytic acid.

2 Phytase Activity in the Flower

By far the highest level of phytase activity to be found in the floral structure resides in mature pollen (Table 2). Unlike the striking localization of substrate phytic acid, all parts of the flower showed some phytase activity. Some difficulty lies in the interpretation of these results since we are uncertain of the specificity of phytase in *Petunia hybrida.* At least three acid phosphatases of unknown but probably quite wide specificity are present in some plant tissue (Tanksley et al. 1981), any of which may hydrolyse phytic acid. However, regardless of the specificity of these acid phosphates the fact remains that these or other enzyme(s) that can hydrolyse phytic acid are present in all parts of the flower tested. Thus no simple explanation relative to phytic acid disappearance via a phytase after pollination suggests itself from these results. However, it is apparent that the S_3 allele could be associated with low phytase in the pistil and the S_1 allele with high pistil phytase levels (Table 2).

Table 2. Relative phytase activity in various parts of the flower in clones W166K, W166H and T_2U

	W116K (S_1S_2)	W116H (S_2S_3)	T_2U (S_3S_3)
Stigma	0.1	0.01	0.03
Upper style	0.1	0.04	0.03
Lower style	0.05	0.03	0.03
Ovary	0.30	0.33	N.D.
Pollen	1.0	1.0	1.0

3 Changes in Phytic Acid Levels after Pollination and Influence of the S Genes

It has recently been established that phytic acid in pollen is rapidly degraded during germination in vitro (Jackson and Linskens 1982a). As shown in Fig. 1, when pollen from clones W166H (S_2S_3) or W166K (S_1S_2) is used to pollinate W166H (S_2S_3) stigmas, phytic acid is similarly reduced to lower levels over a period of several hours. However the pattern of degradation varies depending on whether the pollination is compatible (W166H × W166K) or self incompatible (W166H × W166H). The latter shows a steady reduction, while the compatible combination has a significantly higher phytic acid level at 2 h after pollination than the incompatible one, even though both are reduced to lower levels over a longer period. Other combinations with W166K, W166H and T_2U styles

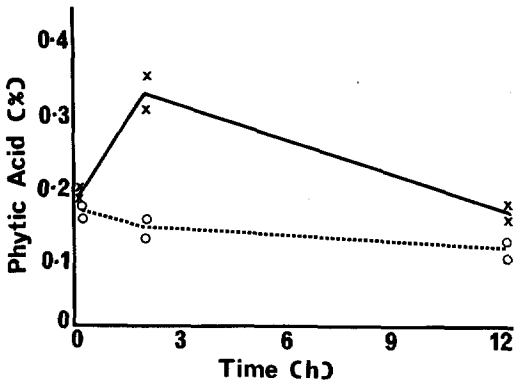

Fig. 1. Phytic acid content of W166H (S_2S_3) pistils after pollination with compatible W166K (S_1 and S_2 pollen) (—×—) or self-incompatible W166H (S_2 and S_3 pollen) (—○—). Pollinations were carried out under standard conditions, and pistils snap frozen at 10 min, 2 h or 12 h after pollination, prior to being freeze dried

showed a similar results at 2 h; thus pistils of the compatible combination contained higher amounts of phytic acid than those of the incompatible one (Table 3). It may well be that some phytic acid is synthesized over the first two hours in the pollen-stigma-style combination in the compatible situation, or perhaps synthesis occurs in both compatible and incompatible combinations and phytic acid is utilized more vigorously in the incompatible one. At present we lean towards the latter hypothesis as it seems to tie in well with the observed patterns of translocation of organic substances within the flower after pollination. Thus in the first few hours after pollination there is a stronger influx into self-pollinated incompatible styles than into cross-pollinated compatible styles (Linskens 1974, 1975 a, b).

4 Sparing Effect of Styles on Pollen Phytic Acid Breakdown

As referred to above, *Petunia hybrida* pollen when germinated in vitro quickly loses contained phytic acid (Jackson and Linskens 1982 a). When W166K styles are added to germinating pollen in vitro, the loss of phytic acid in the pollen after two hours is reduced, particularly with W166H (or compatible) pollen (Table 4). Styles reisolated from the culture solution contained only very small amounts of phytic acid in both cases, as expected, and so differential binding of pollen to styles cannot explain the result.

