Theoretical and Applied Genetics 40, 173—175 (1970)

The Effect of Cycloheximide and 6-Methylpurine on *in vivo* Compatible and Incompatible Pollen Tube Growth in *Lilium longiflorum**

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Summary. Cycloheximide, a potent inhibitor of protein synthesis, placed in styles of *Lilium longiflorum* at 10^{-4} M in stigmatic exudate before, 6, or 12 hr after compatible or incompatible pollination retarded all pollen tube growth. An inhibitor of RNA synthesis, 6-methylpurine, placed in the style at 10^{-4} M in stigmatic exudate before, 6, or 12 hr after pollination restricted compatible pollen tube growth to lengths not significantly different than incompatible pollen tubes in treated or nontreated styles. While pollen tube growth in the style of *L. longiflorum* appears to require protein synthesis, only compatible pollen tube growth requires RNA synthesis. Stigmatic exudate proved to be an excellent carrier of exogenous substances into the style of *L. longiflorum*.

Introduction

The diploid organism with highly differentiated, multicellular organs and an involved life cycle does not lend itself readily to the application of concepts resulting from the molecular biology of microorganisms. However, some of these difficulties can be overcome in angiosperms by investigating gene action in the male gametophyte, a haploid organism which at maturity consists of a pollen tube containing a vegetative nucleus and two sperm. Development of the male gametophyte from the pollen grain involves germination on the stigma, growth down the style to the ovary, and at most, one mitosis, that of the generative cell into sperm.

Superimposed on the relatively simple developmental pattern of the male gametophyte of many angiosperms is gametophytic self-incompatibility. Pollen tubes of gametophytically self-incompatible species follow one of two mutually exclusive developmental pathways, either that of a compatible tube, growing to the ovary and accomplishing fertilization, or that of an incompatible pollen tube, being inhibited in the style before fertilization, depending on the interaction between alleles in pollen and style at the self-incompatibility locus. When the self-incompatibility allele in the pollen matches one of those of the style, the pollen tube is incompatible. Modification of pollen tube growth by the self-incompatibility reaction is irreversible. Incompatible pollen tubes differ from compatible in respiration (Linskens, 1955), protein complement (Linskens, 1955) and amino acid pattern (Linskens and Tupy, 1966), as well as growth rate. The self-incompatibility reaction appears to be an excellent system for studying gene action in higher organisms.

The Easter lily, *Lilium longiflorum*, has a hollow style 100 to 150 millimeters long, lined with specialized epithelial cells on which pollen tubes grow to the ovary. Incompatible pollen tubes in *L. longiflorum* grow about half the length of the style in the time that compatible tubes pass through the style and reach the ovary. Exudate collected from the stigma of the Easter lily can be injected into the style before or after pollination without adversely affecting pollen tube growth or the self-incompatibility reaction (Ascher and Drewlow, 1969). Therefore; inhibitors of RNA and protein synthesis can be added to stigmatic exudate, injected into the style, and the effect on pollen tube growth and the self-incompatibility reaction determined *in vivo*.

Materials and Methods

Lilium longiflorum Thunb. cultivars Ace and Nellie White are self-incompatible but cross-compatible. Pistils from flowers on greenhouse grown plants of cultivar Ace were excised one to 3 days after anthesis for experimental use. Incompatible pollen tubes result from self pollination of these pistils while compatible tubes result from pollination with Nellie White pollen. Stigmatic exudate was harvested from cut flowers of Nellie White maintained in water in the laboratory and was stored until used at −29 °C. The inhibitors cycloheximide and 6-methylpurine were dissolved directly in stigmatic exudate at a concentration of 10^{-3} M and diluted with exudate to 10^{-4} M for injection into the style. Control styles were injected with exudate alone. Injections were made before, 6, or 12 hr after compatible or incompatible pollination. Experiments involving treatment before pollination consisted of 8 replications while those involving post-pollination treatment were replicated 4 times.

To place the exogenous material into the stylar canal, the ovary was snapped from the pistil and the material injected through the stigma with a hypodermic syringe and a 22 gauge needle until a drop of the substance ap-

* Paper number 7047 of the Scientific Journal Series Minn. Agr. Exp. Sta. Research was supported in part by funds provided by the Graduate School, University of Minnesota.

peared at the ovarian end of the style. Styles were incubated at 25 °C for 48 hr after pollination. Pollen tube length was determined by longitudinally bissecting the style with a razor blade, staining with acetocarmine and aqueous aniline blue and measuring the longest pollen tube in each half style to the nearest millimeter.

Results and Discussion

Pollen released from the anther contains sufficient ribosomes for growth of the pollen tube without synthesis of ribosomal RNA (Steffensen, 1966; Jensen, Fisher and Ashton, 1968; Mascarenhas and Bell, 1969). The presence of polyribosomes in ungerminated pollen and the formation of additional polyribosomes within minutes of the placement of pollen into a medium suitable for germination (Linskens, 1967; Mascarenhas and Bell, 1969; Crang and Miles, 1969) suggest that preformed messenger RNA also exists in the resting pollen grain. Inhibition of RNA synthesis with actinomycin-D does not affect pollen tube germination and growth *in vitro* (Mascarenhas, 1966).

