To the Graduate Council:

I am submitting herewith a dissertation written by Duane A. Graves entitled "Protein Expression During Early Cotton Fiber Development." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

James McD. Stewart, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of The Graduate School

PROTEIN EXPRESSION DURING EARLY COTTON FIBER DEVELOPMENT

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Duane A. Graves

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ABSTRACT

The total protein composition of developing cotton (Gossypium hirsutum L.) ovules and fibers was examined at 6, 3 and 1 day preanthesis, anthesis and 2, 6, 10, 16 and 20 days postanthesis (-6, -3, -1, 0, 2, 6, 10, 16 and 20 dpa) using two-dimensional (2D) gel electrophoresis. Ovule and fiber proteins from three fiber mutants were also examined. Fiber-specific and fiber-enriched proteins were identified by probing western blots of 2D gels with a fiber-specific serum. In addition, in vitro ovule culture was used to test the ability of preanthesis ovules to grow fibers.

Results indicate that cotton ovules express an early group of proteins from -3 through 2 dpa and a late group from 6 or 10 dpa through 20 dpa. The transition from early to late protein expression occurs between 2 and 10 dpa. Also, a constitutively expressed group of proteins is present.

Using the fiber-specific serum, 35 proteins were detected on western blots of total proteins from -6 to 20 dpa, 9 are fiber-specific, occurring only in the fibers and not in the ovules, and 9 are fiber-enriched, occurring at a higher concentration or for a longer time in fibers versus ovules. The others are common to both fibers and ovules. Immunodetected proteins show early and late expression patterns similar to the expression trends seen with total proteins. Expression of early proteins coincides with initiation and elongation, and the expression of late proteins coincides with secondary cell wall synthesis.

Based on early protein expression patterns, the "initiation plateau hypothesis" was proposed. According to the hypothesis, epidermal cells are potentiated to fiber cells as early as -3 dpa; initiation of prefiber cells is triggered by phytohormonally; and initiation can occur any time following potentiation. By manipulating the hormonal environment *in vitro*, preanthesis ovules were shown to acquire the ability to produce fibers between -3 and -2 dpa. Preanthesis ovules (-2 or -1 dpa) maintain the ability to produce fibers in response to phytohormones after culture in hormone-free medium through the *in vivo* scheduled day of anthesis but not beyond. These data indicate the preprogramming of fiber cells between -3 and -2 dpa and a link between the preprogramming event and anthesis.

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CHAPTER I

INTRODUCTION

One of the most exciting areas of biological research is that of gene regulation and the consequences regulation has on the cell. The temporal and tissue specific regulation of gene expression is an event which holds the secrets of cellular development, tissue morphogenesis and cell transformation. Several levels of control exist which can modulate the level of gene expression (7,13).

Expression is frequently controlled at the level of transcription. However, within the area of transcriptional control there are several ways by which gene expression is known to be modulated (7). Initiation of mRNA synthesis can limit the level of expression. Initiation may be modulated by hormonal stimuli, feedback of gene products and initiation site selection for gene transcription. DNA rearrangements may also determine which genes are available for initiation of RNA synthesis. Termination of RNA synthesis has been shown to play a role in the expression of some genes, particularly viral genes (7).

Although transcription is considered to be the major point of control of gene expression, cases of posttranscriptional control have been documented (7,13). Control may occur in the differential processing of mRNA's, which can lead to different forms of the same basic molecule as in immunoglobulin μ heavy chain. The transport

of mRNA's out of the nucleus may limit translation, as may the stability of the mRNA in the cytoplasm. The translational activity of mRNA may restrict the rate at which translation occurs. One final point of dictation is posttranslational control where proteolytic cleavage, glycosylation, turnover rate or allosteric effectors alter the concentration or activity of a protein in the cell.

These same general controls are presumed to function in plants. In addition to the above, plants appear to have some unique control mechanisms. For example, plant and animal hormones are chemically very different. The mode of action of phytohormones is generally obscure and the entire process seems different from that seen with animal hormones. Plants also respond to a number of oligosaccharides. These compounds have been shown to produce hypersensitive responses to pathogen invasion and, more remarkably, to elicit developmental responses such as flower differentiation and plantlet formation from somatic cells (48). Most plants produce a number of secondary metabolites; some are derived from the shikimic acid pathway and are derivatized and polymerized phenolic compounds. Any activity that these compounds have in plants is unknown; however, some are known to have biological activity in plant-microorganism interactions. Acetosyringone and other phenolics stimulate the expression of *vir* genes in *Agrobacterium tumefaciens* (5,41). A similar situation occurs with a phenolic compound from soybeans that stimulates expression of certain *nod* genes in *Rhizobium japonicum* (40). IAA oxidase activity in cotton has been reported to be inhibited by o-diphenols (2).

Clearly, plant development and the control mechanisms involved to maintain an orderly progression of events hold many secrets.

Cotton fiber was used in this study as a model system for examining angiosperm cell development. Cotton fiber has four advantages which make it a valuable research model.

1. A fiber results from the differentiation of a single epidermal cell (2,44) allowing research to be focused on the activity of a single cell type rather than multiple cell types. Confusion due to the synergism of several different cell types working in concert to produce observable responses can be minimized using cotton fiber as a model. Stimuli are undoubtedly transmitted from the ovule to the fiber via phytohormones and the ovule also provides carbon for the growing fiber (2,26,44). Nonetheless, all macromolecular synthesis apparently originates within the fiber cell.

2. Extensive studies on fiber growth, isozyme expression, enzyme levels, nucleolar activity, cell wall composition, gross morphology and ultrastructure indicate four distinct periods in fiber growth; initiation, elongation, secondary cell wall synthesis and maturation (1,2,8,21,22,30,32,35,36,37,38,44,46,49).

Initiation, characterized by the ballooning of certain epidermal cells at anthesis, marks the beginning of fiber growth. These ballooning cells are destined to become fibers. The exact time that potential fiber cells are set apart from other cells is not known although these cells can be discerned from other epidermal cells as early as 16 hours preanthesis based on ultrastructure (35).

Initiation occurs at the chalazal end of the ovule and proceeds "wave-like" toward the micropyle (43). The initiation "wave" occurs more than once with the first set of fibers becoming lint, the long fibers used in textiles, and the last set becoming fuzz, very short fibers. Constraints on initiation are unknown although fuzz initiates at about the same time that epidermal cell division ceases (about 6 dpa) (39,44). Initiation may be linked to epidermal cell division since no additional fibers initiate after cessation of cell division. The distribution of fiber initials on the epidermis suggests that initiation could be controlled by "nearest neighbor inhibition" (Stewart and Gross, unpublished) where initiated cells are suggested to inhibit the initiation of adjacent cells.

The next stage of fiber growth is elongation. Fiber initials rapidly elongate to reach a final length of 2 to 3 cm in about 25 days. The elongating cells are characterized by being highly vacuolated with a central nucleus containing a large nucleolus. Cell elongation is driven by negative water potential generated by a high intravacuolar concentration of potassium malate (10). The maximum rate of elongation occurs at about 10 dpa.

