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Disc-Electrophoresis of Proteins and Enzymes from Styles, Pollen and Pollen Tubes of Self-Incompatible Cultivars of *Lilium longiflorum* *

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Summary. *Lilium longiflorum* possesses several advantages for investigating the nature of self-incompatibility. The large hollow styles permit analyses of proteins and enzymes from pollen tubes removed from the styles after selfing or crossing along with their corresponding styles, as well as from pollen grains and from styles of unpollinated flowers. Biochemical methods have been developed for protein extractions and subsequent analyses by disc electrophoresis. These techniques have been used to demonstrate changing protein and esterase patterns in pollen and styles from four cultivars. No apparent association between the self- incompatibility reaction and proteins which migrate at pH 8.3 in 7½% acryamide gel was noted. These analyses included soluble proteins, esterases, peroxidases and several dehydrogenases.

I. Introduction

The incompatibility reaction between pollen and styles with similar S alleles is an effective means of preventing fertilization in many flowering plants. Several hypotheses have been proposed which speculate on the substances and the type of interactions involved in the reaction, however, no specific biochemical mechanism has been elucidated. Generally proteins have been implicated as one of the principal incompatibility substances, either directly in the reaction or indirectly as an enzyme affecting an end product for the reaction.

Two genera, *Oenothera* and *Petunia*, frequently used in biochemical studies of incompatibility possess the gametophytic system of self-incompatibility. In this system pollen tubes are usually retarded as they grow through solid styles. Therefore, it is difficult to separate pollen tubes from surrounding stylar cells; thus most biochemical analyses have been of substances present in mature pollen or styles.

Lilium longiflorum offers an advantage for biochemical studies of the incompatibility reaction not provided by *Petunia* or *Oenothera*. It possesses a hollow style which allows pollen tubes to be easily removed from the style at various stages of growth or following various treatments. It is therefore possible to allow pollen tubes and styles to interact and still separate them for biochemical analyses. The purpose of this study was to use disc electrophoresis to examine total soluble proteins and specific enzymes from pollen, styles of various ages from unpollinated flowers, pollen tubes removed from styles, and corresponding styles.

II. Materials and Methods

Lilium longiflorum Thunb. cultivars Ace, Croft, Georgia, and Nellie White were the source of pollen and styles. The detached style technique of ASCHER and PELOQUIN (1966b) was employed to maintain styles following pollination. Pollen was taken directly from flowers. Pollen tubes were removed by slicing styles longitudinally and gently lifting them out with fine forceps.

Several methods of tissue maceration and subsequent release of cell contents, measured by proteins discernible with disc electrophoresis, were compared. Cold buffer extractions, tris, phosphate or acetate from pH 6.4 to 8.4 were not efficient either with abrasives using mortor and pestle or without abrasives using glass homogenizing tubes. Presumably degradative enzymes and phenoloxidase activity is not suitably prevented when only pH is maintained. Addition of sodium hydrosulfite to the extraction buffer did not noticeably improve stability. Cold acetone precipitates followed by cold ether wash yielded "proteins" which apparently did not retain their electrophoretic mobility. Dialysis of the buffer extracts against PVP or aquacide were reasonably successful, however this was time consuming and for very small samples resulted in a loss of material. Freezing and thawing tissue directly or following submergence in buffer did not break an adequate number of cells. Sugar concentration was varied and found to be optimal at five percent, or above, as determined by cell rupture.

The procedure which gave the most consistent results and greatest number of proteins involved a more complex extraction medium. Extracts were prepared by macerating styles or pollen in cold ground-glass homogenizing tubes containing the following media: 0.01 M potassium phosphate buffer pH 6.4, 250 μM cysteine, mercaptoethanol or dithiothreitol, 5% sucrose, 6 μM magnesium chloride, 1% deoxycholate and 10% PVP. Pollen tubes were placed directly in this medium in depression slides and macerated with a small glass stopper. Pollen tube samples were applied directly to the gels. The whole pollen and styles samples were centrifuged before application. The amount of extraction media used generally was 0.1 ml. per style, 1 ml. per pollen from six anthers, and 0.2 ml. per pollen tubes from six styles.