Table 3. Phytic acid content of pistils 2 h after pollination in various crosses between the clones. Two different repetitions

Style	Pollen	Phytic acid (% by wt)
W166K (S_1S_2)	W166K (S_1, S_2)	0.20; 0.15
W166K (S_1S_2)	T_2U (S_3, S_3)	0.40; 0.40
T_2U (S_3S_3)	T_2U (S_3, S_3)	0.40; 0.35
T_2U (S_3S_3)	W166H (S_2, S_3)	0.50; 0.55
W166H (S_2S_3)	W166H (S_2, S_3)	0.50; 0.45
W166H (S_2S_3)	T_2U (S_3, S_3)	0.55; 0.55

Table 4. Sparing effect of styles on phytic acid degradation during pollen germination in vitro for 2 h

Pollen	Style[a, b]	Phytic acid (% by wt)
W166H (S_2S_3)	–	0.15, 0.15
W166H (S_2S_3)	W166K (S_1S_2)	0.35, 0.30
W166K (S_1S_2)	–	0.05, 0.07
W166K (S_1S_2)	W166K (S_1S_2)	0.05, 0.10

[a] Styles had 0.02% phytic acid after the incubation
[b] 3 Styles per 35 mg pollen in 5 ml medium

5 Other Factors Involving Stability of Phytic Acid in Pollen

Germination of *Petunia hybrida* pollen in vitro is greatly improved by prior hydration of the pollen at high relative humidity (Gilissen 1977). We observed no breakdown in pollen phytic acid as a result of this treatment (up to 48 h at 100% relative humidity, 20 °C). Breakdown of phytic acid subsequently takes place in pollen from all three clones when shaken (or merely stood) in 10% sucrose solutions with or without added boron. When a wheat germ phytase preparation (2 mg per 35 mg pollen) was added to germinating pollen cultures, there was no acceleration of phytic acid degradation after 1 h, nor was there any subsequent effect on the germination rate or average tube length achieved after 14 h for any of the three clones tested.

Discussion

Earlier investigation into the physiology of phytic acid dealt mainly with the development of the seed after fertilization and the concentration of phytic acid in the aleurone layer of the monocotyledenous seed (Cosgrove 1980; Jennings and Morton 1963; Tanaka et al. 1973; Tanaka et al. 1974). Recently we have looked for phytic acid in reproductive tissue before fertilization and investigated as wide a range of species as possible. We found that phytic acid occurs in pollen (Jackson et al. 1982; Jackson and Linskens 1982 b), particularly in dicotyledenous species with longer styles, and that in *Petunia hybrida* at least, phytic acid is degraded during in vitro pollen germination (Jackson and Linskens 1982 a).

The present investigations with *Petunia hybrida* reproductive tissue made before fertilization builds on the previous observations that phytic acid occurs in high concentration in the pollen of species showing gametophytic incompatibility and demonstrates that it is found only in those parts of the plant taking part in the incompatibility reaction. Thus we found that phytic acid is localized before pollination in the pollen, stigma and style in amounts which vary according to the S alleles present, and that the same alleles influence the changes in phytic acid levels that occur after pollination. Incompatible reactions lead to a more rapid decrease of phytic acid than with compatible alleles, which could in turn imply greater utilization by the incompatible combination. In germinating lily pollen myo-inositol is taken up readily for polysaccharide biosynthesis (Loewus et al. 1978) where the myo-inositol oxidation pathway is operational for pectin synthesis (Loewus and Loewus 1980). Assuming a similar pathway in *Petunia hybrida*, then the products of phytic acid degradation, myo-inositol or a phosphorylated derivative (Cosgrove 1980), could well be utilized for the biosynthesis of pectin or other polysaccharides. Recently Kroh and Knuiman (1982) found that the secondary wall thickening and the plugs of pollen contain pectin in addition to callose and cellulose. Linskens (1975a) pointed out that in the incompatible pollen tube a wall thickening occurs and more "callose" plugs are formed. It is tempting to suggest that the more rapid decrease of phytic acid observed in our studies after an incompatible pollination is in fact due to increased utilization of phytic acid for the formation of inositol which in turn is in greater demand for pectin biosynthesis in the early stages of incompatible pollen germination and tube growth. This agrees well with the differences in flow of organic substances described by Linskens (1974, 1975a, b) for incompatible and compatible pollinations. Later after pollination one would expect the difference in phytic acid levels between incompatible and compatible combinations to decrease owing to steady uptake for pectin synthesis in the longer tubes developing as a result of compatible pollination after incompatible tube growth has ceased (see Linskens 1975a, b).

We have shown here several points of interaction of phytic acid localization and metabolism with the S alleles. Further biochemical investigation will be needed to verify our interpretation in terms of pectin biosynthesis and its control by incompatibility alleles.

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