Secondary cell wall synthesis is generally considered to begin at 16 dpa (2,44); however, it actually seems to begin at about 12 dpa but at a low rate (30). Since elongation is occurring at a high rate at 12 dpa, the contribution of cellulose to the total weight increase is negligible. At 16 dpa a sharp rise in the rate of cellulose synthesis occurs (30) and secondary cell wall deposition

becomes more apparent. Another, greater increase in rate occurs around 28 dpa (30). The entire period of secondary wall formation is marked by the deposition of pure cellulose in the cell wall. Elongation continues for a few days after secondary cell wall synthesis begins but at a much lower rate. Until the beginning of this period the cell wall contains a variety of sugars and proteins, but with the onset of secondary cell wall deposition noncellulosic components of the wall become much less prominent (30). Also at this stage of fiber growth, changes in protein composition are seen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of *in vitro* translation products (1). Some protein bands increase in intensity while others decrease. Some of these proteins are possibly involved with secondary wall formation.

The last period of development is maturation which occurs 40 to 50 dpa and is marked by boll opening. The mature fibers dry and collapse to give the appearance of twisted ribbons (2,44). At this point the cotton fiber is ready for harvesting and processing for commercial use.

3. Cotton fiber can be grown on ovules *in vitro* (4). *In vitro* grown fibers are apparently normal (9) although little research has been directed toward the differences between *in vitro* and *in vivo* grown fibers. The ability to grow fibers on ovules *in vitro* is a valuable asset as the culture technique provides an easy method for radiolabeling proteins and nucleic acids in developing fibers. The investigation of phytohormonal involvement in fiber growth is also facilitated by *in vitro* culture.

4. Four mutants are available which show various abnormalities in their fibers (17,24).

a. A fiberless mutant produces completely naked seed in the homozygous state and heterozygotes produce differing amounts of lint fibers but no fuzz fibers (17,23). Genetic analysis of this mutant indicates that the trait is controlled by one gene which segregates in the classical Mendelian fashion (16). The variation observed in heterozygotes has not been addressed and the mechanism of variation is unknown.

b. The mutant Ligon lintless produces very short, thick walled fibers and is caused by an incompletely dominant mutation (24). This mutant also causes the plant to have contorted leaf laminae and twisted stems (24).

c. Immature fiber produces lint and fuzz but the lint is weak (immature) suggesting a defect in the process of secondary cell wall formation. This trait is controlled by a single recessive mutation (24) which apparently affects only the fiber as the plant appears normal in all other aspects.

d. Pilose is characterized by having shortened, coarse fibers and dense pubescence on the plant. This trait is encoded by a single gene whose alleles are apparently incompletely dominant as heterozygotes show an intermediate phenotype (41).

These mutants may prove to be useful in determining the function of various proteins in the developmental process. Although exact enzymatic or structural function can not be unambiguously

assigned to any protein based on the study of these mutants, phenotypic characteristics may be correlated with the presence, absence, increase, decrease or alteration of various proteins or protein populations when proteins from mutant fibers are compared to those of normal fibers. These mutants will likely provide many surprises when the molecular basis of their phenotypes is investigated.

In briefly reviewing four key characteristics of cotton fiber, one realizes that many plants possess one or more of these general characteristics, but very few contain the combination that cotton does. Because of the nature of fiber growth, the research area initiated by this work will likely provide substantial and diverse information regarding many facets of plant development.

The goal of this research has been to investigate the events involved in fiber differentiation at the level of protein expression. The research plan was defined to provide a foundation for subsequent research, and as such, it was structured to examine general parameters and developmental events which are reflected in the protein constituency of ovules and fibers. The specific objectives of this research are as follows:

1. Analysis of protein expression in -6 to 20 dpa ovules or isolated fibers using two-dimensional (2D) gel electrophoresis.

2. Examination of fibers for proteins specifically expressed in fibers and not in ovules. Analysis of the expression regime of fiber-specific proteins.

3. Examination of fiber initiation on preanthesis ovules.

4. Comparison of the protein constituency of developing ovules and fibers from Immature fiber, Pilose and Ligon lintless mutants with that of normal ovules and fibers.

The accomplishment of these objectives provides a base of information which will stimulate further research regarding fiber development at the molecular, cellular and whole plant levels.

CHAPTER II

ANALYSIS OF THE PROTEIN CONSTITUENCY OF DEVELOPING COTTON FIBERS USING TWO-DIMENSIONAL GEL ELECTROPHORESIS

Rationale and Experimental Approach

A logical first step to begin this investigation of the biology of cotton fiber was to examine the total protein constituency of fibers and ovules over time. This approach gave a global and non-specific view of the protein species composition of intact young ovules or fibers. By matching the protein composition of one age, relative to anthesis, with the composition of other ages, trends and general temporal changes in the total protein species composition were detected. The shifts or changes in the total protein population were then associated with known developmental events.

In order to achieve maximum resolution of the total protein composition, 2D gel electrophoresis after O'Farrell (33) was employed. (A detailed protocol for 2D gel electrophoresis is given in the Appendix, Section 5.) A broad linear pH gradient (pH 4.5 to 8.0) was established and used for the first dimensional separation of proteins by isoelectric focusing. The second dimensional separation was conducted by SDS-PAGE (procedure in Appendix, Section 4) using a 10% total acrylamide resolving gel (27). Following electrophoresis, gels were silver stained according to Merril

(31) except that the fixation time in acetic acid-methanol was increased from 20 min to 18 hrs to facilitate removal of phenolic compounds which caused a dark background on the gels. (See Appendix, Section 6 for the staining procedure.)

Cotton ovules were collected from Gossypium hirsutum L. c.v. "Deltapine 61" (DPL-61) (Delta and Pineland Co., Scott, Miss.). Ovules 6, 3 and 1 day preanthesis (-6, -3 and -1 dpa), ovules at anthesis (0 dpa) and at 2, 6, 10, 16 and 20 days postanthesis (2, 6, 10, 16 and 20 dpa) were collected from field grown plants, lyophilized and stored as described in the Appendix, Section 1.

Total protein was extracted from -6 to 6 dpa ovules. Fibers from 10 to 20 dpa ovules were isolated by breaking freeze-dried locules apart and removing the seed (ovule) intact. Insoluble polyvinylpyrrolidone (Polyclar AT) and extraction buffer were added to the tissue and the mixture was ground in a Polytron. The mixture was heated to 100° C, centrifuged to remove debris, the supernatant was removed and proteins were precipitated with -20° C acetone. The precipitated protein was collected and redissolved in an appropriate buffer. (See Appendix, Section 2 for a complete description of the extraction procedure.) The quantity of protein loaded onto the IEF tube gels was determined by the method of Esen using BSA as a standard (12) (Details in Appendix, Section 3). Unless otherwise stated, 250 µg of total protein was loaded onto the first dimensional gels.