Samples from pollen grains (PG), styles from unpollinated flowers (US), pollen tubes removed from

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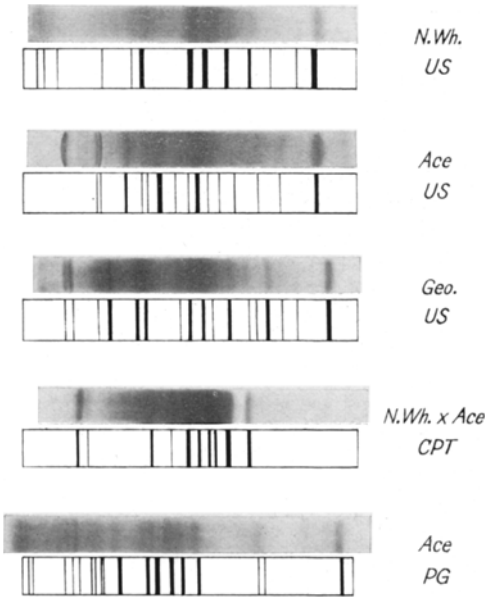

Fig. 1. Representative photographs and diagrams of protein patterns from extracts of styles of Nellie White buds (160 mm), Ace flowers two days after anthesis, Georgia flowers nine days after anthesis and of pollen tubes removed from Nellie White styles 42 hours after pollination with Ace pollen, and Ace pollen grains

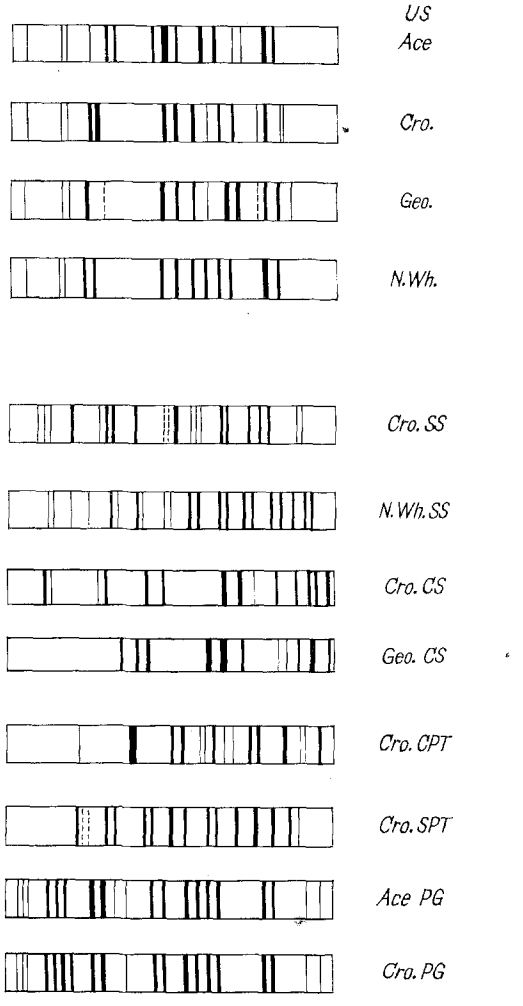

Fig. 2. Representative diagrams of protein patterns from extracts of styles from flowers at anthesis of Ace, Croft, Georgia and Nellie White and from pollen, pollen tubes, and styles

styles following self (SPT) or cross (CPT) pollination, and the corresponding styles from which pollen tubes have been removed after self (SS) or cross (CS) pollination were prepared. The number of styles used for an individual sample varied from four to twelve (a few of one or two were included); in general six styles were pooled for each sample. Correspondingly, pollen tubes from six styles were usually pooled. PG, US, SPT, CPT, SS and CS samples were analysed directly by electrophoresis or frozen and then analysed. No differences were noted between fresh and frozen samples, therefore, samples could be stored at −20 °C. until used.