Table 1. *The length of pollen tubes in millimeters after 48 hr growth in L. longiflorum styles filled before pollination with 10^{-4} M cycloheximide in stigmatic exudate*

Pollination	Cycloheximide in stigmatic exudate	Stigmatic exudate alone (control)
Incompatible	6.0 a*	62.1 b
Compatible	6.6 a	97.7 c

* Means followed by different letters are significantly different at the 1% level (Duncan's New Multiple Range Test).

Cycloheximide placed in the style of *L. longiflorum* before pollination severely retarded pollen tube growth (Table 1). The morphology of the inhibited pollen tubes deviated considerably from normal: tubes in cycloheximide treated styles grew in a corkscrew fashion, bulged on the sides and tip, and appeared to be disoriented in the style, often growing across rather than down the stylar canal. In morphology and length, these pollen tubes resembled the inhibited tubes resulting from intrasectional, interspecific pollination in *Lilium* (Ascher and Peloquin, 1968). Treatment of styles 6 or 12 hr after pollination with 10^{-4} M cycloheximide did not alter the pattern of pollen tube growth from that observed when the inhibitor was placed in the style before pollination (Table 2). The greater length reached by pollen tubes in styles treated 12 hr after pollination can be explained by the growth of tubes before treatment. For the first 12 hr after pollination, pollen tubes of *L. longiflorum* grow at a rate of about one millimeter an hour (Ascher, unpublished). Therefore, treatment with cycloheximide 6 or 12 hr after pollination apparently inhibited pollen tube growth immediately.

Table 2. *Length of pollen tubes in millimeters after 48 hr growth in L. longiflorum styles injected 6 or 12 hr after pollination with stigmatic exudate containing 10^{-4} M cycloheximide*

Pollination	Injection 6 hr after pollination	Injection 12 hr after pollination
Incompatible	6.8	10.2
Compatible	6.5	13.1

Cycloheximide inhibits protein synthesis by interfering with the transfer of amino acids from aminoacyl-sRNA to nascent polypeptide chains (Ennis and Lubin, 1964; Siegel and Sisler, 1964) and by inhibiting the initiation of new polypeptide chains on ribosomes (Lin, Mosteller, and Hardesty, 1966). Since pollen of *L. longiflorum* germinates in hanging drops of stigmatic exudate containing 10^{-4} M cycloheximide (Ascher, unpublished) and pollen tubes grew into the stylar canal of styles treated with cycloheximide before pollination, pollen of the Easter lily must contain the protein necessary for germination as well as stores of ribosomes, polyribosomes and mRNA. The rapid inhibition of pollen tube growth by cycloheximide injected after pollination and the limited growth of tubes in styles treated before pollination suggest a dependence of pollen tube growth in the style on continued protein synthesis.

6-methylpurine injected into Easter lily styles before pollination restricted the growth of pollen tubes to lengths not significantly different than those of incompatible pollen tubes growing in styles containing stigmatic exudate only (Table 3). The stimulation of pollen tube growth attributed to the presence of stigmatic exudate in the style before pollination (Ascher and Drewlow, 1969) was not affected by 6-methylpurine. Retardation of compatible pollen tube growth to a rate typical of incompatible tubes also occurred when the inhibitor was applied to the style 6 or 12 hr after pollination (Table 4). Morpho-

Table 3. *Length of pollen tubes (mm) after 48 hr growth in L. longiflorum styles injected before pollination with 10^{-4} M 6-methylpurine in stigmatic exudate*

Pollination	6-methylpurine in stigmatic exudate	Stigmatic exudate alone (control)
Incompatible	74.1 a*	77.1 a
Compatible	75.5 a	96.5 b

* Means followed by different letters are significantly different at the 1% level (Duncan's New Multiple Range Test).

Table 4. *Length of pollen tubes (mm) after 48 hr growth in L. longiflorum styles injected 6 or 12 hr after pollination with stigmatic exudate containing 10^{-4} M 6-methylpurine (6-mp) or exudate alone (control)*

Pollination	Injection 6 hr after pollination		Injection 12 hr after pollination	
	6-mp	control	6-mp	control
Incompatible	57.0	55.7	57.6	56.4
Compatible	57.0	83.5	55.5	73.7

logically, pollen tubes in 6-methylpurine-treated styles did not differ from tubes in noninjected styles.