Gels were reproducible based on comparison of gels of the same age run on different days with the same and different protein

extracts. Also, when gels of different ages were compared, protein spots common to both were easily matched. When gels were divided into imaginary quadrants, protein spots within a quadrant were usually within 1 mm of the corresponding spots on other gels. The reproducibility of spot position was not as good across the entire gel. For example, if a spot on one gel and the corresponding spot on another, both occurring in the high MW acidic region of the gels, were matched to each other then two corresponding spots on the low MW basic region of the gels occasionally deviated by as much as 5 mm. Because the fidelity of visually matching spot patterns between two gels is highly dependent on the experience of the observer and can become overly subjective, a conservative approach was adopted. Only changes in spot patterns that were judged obvious were used to analyze the overall trends and changes in protein expression.

Black and white photographic reproduction of silver-stained 2D gels decreased clarity and resolution of the gels. Photographic reproduction makes changes in individual spots more difficult to see compared to original gels. Protein spots were identified on and among gels by their positions relative to other spots or constellations of spots and by the color of the spot. Silver staining yielded black, brown, blue, red, gray and negatively stained spots. Colored proteins were useful position markers. However, even on original gels the complexity of some regions was such that those areas could not be accurately compared at all points. For this reason numerical quantitation of spots in expression groups was not performed

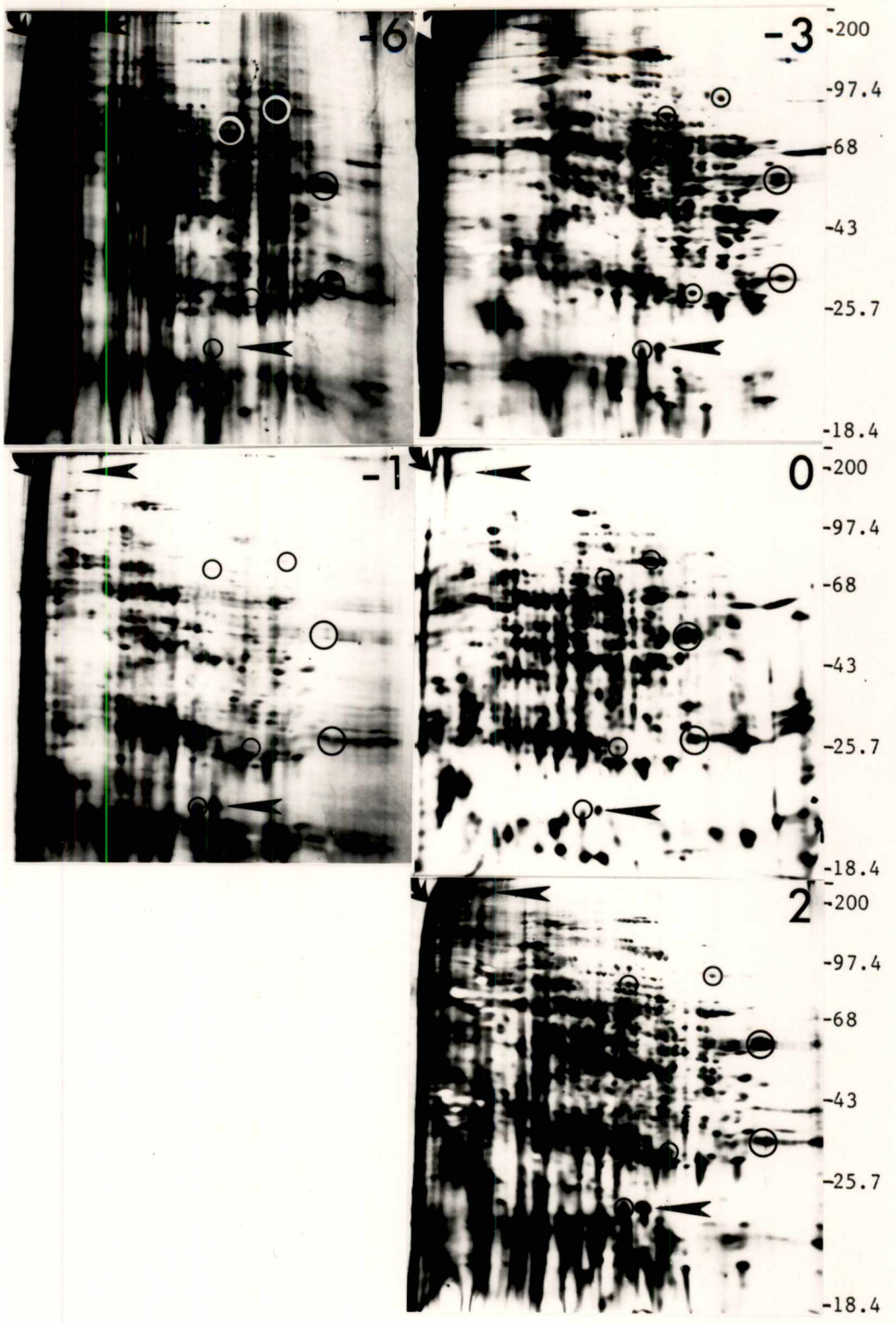
as the data would be incomplete and inaccurate. The value of the 2D gels is in exposing general trends in protein expression rather than in mapping individual protein spots.

Results

Cotton ovules contain significant quantities of polyphenolic compounds. These compounds co-extracted and co-precipitated with proteins and caused substantial dark background staining when gels were silver stained. This problem was partially but not completely alleviated by adding large quantities of Polyclar AT to the initial extraction mixture. Polyclar AT acts by providing reactive sites for ionic bonding with polyphenolics (28).

Acidic alcohol solutions have been commonly used to extract phenolic compounds from a variety of plant tissues (A. Bell, personal communication). To further reduce background staining, gels were soaked for at least 18 hrs in acidic methanol (50% methanol, 12% acetic acid) prior to silver staining. However, extended fixation did not completely eliminate this reaction. Several gels in Fig. II.1 have a dark smear at their acidic edge. These smears appear by staining characteristics and color to be phenolic in nature. Polyphenolics are known to bind to proteins (28), and this reaction may be the result of protein-phenolic complexes. Unbound polyphenolics, on the other hand, seemed to cause a general background throughout the top half of the 2D gels; this reaction was successfully eliminated by extended fixation (data not shown).

Figure II.1 2D gels of extracted intact ovule proteins -6 to 2 dpa. Ovule age is indicated in the top right corner of each gel. The large arrows (top left corner) indicate the acidic end of the gels and point to the polyphenolic smear. Indicated are six representative early proteins (○) and two constitutively expressed proteins (⟵). MW is given as 10^{-3}.


-6
-3
-1
0
2
-200
-97.4
-68
-43
-25.7
-18.4

The exact nature of the acidic smear seen on Fig. II.1 merits further investigation because of the temporal changes in its presence. The smear gradually diminished from -6 to 0 dpa, resurged at 2 dpa and disappeared by 6 dpa (Fig. II.1, compare gels -3 and 2 with -1 and 0, and Fig. II.2, compare 2 and 6 dpa gels). The decline of the smear coincides with fiber initiation, early elongation and fertilization.