Soluble proteins and enzymes were separated by disc electrophoresis as described in DESBOROUGH and PELOQUIN (1966). Electrophoresis was carried out at 5 °C for 1 to 2 hours and protein staining was with Aniline Blue Black. Attempts were made to determine a number of specific enzymes. Methods detecting esterases (DESBOROUGH and PELOQUIN, 1967) and peroxidases (DESBOROUGH and PELOQUIN, 1968) were the most reliable. Tests for catalases, amylases, phosphatases, cytochrome oxidase and leucine aminopeptidase were not reliable. The failure to detect these enzymes could be due to either low concentrations within the samples or possibly the methods for enzyme determination were not valid. Dehydrogenase activity for the following substrates-lactate, malate, oxalsuccinate, pyruvate, glucose-6-phosphate, 6-gluconic phosphate and l-glutamate was investigated. The basic solution contained 3 mg $MgCl_2$, 0.6 mg TPN, 2 mg Nitro Blue Tetrazolium, 1 mg Phenazine Methosulfate, 10 ml tris pH 7.5, and 30 mg substrate. Only enzymes active on the latter three substrates given above were detected in high enough activity to be considered.

III. Results

Soluble proteins were separated by disc electrophoresis from styles of unpollinated flowers ranging in age from 3 days prior to anthesis to 12 days after anthesis. All four cultivars, Ace, Croft, Georgia and Nellie White, revealed many minor differences in the number of protein bands and in their banding patterns at various style ages. No simple difference appeared cultivar specific or correlated with specific floral age. Representative gel photos and diagrams of three style sample are given (Fig. 1); proteins of Georgia are more widely spaced because electrophoretic separation was for a longer time. A maximum of twenty proteins and a minimum of twelve proteins were observed in gels from styles. The group of proteins near the gel center were usually most prominant. Gels from styles of the four cultivars taken from flowers at anthesis are represented by diagrams (Fig. 2). It should be emphasized that these are representative of only a few samples, many floral ages and various style numbers were included in analyses of other samples.

Comparisons of US with either SS or CS samples were made between flowers of the same age from the same cultivar. No consistent differences were noted in proteins attributable to type of pollination. Also, no effect of floral age at time of pollination on protein patterns was evident. Two examples of SS and CS

samples are given (Fig. 2); these diagrams should not be compared with the top four because their protein extracts were done in other media and running time was longer. SS and CS samples were also examined for possible differences due to length of time of PT growth. After one, two or three days of SPT or CPT growth, no striking changes were realized in SS or CS samples.

Likewise, no large protein differences were detected in SPT or CPT samples after one, two or three days growth or between SPT and CPT samples. SPT and CPT samples contained about 10 to 20 proteins (Fig. 1 and 2). These samples revealed minor variations within any one experiment, but no single protein or group of proteins could be associated with either SPT or CPT samples. The extraction procedure for pollen tubes was the most unreliable probably because external effects on such small samples were more difficult to control.

PG extracts from four cultivars yielded a greater number of protein bands, up to 25, than pollen tubes or styles. PG from all cultivars appeared to have similar protein patterns (Fig. 1 and 2). The additional four bands in Ace pollen (Fig. 1 vs Fig. 2) indicates improvement in extraction technique. The bands near the top of PG gels appear to be lipoproteins not observed in pollen tubes.

A summary of protein analyses of PG, US, SPT, CPT, SS and CS samples, which were considered successful, is given in Table 1. The floral ages and length of time after pollination are not detailed since no major differences dependent on any one factor were detected. The resultant protein patterns from these samples represent about 70% of the experiments.