As an analog of adenine, 6-methylpurine inhibits RNA synthesis by depressing incorporation of adenine into RNA and inhibiting the synthesis of guanylic acid from adenylic acid (Miller and Kempner, 1963). The effect of 6-methylpurine on pollen tube growth in *L. longiflorum* styles suggests that compatible pollen tube growth depends on an RNA synthesis unnecessary for incompatible pollen tube growth. Pollen tubes of the Easter lily appear to be capable of growth as incompatible tubes on the RNA stored in the pollen grain. The requirement of RNA synthesis, presumably for the elaboration of a new protein complement, indicates that compatible pollen tubes grow utilizing a metabolic pathway different than that of incompatible tubes. Synthesis of RNA necessary for compatible pollen tube growth is not completed before 12 hr after pollination.

A gene action modeling explaining self-incompatibility (Ascher, 1966) proposed the existence of two metabolic pathways in pollen tube growth: one conferring limited growth potential at a slow velocity, typical of incompatible pollen tubes, and the second resulting in an extensive growth potential at a faster rate, typical of compatible pollen tubes. Identity of self-incompatibility alleles in pollen and style would prevent the pollen tube, which germinated and began growth utilizing the slow growth metabolism, from switching to the fast growth metabolism, and would result in an incompatible tube. This hypothesis requires the pollen tube to synthesize the enzymatic machinery for the fast growth metabolism, that is, to transcribe DNA message into mRNA and translate the mRNA message into protein, after pollen germination. The effect of an inhibitor of RNA synthesis, 6-methylpurine, on compatible pollen tube growth in *L. longiflorum* styles supports this model.

The successful utilization of stigmatic exudate as a carrier of exogenous substances into the environment of growing pollen tubes in *L. longiflorum* suggests many new avenues of research. With this technique pollen tube metabolism can be studied *in vivo* with isotopic tracers, antimetabolites and inhibitors.

The authors wish to thank the United Bulb Co., Mount Clemens, Mich. for lily bulbs and L. H. Fuchigami and L. V. Gusta, Dept. of Hort. Sci., Univ. of Minn. for advice on use of the inhibitors. Mr. Drewlow is a National Science Foundation predoctoral trainee.

References

1. Ascher, P. D.: A gene action model to explain gametophytic self-incompatibility. Euphytica **15**, 179 to 183 (1966). — 2. Ascher, P. D., Drewlow, L. W.: Effect of stigmatic exudate injected into the stylar canal on compatible and incompatible pollen tube growth in *Lilium longiflorum* Thunb. International Conference on Pollen Physiology, Pullman Wash. (1969). — 3. Ascher, P. D., Peloquin, S. J.: Pollen tube growth and incompatibility following intra and interspecific pollinations in *Lilium longiflorum*. Amer. J. Bot. **55**, 1230—1234 (1968). — 4. Crang, R. E., Miles, G. B.: An electron microscope study of germinating *Lychnis alba* pollen. Amer. J. Bot. **56**, 398—405 (1969). — 5. Ennis, H. L., Lubin, M.: Cycloheximide and acetoxycycloheximide: Inhibitors of transfer of amino acid from aminoacyl-s-RNA to polypeptide in mammalian cells. Fed. Proc. **23**, 269 (1964). — 6. Jensen, W. A., Fisher, D. B., Ashton, M. E.: Cotton embryogenesis: The pollen cytoplasm. Planta **81**, 206—228 (1968). — 7. Lin, S. Y., Mosteller, R. D., Hardesty, B.: The mechanism of sodium fluoride and cycloheximide inhibition of hemoglobin biosynthesis in the cell free reticulocyte system. J. Mol. Biol. **21**, 51—69 (1966). — 8. Linskens, H. F.: Physiologische Untersuchungen der Pollenschlauch-Hemmung selbststeriler Petunien. Z. Bot. **43**, 1—44 (1955). — 9. Linskens, H. F.: Isolation of ribosomes from pollen. Planta **73**, 194—200 (1967). — 10. Linskens, H. F., Tupý, J.: The amino acids pool in the style of self-incompatible strains of *Petunia* after self and cross-pollination. Der Züchter **36**, 151—158 (1966). — 11. Mascarenhas, J. P.: Pollen tube growth and ribonucleic acid synthesis by vegetative and generative nuclei of *Tradescantia*. Amer. J. Bot. **53**, 563—569 (1966). — 12. Mascarenhas, J. P., Bell, E.: Protein synthesis during germination of pollen, studies on polyribosome formation. Biochim. Biophys. Acta **179**, 199—203 (1969). — 13. Miller, J. H., Kempner, E. S.: Effects of an adenine analog on yeast metabolism. Biochim. Biophys. Acta **76**, 333—340 (1963). — 14. Siegel, M. R., Sisler, H. D.: Site of action of cycloheximide in cells of *Saccharomyces pastorianus* II. The nature of inhibition of protein synthesis in a cell free system. Biochim. Biophys. Acta **87**, 83—89 (1964). — 15. Steffensen, D. M.: Synthesis of ribosomal RNA during growth and division in *Lilium*. Exp. Cell Res. **44**, 1—12 (1966).

Received January 20, 1970.

Communicated by H. F. Linskens

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