Global examination of all gels indicated substantial changes in the species composition of the protein population over time. Examination of individual protein spots and total protein patterns indicated three major protein expression groups. 1) Constitutive proteins were present from -6 or -3 to 20 dpa. 2) Early proteins were present from -6 or -3 through 2 dpa and disappeared by 6 or 10 dpa. 3) Late proteins were present from 6 or 10 dpa through 20 dpa. Some proteins in all groups showed temporal modulation in staining intensity. Based upon these observations, 2D gels representing ovules at -6 to 6 dpa and fibers at 10 to 20 dpa were divided into three groups comprising the early proteins (Fig. II.1), the transition period between early and late (Fig. II.2) and the late proteins (Fig. II.3).

Comparison of gel spot patterns and species composition of ovular proteins at -6 through 2 dpa suggested that a base population of proteins was established and maintained. Representative members of this population are circled in Fig. II.1. Ovules at -6 dpa contained an incomplete base population, but some homology in spot

Fig. II.2. 2D gels of whole ovule or isolated fiber protein showing the transition from early to late protein expression. Ovule or fiber age is indicated in the top right corner of each gel. Gels 2 and 6 dpa contain proteins from intact ovules, and the 10 dpa gel contains proteins from isolated fibers. The large arrow (top left corner) marks the acidic edge of the gels. Six representative early (○), six late (□), and two constitutively expressed proteins (◄) are marked. MW is given as 10^{-3}.

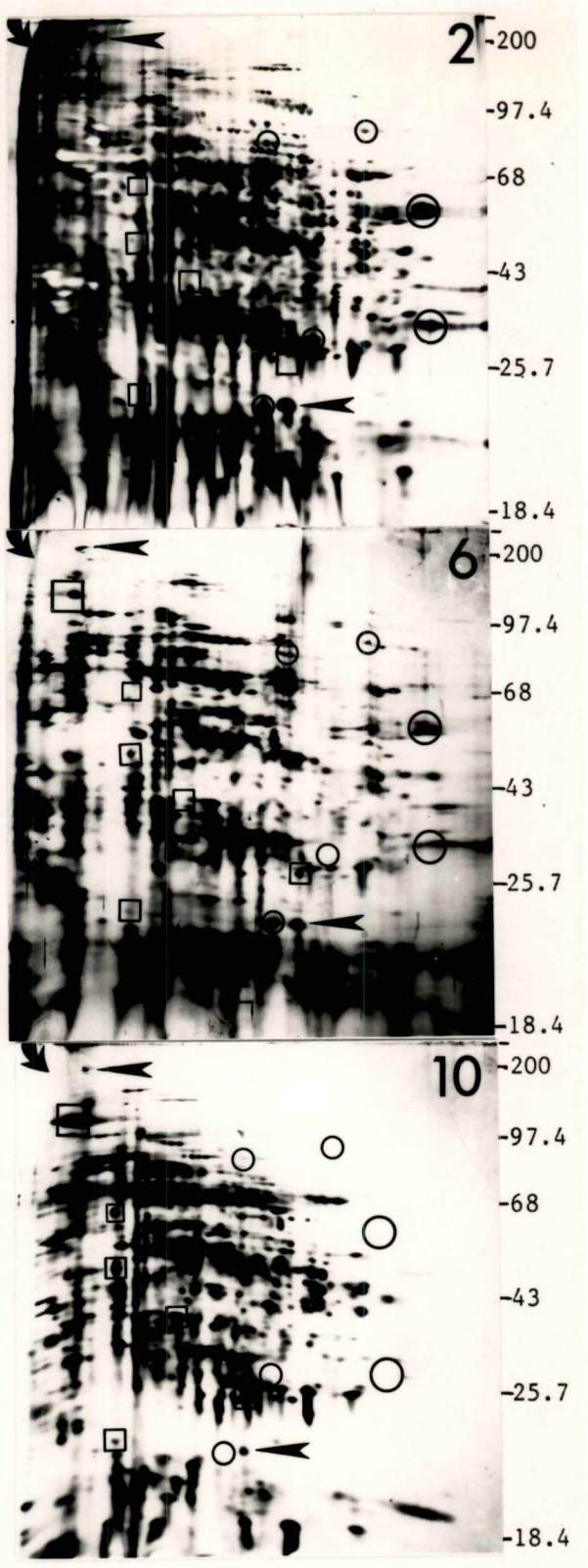
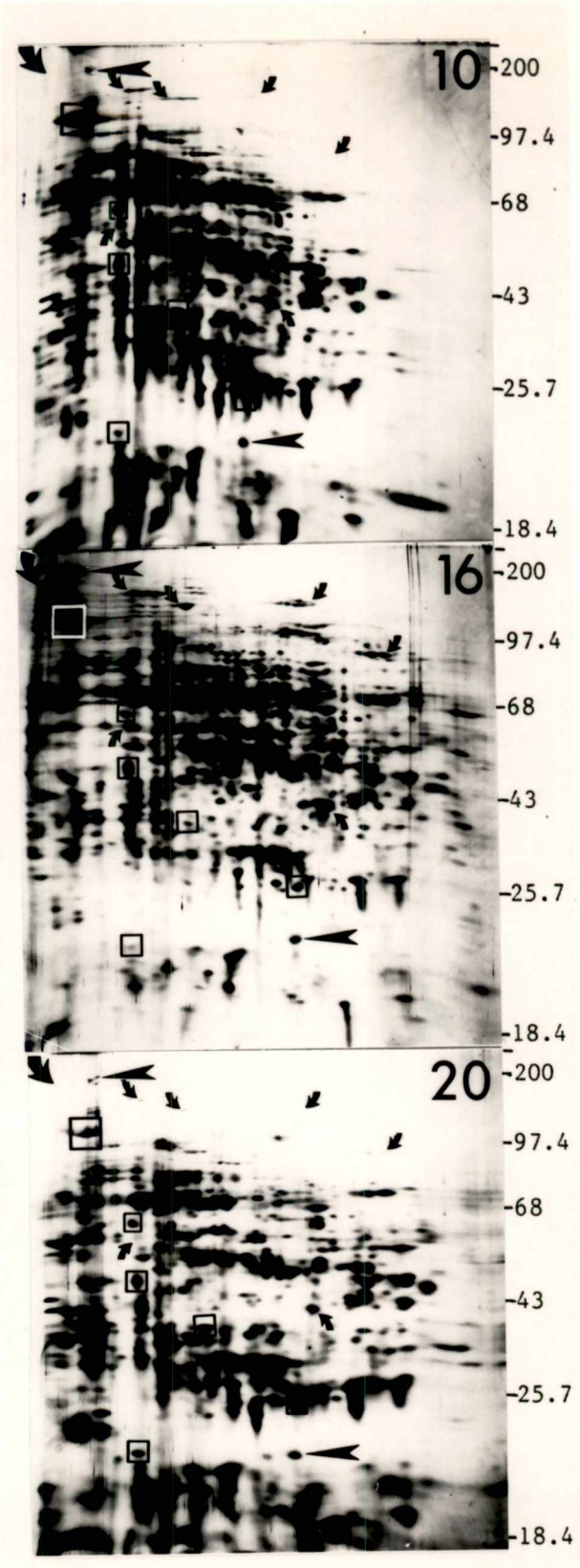