Table 1. *Number of successful protein determinations from extracts of various types of samples by disc electrophoresis*

Types of Samples	Cultivars			
	Ace	Croft	Georgia	Nellie White
US	24	23	19	25
SS	13	19	15	13
SPT	18	10	4	6
PG	8	5	3	4
CS	total of 13			
CPT	total of 13			

The protein patterns from US, CS, SS, CPT, SPT and PG did have bands in common, i.e. proteins with the same electrophoretic mobility. The extraction procedures, or more likely biochemical events occurring in the plant material, produced minor changes in proteins that appear to vary within styles and pollen or pollen tubes.

The inability to detect specific protein differences could be due to several causes but the main drawback in electrophoresis seems to be lack of a sensitive protein stain (in some instances complete inability to form a protein-dye complex). The zymogram method determining enzyme systems was more sensitive, that is enzymes could be made visible in gel regions which remain clear after protein staining. In addition, the possibility of noticing minor changes in banding patterns is increased when fewer bands can be compared. For these studies it was more

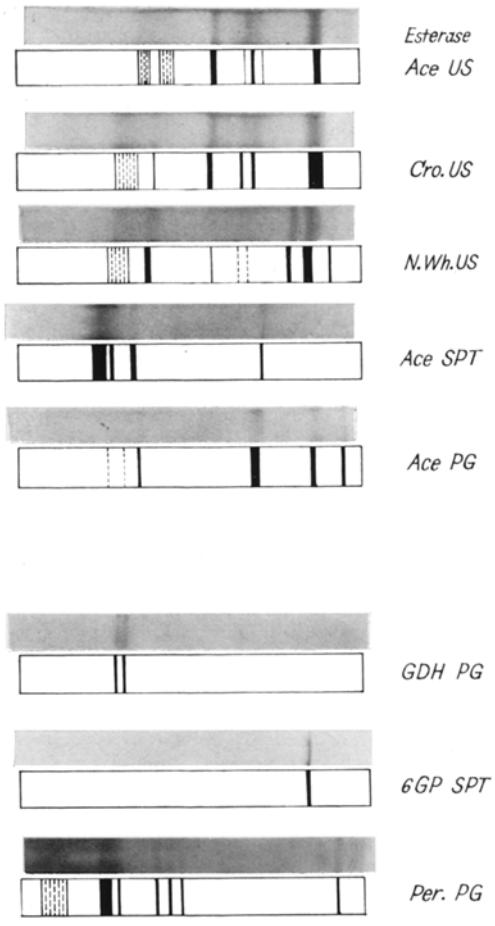

Fig. 3. Representative isozyme photographs and diagrams of esterase, glutamic dehydrogenase (GDH), 6-gluconic phosphate dehydrogenase (6GP) and peroxidase (Per.) from extracts of styles, pollen tubes and pollen grains

meaningful to identify proteins as specific enzymes that may be affected by the incompatibility reaction. Therefore experiments to designate certain proteins as esterases were next initiated.

Esterases active on alpha naphthyl acetate are stable in the extraction media and all samples examined contained at least one isozyme. Several isozymes were observed which could be compared to protein patterns for identification of specific proteins as esterases. One of a pair of gels from a style extract was stained for protein and the other for esterase. The style samples exhibited esterase isozymes which migrated to three gel locations. In Ace and Croft styles a single isozyme had the fastest rate, an intermediate group of isozymes varied from 1 to 4 and a slower group varied from 2 to 4 (Fig. 3). Ace Croft and Nellie White styles were analyzed at various floral ages to determine if esterase isozymes revealed variations similar to those of proteins. The esterase isozyme patterns were different in Ace and Nellie White styles; styles taken daily from flowers one day prior to anthesis through 12 days old did reflect changes in these isozymes patterns (Fig. 3). The enzyme variations parallel protein variation observed in styles of different floral ages.