2
-200
-97.4
-68
-43
-25.7
-18.4
6
-200
-97.4
-68
-43
-25.7
-18.4
10
-200
-97.4
-68
-43
-25.7
-18.4

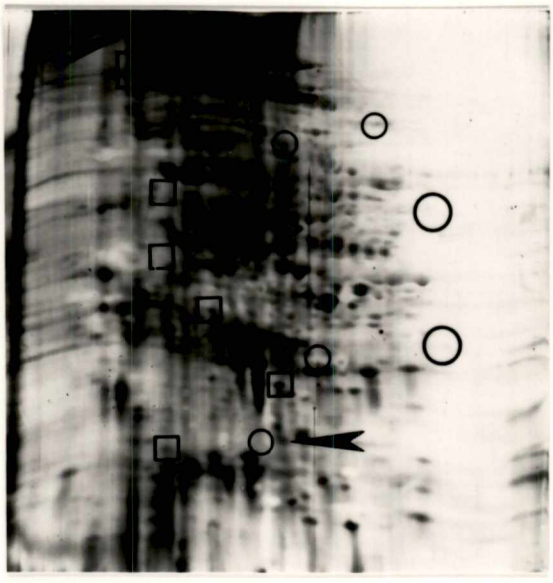
Fig. II.3. 2D gels of total protein from isolated fibers 10 to 20 dpa. Fiber age is indicated in the top right corner of each gel. Large arrows (top left corner) indicate the acidic edge of the gels. Marked are six representative late proteins (□), two constitutive proteins (◄) and six proteins which show increased spot intensity at 16 dpa (↘). MW is given as 10^{-3}.


10
200
97.4
68
43
25.7
18.4
16
200
97.4
68
43
25.7
18.4
20
200
97.4
68
43
25.7
18.4

pattern existed. By -3 dpa the base population had been established. This population was maintained through 2 dpa. When -3 dpa was compared to 2 dpa, substantial similarity was observed; however, -1 and 0 dpa gels were less similar to either -3 or 2 dpa gels. There appeared to be short term changes in protein synthesis between -3 and 2 dpa as evidenced by the -1 and 0 dpa gels. The general expression reached a peak at -3, declined, and peaked again at 2 dpa. This observation gave the impression of specific patterns of protein expression similar to the appearance and disappearance of the polyphenolic smear described earlier. However, the two occurrences did not seem to be tightly synchronized.

Fig. II.2 shows 2D gels of proteins from 2 and 6 dpa ovules and 10 dpa fibers. Comparison of 2 and 10 dpa gels showed significant dissimilarity in protein spot pattern. Some of the changes could be credited to the fact that the 10 dpa gel contained only proteins from fibers whereas the 2 dpa gel contained both fiber and ovule proteins. This possibility was addressed by examining the 2D protein pattern of 10 dpa depilated ovules. Protein patterns on 10 dpa ovules and 10 dpa fibers had many common proteins; however, several proteins were not shared by the two tissues. Comparison of 2 and 6 dpa proteins with both 10 dpa fiber (Fig. II.2) and depilated ovule proteins (Fig. II.4) indicated that some proteins disappeared while others appeared. Examination of both 10 dpa ovule and fiber proteins showed that the transition from early to late protein expression was not an artifact due to the enrichment of fiber/integument proteins or

Fig. II.4. 2D gel of depilated 10 dpa ovule proteins. The positions of six representative early (○), six late (□) and two constitutive (◄) proteins from Figs. II.1, 2 and 3 are shown.



the removal of a major subset of the total protein population as several proteins present at 2 dpa were absent on both 10 dpa ovule and fiber gels. Therefore, the general theme seen between 2 and 10 dpa indicated that a transition from the early protein population (Fig. II.1, page 13) to the late population (Fig. II.3, page 18) occurred. Representative early and late proteins are indicated in Fig. II.2, page 16.

Fig. II.3 shows 10, 16 and 20 dpa fiber proteins. The gels show a slight decrease (20%) in the total number of protein spots from 10 to 20 dpa. More significantly, a modulation in protein spot intensity was seen in these gels. Several protein spots show more intense stain at 16 dpa than at 10 and 20 dpa. These shifts in intensity coincide with changes in the rate of cellulose synthesis (30).

The increase in intensity seen in several protein spots on the 20 dpa gel versus the 16 dpa gel was explained by the fact that equal amounts of total protein were added per gel. A decrease in intensity of some spots must cause a general increase in the others. This kind of intensity shift was seen with several low molecular weight proteins on the 20 dpa gel (Fig. II.3, page 18, compare 16 and 20 dpa gels). The effect was enhanced on low MW proteins due to inefficient stacking and spot diffusion in the 10% T gels.

The preceding description of results focuses on the general occurrences and trends seen during the temporal period of study. Other, more subtle changes were observed, but they were not a major

factor in the analysis and interpretation of the data. Nevertheless, most gels appeared to have some unique protein spots. No significance can be assigned to these proteins at present.

Discussion

Anthesis marks the time when cotton fibers initiate; however, the biochemical mechanisms underlying initiation are not clear. Initiation is apparently triggered hormonally (2,44). IAA, GA, or both together are likely candidates as indicated by their effect on fiber production on in vitro grown ovules. Based upon observation of protein expression patterns, prefiber cells may be determined or set aside from the rest of the epidermal cells a few days prior to anthesis. The similarity of protein composition of ovules at -3 through 2 dpa suggests that the differentiation process may occur as early as -3 dpa and that these prefiber cells are in a static mode awaiting the hormonal stimulus which triggers the next stage of differentiation. Ultrastructural changes can be identified in prefiber cells at 16 hrs preanthesis (35); however, fiber production on 0 dpa ovules requires a hormonal supplement (3). This indicated that even though morphological differentiation is occurring, the process is not self-sustaining and requires an extracellular, likely extraovular, supply of hormones. Prefiber cells are obviously potentiated or determined prior to anthesis. If protein expression and the presence of the early population of proteins is related to prefiber differentiation, then it may be possible to induce prefibers

to initiate prior to the in vivo scheduled time of anthesis by providing the proper hormonal environment.

The study of fiber initiation is complicated by the fact that initiation occurs in cycles. One cycle is simply explained by the transport and diffusion of hormones along the outer integument starting at the chalaza and going toward the micropyle. The subsequent cycle(s) which produces fuzz fibers is more difficult to explain. Stewart and Gross (unpublished) suggested in their "nearest neighbor inhibition" hypothesis that all outer epidermal cells are prefiber cells, but the initial stimulation of one cell causes an inhibition of adjacent cells so that they can not become fibers. When these hypothesized events occur is neither understood nor predicted by that model. The initiation of fuzz fiber is explained in the model by the division of nonfiber epidermal cells. Division allows some of the cells to be relieved from inhibition by their increased distance from existing fibers. These cells are now free to develop as fibers; however, the hormonal environment has changed by this time (5 to 6 dpa) and the newly initiated fibers do not elongate like the first wave fibers.