Extracts of immature pollen or pollen grains from Ace and Croft were examined for possible esterase isozyme variations (Fig. 3). A single esterase isozyme, comparable to the intermediate group of isozymes

from styles, was present in immature pollen from Ace anthers 15—32 mm long; while mature pollen had four isozymes, one in each migration group and one additional fast one. Croft pollen had these same esterases, but immature pollen showed 3 or 4 additional minor bands in the intermediate gel region.

A few esterase determinations were made from styles and pollen tubes after crossing and selfing. All esterases from SPT or CPT were not clearly resolved, but their patterns were similar. An intermediate migrating isozyme and one distinct one in the slower group plus a few minor ones which did not separate into definite bands were seen (Fig. 3). The styles of Ace or Nellie White after selfing revealed no significant changes compared to controls. Eight earlier analyses of styles plus the pollen tubes growing in them, indicated additive patterns of isozymes. Table 2 summarizes the total successful determinations of esterase isozymes in all samples. The data accumulated thus far indicate no apparent correlation of esterases with the incompatibility reaction.

Table 2. *Number of successful esterase determinations from extracts of various types of samples by disc electrophoresis*

Types of Samples	Cultivars			
	Ace	Croft	Georgia	Nellie White
US	17	13	13	9
SS	3			1
SPT	4			1
PG	3			4
CS		1		1
CPT				1
CS & CPT	8			

Peroxidases and 1-glutamate (deamin) glucose-6-phosphate and 6-gluconic phosphate dehydrogenases were tested for a few samples. The peroxidase isozyme system (Per.) appears to be the most complex yet detected. Both styles and pollen have a complex of bands which migrate at a very slow rate, two or three clear bands near the center of the gel and two or three with fast migration rates (Fig. 3). The glutamic dehydrogenase (GDH) system in styles consists of one or two bands, however in addition to these, pollen has a slow complex of 3 to 5 bands. Thus far a single 6-gluconic phosphate dehydrogenase (6GP) and two glucose-6-phosphate dehydrogenases (G6P) have been detected in pollen (Fig. 3). These enzyme systems have not been extensively studied at the various floral ages or in style-pollen combinations. The simple isozymes systems will be the ones of choice for future analyses.

IV. Discussion

Methods for detection of enzymes known to be present in pollen and styles are essential for incompatibility studies, especially those enzymes correlated with specific S alleles. Techniques for this preliminary work have been developed for *Petunia, Oenothera* and *Lilium* by a number of workers. All these studies appear to support the concept of a protein = enzyme system involved in the incompatibility reaction. Several studies of *in vitro* systems have provided biochemical information about pollen and styles. STANLEY and LINSKENS (1965) detected an enzyme which breaks down sucrose in media containing *Petunia* pollen. Respiratory changes in germinating lily pollen were found to have a three-phase pattern by DICKINSON (1967); this is a direct reflection of increased enzyme activities involved in respiratory pathways. He was also able to demonstrate starch synthesis associated with increased respiration as pollen tubes lengthen (DICKINSON 1968). Immunological methods employed by LEWIS, BURRAGE and WALLS (1967) could detect protein diffusion from single intact pollen grains of *Oenothera*. These proteins were precipitated only by antiserum prepared against the same S-genotype. Amylase and invertase were also detected in diffusates, but could not be correlated with the S-protein. Additional enzymes were found to diffuse out of *Oenothera* pollen grains by MÄKINEN and BREWBAKER (1967). These were esterase, leucine amino peptidase, catalases, and acid phosphatases from a single incompatible clone. PANDEY (1967) using *Oenothera* style extracts for zymograms characterized peroxidase isozymes. He believes peroxidase isozymes may be involved in the expression of multiple alleles of the S-gene cistron. He found certain S allelic styles contained particular patterns of isozymes and suggested the S-gene cistron specified these in adequate complexity to account for the large number of S alleles.