The results presented here do not directly address the "nearest neighbor inhibition" model, but the results are consistent with events predicted by the model. An apparent increase in the level or diversity of proteins being expressed was seen at -3 dpa and again at 2 dpa (Fig. II.1, page 13, note general differences between -3, -1, 0 and 2 dpa gels). This observation suggested that

prefiber cells are determined twice, once at -3 dpa and again at 2 dpa. The time between the first putative cycle of protein expression and in vivo fiber initiation is 3 days. The time between the second cycle of protein expression and the postulated time of fuzz initiation is 3 or 4 days. A functional relationship may exist between the observed changes in protein expression and fiber initiation as suggested by the timing of protein expression and fiber initiation. Further research is needed to test this relationship.

The apparent undulation of protein expression seen in the early protein pattern at -3 and 2 dpa seems to be reflected also in the polyphenolic content of the protein extracts. Qualitatively, the phenolic smear seen at the acidic edge of the gels in Fig. II.1, page 13, declines from -6 to 0 dpa and then increases at 2 dpa and disappears by 6 dpa (Fig. II.2, page 16). Phenolic compounds have been suggested to have a role in posttranslational regulation of protein function (2). Whether the putative polyphenolic-protein smear seen on these gels is an artifact caused by the release of polyphenolics and subsequent binding to proteins or whether the proteins are already bound to polyphenolics in vivo is not clear. It is clear that the situation does not exist at 0 dpa and at 6 dpa and beyond. Given that initiation occurs at 0 and 6 dpa, the absence of the smear at 0 dpa, its reappearance at 2 dpa and its disappearance by 6 dpa may have some significance in initiation or elongation of lint and fuzz fibers. Fuzz fibers are known to contain more phenolics than lint fibers, yet the putative polyphenolic smear

disappears at the time when fuzz reportedly initiates. This fact seems to conflict with our observations on the disappearance of the smear. However, a change in the type of phenolic compounds present, a change in their ability to bind proteins or a change in the subcellular localization of these compounds could cause the disappearance of the smear even though the cells still contain phenolic compounds. Clearly, further research is needed in this area.

The close parallel between these results and known physical and biochemical events led to the term "initiation plateau" to describe the region of time -3 to 2 dpa. Although correlations can be drawn between fiber growth and protein patterns, the presence of ovular proteins confounds interpretation of the results. Even though the ovule makes virtually no contribution in dry weight gain until 6 dpa (44), the protein synthesizing activity of the ovule has not been determined. The ovule certainly contributes proteins, but because of lack of knowledge concerning the development of the ovule around anthesis, its effect on the total protein population can not be predicted.

The major activity in the development of fiber from 6 to 10 dpa is elongation. It is difficult to associate any protein with this phase of development since the data indicated an apparent transition period in protein expression. Also, as elongation had been occurring since shortly after anthesis, any necessary proteins must have been present relatively early (0 dpa). Hence, there was no time during fiber and ovule development when elongation proteins

were clearly present in the absence of initiation proteins or secondary wall synthesis proteins. For this reason no proteins or protein spot patterns have been associated with elongation.

Secondary wall synthesis coincides with the later stages of elongation. The secondary wall is essentially pure cellulose; therefore, the fiber must have an abundant supply of glucose, an active mechanism for activating the sugar and adding UDP and recycling UDP to UTP, cellulose synthesizing complexes, and the ability to maintain and replenish these systems. Due to the great amount of secondary wall formed, the cellulose synthesizing mechanism must be very active, present in large amounts, or both. In any case, it was plausible to assume that at least some of the proteins present from 10 to 20 dpa were associated with cellulose synthesis, although no individual proteins were assigned to such a role. Pulse labeling fiber proteins during this period may help to determine which proteins are being most actively translated.

Secondary wall synthesis is first detectable at 12 dpa (30). The rate of deposition is slow, and the fibers are elongating so rapidly that deposition has little bearing in the total cellulose content of the fiber or change in dry weight. Our results suggest that most or all of the cellulose-synthesizing proteins are present by 10 dpa. Whether these proteins begin to function immediately or wait for some stimulus at 12 dpa is not known. Meinert and Delmer (30) examined the composition of cotton fibers during development and found a sharp increase in the rate of cellulose deposition at

16 dpa which peaked at 17 dpa, and then declined by 18 dpa. The rate of synthesis was still low at 20 dpa, but from 21 to 25 dpa the fibers underwent a steady increase in the rate of synthesis. At 26 dpa another sharp increase in rate occurred which peaked at 28 dpa and then decreased drastically (30). The increase in staining intensity of several proteins at 16 dpa followed by the less intense staining at 20 dpa parallels the change in rate of cellulose synthesis. Data collected did not include ovules older than 20 dpa; therefore, whether the protein pattern seen at 16 dpa will repeat itself at 26 and 28 dpa is unknown, although this would be predicted, if the changes at 16 dpa are associated with cellulose synthesis.

This chapter presents the protein profile of developing cotton ovules and fibers. The investigation provides a global and non-specific step toward examining the mechanisms by which fiber development is regulated and coordinated with other events occurring in the growing cotton fruit. Fig. II.5 summarizes the developmental scheme that was suggested by the protein patterns seen on 2D gels. These suggestions do not negate the accepted timing of developmental events (Fig. II.5), but rather they provide a picture of the protein constituency that corresponds to and, in some cases, seemed to precede these events. Major areas for future research include the possible role of phenolics in fiber initiation and elongation; the timing of the potentiation of epidermal cells to prefiber cells; control mechanisms or hormonal triggers that coordinate shifts in protein expression; the regulation and significance of protein expression at 16 dpa; and the role of the 16 dpa proteins.

Fig. II.5. The temporal sequence of events which directly relate to fiber development. Time is measured in days relative to anthesis (0) and the line is to scale from -6 to 28 days. The extreme time points (-40 and 45 d) mark the beginning and end of cotton boll development. The heavy portion of the line indicates the period under examination. Information above the line shows the currently accepted events during fiber development. Excepting the rate peaks for cellulose synthesis, the data have been generated primarily by morphological and ultrastructural studies. The information below the line depicts our interpretation of fiber development based on this study.

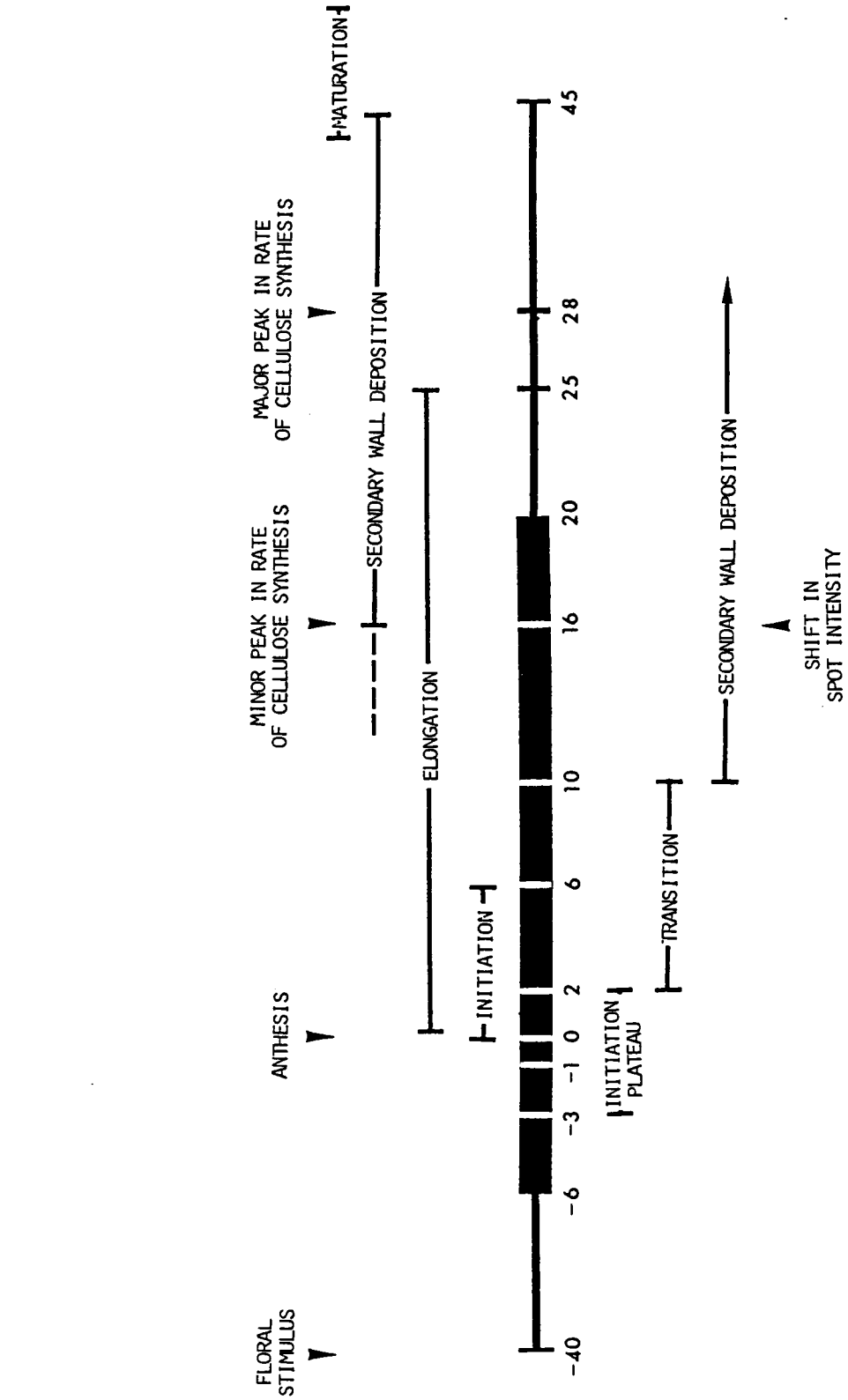

FLORAL STIMULUS
ANTHESIS
MINOR PEAK IN RATE OF CELLULOSE SYNTHESIS
MAJOR PEAK IN RATE OF CELLULOSE SYNTHESIS
MATURATION
SECONDARY WALL DEPOSITION
ELONGATION
INITIATION
INITIATION PLATEAU
TRANSITION
SECONDARY WALL DEPOSITION
SHIFT IN SPOT INTENSITY
-40
-6
-3
-1
0
2
6
10
16
20
25
28
45

Fig. II.4

CHAPTER III

DISCOVERY OF FIBER-SPECIFIC PROTEINS AND THEIR TEMPORAL EXPRESSION IN DEVELOPING COTTON FIBERS

Rationale and Experimental Approach

The complexity of the protein spot patterns seen on 2D gels of total ovule and fiber proteins would not allow the definitive detection of proteins uniquely associated with fiber cells. To examine the possibility that proteins exist which are specifically expressed in the fiber or substantially enriched in the fiber, an antiserum specific for such fiber proteins was developed. The "fiber-specific" serum was also used to track the developmentally regulated expression of fiber-specific and fiber-enriched proteins. Mapping expression patterns of a selected population of proteins revealed important information about fiber development that was obscured by the complexity of the total protein population. Identification of fiber-specific proteins also provided targets for future studies.

To initiate this investigation, an antiserum against total DPL-61 ovule and fiber proteins (0 to 20 dpa) was raised in rabbits. (For experimental protocol, see Appendix, Section 7.) A fiberless mutant (17,23), in a G. hirsutum cultivar unrelated to DPL-61, produces no fibers (Fig. III.1). The assumption was made that no fiber-specific proteins would be expressed in mutant ovules and that a

Fig. III.1. Mature normal and fiberless mutant ovules.

full complement of ovule proteins would be expressed as the mutant produces normal, viable seeds. This assumption was the basis for the purification scheme used to generate the fiber-specific serum. The IgG fraction of the crude serum was affinity purified over a Protein A-agarose column (details in Appendix, Section 8). The IgG was further fractionated into fiber-specific and nonfiber-specific components by affinity chromatography. Total protein from 0 to 20 dpa fiberless mutant ovules plus protein from manually depilated DPL-61 ovules, 10 to 20 dpa, was bound to activated Affigel 10 and 15 (Bio-Rad Laboratories) (see Appendix, Section 9, for protocol). The affinity matrix containing ovule proteins was used via column chromatography to bind and remove from solution all antibodies which could recognize a bound antigen. The IgG molecules which did not bind to the column were eluted and after a second passage through the column were used as the fiber-specific serum.

Fiber-specific proteins were detected by an indirect enzyme immunoassay (EIA). Two-dimensional gels of total protein from DPL-61 ovules or fibers at various ages as described in Chapter II were electroblotted onto nitrocellulose sheets (Appendix, Section 10). The blots were reacted with fiber-specific serum which recognized and bound to certain proteins. The blots were then reacted with horseradish peroxidase-conjugated goat anti-rabbit IgG (GAR-HRP, Bio-Rad) which recognized the bound rabbit IgG. A chromogenic substrate of HRP was added and blue spots appeared wherever fiber-specific proteins were located on the blot. (The protocol for EIA is given in the Appendix, Section 11.)

Results

The reaction of crude anti-ovule/fiber IgG to total protein from fiberless mutant ovules, DPL-61 ovules and DPL-61 leaves is shown in Fig. III.2A. The total IgG fraction reacted strongly to all protein extracts. Affinity purification greatly reduced reactivity. Fig. III.2B and C shows the reaction of the antiserum after 1 and 2 passes through the affinity column, respectively. Background staining was not completely eliminated by affinity purification of fiber-specific IgG because antigenic phenolic compounds are not bound by activated Affigel. The chemical crosslinking between activated Affigel and proteins is through primary amine groups, since phenolics have no amine groups, they were not linked to the affinity matrix, and antibodies to phenolics were not removed by this purification step. However, the addition of total extracted proteins provided a sufficient concentration of the phenolic compounds to inactivate the antibodies against phenolics and eliminate background staining (Compare Figs. III.2C and D). Results identical to Fig. III.2D were achieved by adding excess protein from the fiberless mutant to the unfractionated IgG or crude serum. This approach was expensive in terms of the quantity of fiberless mutant protein needed to cross absorb aliquots of the crude serum; therefore, affinity fractionated IgG was used exclusively for subsequent EIA of protein blots. Preimmune serum did not react with any cotton proteins (Fig. III.2F).