Background information, of the type discussed above, about enzymes present in mature styles and pollen is necessary ; however, *semi-in vivo* systems such as pollen tube growth in compatible and incompatible styles could supply more critical data. A cut-style technique was employed by KWACK (1965) to measure pollen tube growth in *Oenothera* after selfing or crossing. Pollen tubes were grown in cut styles that had been pre-soaked in media with calcium, pre-treated with heat (50 °C) or during cold temperature (5°). She observed added calcium partially overcame the incompatibility reaction while heat or cold treatment did so to a lesser extent; she concluded a protein was probably being affected. Another *semi-in vivo* method, detached styles, was used by ASCHER and PELOQUIN (1966b) for measurement of pollen tube growth in *Lilium*. They found high temperature effects on styles permitting incompatible pollen tubes to grow at the same magnitude as compatible ones. The most extensive study has been done by ROGGEN (1967), who has investigated interactions of pollen tubes growing in *Petunia* styles compared to pollen and stylar extracts. Decrease in one enzyme activity was accompanied by increase in seven other enzyme activities as the pollen tubes grew through the style. He was able to attribute these increases to either styles or pollen tubes or to the combination of them. The 1-glutamate dehydrogenase (amin.) (or alpha ketoglutarate dehydrogenase) isozymes were found to exhibit the greatest increase during pollen tube growth. It was significant that S genotype of the style could be correlated with a specific GDH isozyme and a pollen GDH isozyme is unique and has an activator for the style GDH isozyme.

Methods presented here demonstrate the feasibility and advantage of lilies for comparing protein and

enzyme patterns from pollen, styles, styles from flowers following pollination, and pollen tubes removed from these styles. Variations in proteins and esterase isozymes were found in styles from four lily cultivars, Ace, Croft, Georgia and Nellie White to occur during floral aging. These results suggest a parallel of the effect of floral aging on compatible and incompatible pollen tube growth realized by ASCHER and PELOQUIN (1966a). They observed similar pollen tube length of both compatible and incompatible tubes was obtainable in flowers prior to anthesis or about 10 days after anthesis. The length differences between compatible and incompatible tubes were most pronounced when growth occurred in styles from flowers taken less than five days after anthesis. Although results reported here do not characterize a protein or group of proteins correlated with floral age, they do reflect metabolic changes during aging and the importance of this knowledge in incompatibility studies. Esterase isozymes from immature and mature pollen grains varied between anther ages in the two cultivars examined. These results agree with those observed in *Lilium henryi* during pollen development by LINSKENS (1966); he found proteins and six enzyme systems reflected the various metabolic states of microsporocytes and tapetal cells.

In vivo systems where pollen tubes and styles of known S genotypes can be directly analysed for enzyme changes during compatible and incompatible pollinations are required for more critical investigations. Until this is possible a combination of *Petunia*, *Oenothera* and *Lilium* data may yet give clues to the nature of the S-protein or enzyme.

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Zusammenfassung

Blüten von *Lilium longiflorum* bieten mehrere Vorteile bei Untersuchungen über die Natur der Selbstinkompatibilität: Die großen, hohlen Griffel lassen Protein- und Enzymanalysen sowohl von aus den Griffeln isolierten Pollen-Schläuchen nach Selbstung oder Fremdung zu, als auch von Pollen-Körnern und von bestäubten und unbestäubten Griffeln. Zur Extraktion von Proteinen und ihrer anschließenden Analyse mittels Disk-Elektrophorese wurden von uns biochemische Methoden entwickelt, die zur Demonstration von Veränderungen der Protein- und Esterase-Muster in Pollen und Griffeln von vier Klonen (cultivars) angewendet wurden. Dabei konnte kein offensichtlicher Zusammenhang zwischen der Selbstinkompatibilitätsreaktion und den bei pH 8.3 in 7½% Acrylamid-Gel wandernden Proteinen festgestellt werden. Die Analysen erstreckten sich auf lösliche Proteine, Esterasen, Peroxydasen und einige Dehydrogenasen.

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