The affinity fractionation technique was not completely effective. The purified IgG reacted with a few proteins on 2D gels

Fig. III.2. Control blots of SDS-polyacrylamide gels of proteins from fiberless mutant ovules, DPL-61 ovules and fibers and DPL-61 leaves. Lanes 1, 2 and 3 contain total protein from the fiberless mutant ovule, DPL-61 ovule and fiber and DPL-61 leaves, respectively. Ten µg of protein were loaded per lane. Each blot was treated with a different primary antiserum followed by treatment with GAR-HRP and color development.

A. Total IgG.

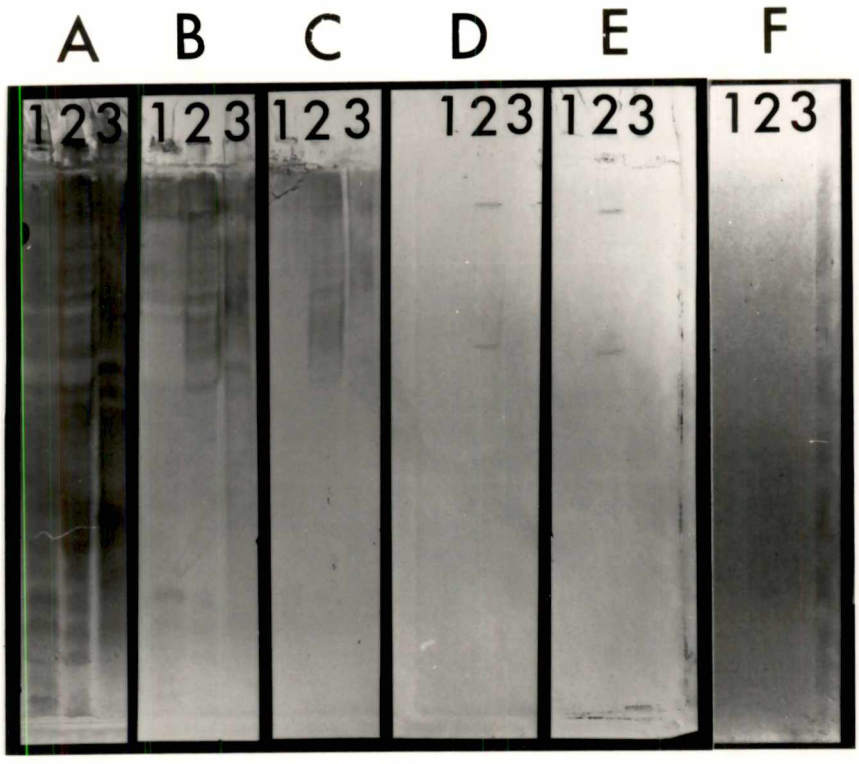
B. Affinity fractionated IgG, first pass through the affinity column.

C. Affinity fractionated IgG, second pass through column.

D. Affinity fractionated IgG, second pass and cross absorbed with total fiberless ovule protein.

E. Whole serum cross absorbed with approximately 2 mg of fiberless ovule protein.

F. Preimmune serum.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of proteins from fiberless ovules (Fig. III.3), depilated DPL-61 ovules (10 to 20 dpa) and leaves (Fig. III.4). Comparison of silver-stained 2D gels revealed that many major proteins were not detected on the blots (see Chapter II), indicating that the IgG's against these proteins were completely removed, whereas the IgG's against some of the less abundant proteins were not. This result was observed in both batch absorbed and affinity purified IgG. No satisfactory explanation was immediately apparent for the incomplete absorption. Although some cross reactivity with fiberless mutant proteins occurred, the fiber-specific serum was useful when controls were included (immunoblots of protein from the fiberless mutant and depilated DPL-61 ovules and DPL-61 leaves).

Fig. III.5 shows a series of immunoblots of proteins from intact DPL-61 ovules (-6 to 6 dpa) and fibers (10 to 20 dpa). Because photographic reproduction of some of the fainter spots was unsatisfactory, a sketch of each blot was included to indicate all detected proteins more clearly. Each unique spot seen among the blots in Fig. III.5 was given an arbitrary numerical designation. Matching proteins on control blots (Figs. III.3 and 4) were also numbered so all blots could be cross-referenced.

Comparison of blots (Figs. III.3, 4 and 5) revealed three groups of proteins. Common proteins were found on blots of DPL-61 ovules and fibers, depilated DPL-61 ovules, fiberless mutant ovules and, in some cases, DPL-61 leaves. Some proteins were apparently enriched in the fibers. Fiber-enriched proteins were defined as

Fig. III.3. Immunoblot of a 2D gel of proteins from the ovules of the fiberless mutant. The gel contains pooled total protein from 0 through 20 dpa fiberless ovules. The blot was reacted with the fiber-specific serum. Protein spots that matched spots on other blots are cross-referenced by number.

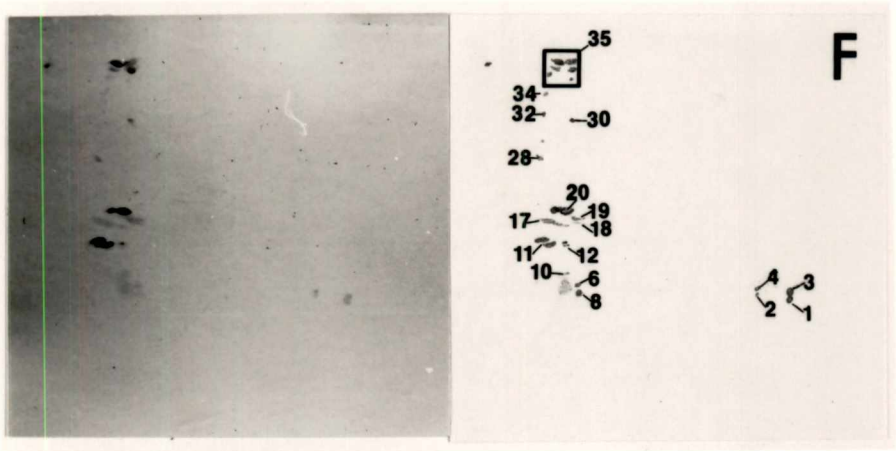
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Fig. III.4. Immunoblots of depilated DPL-61 ovule proteins and DPL-61 leaf proteins. Each blot is accompanied by a sketch clearly showing each detected protein. Large numbers in the right top corner of each sketch indicates the age of the ovules or tissue source (L-leaf). Numbered protein spots are cross-referenced with all other blots.

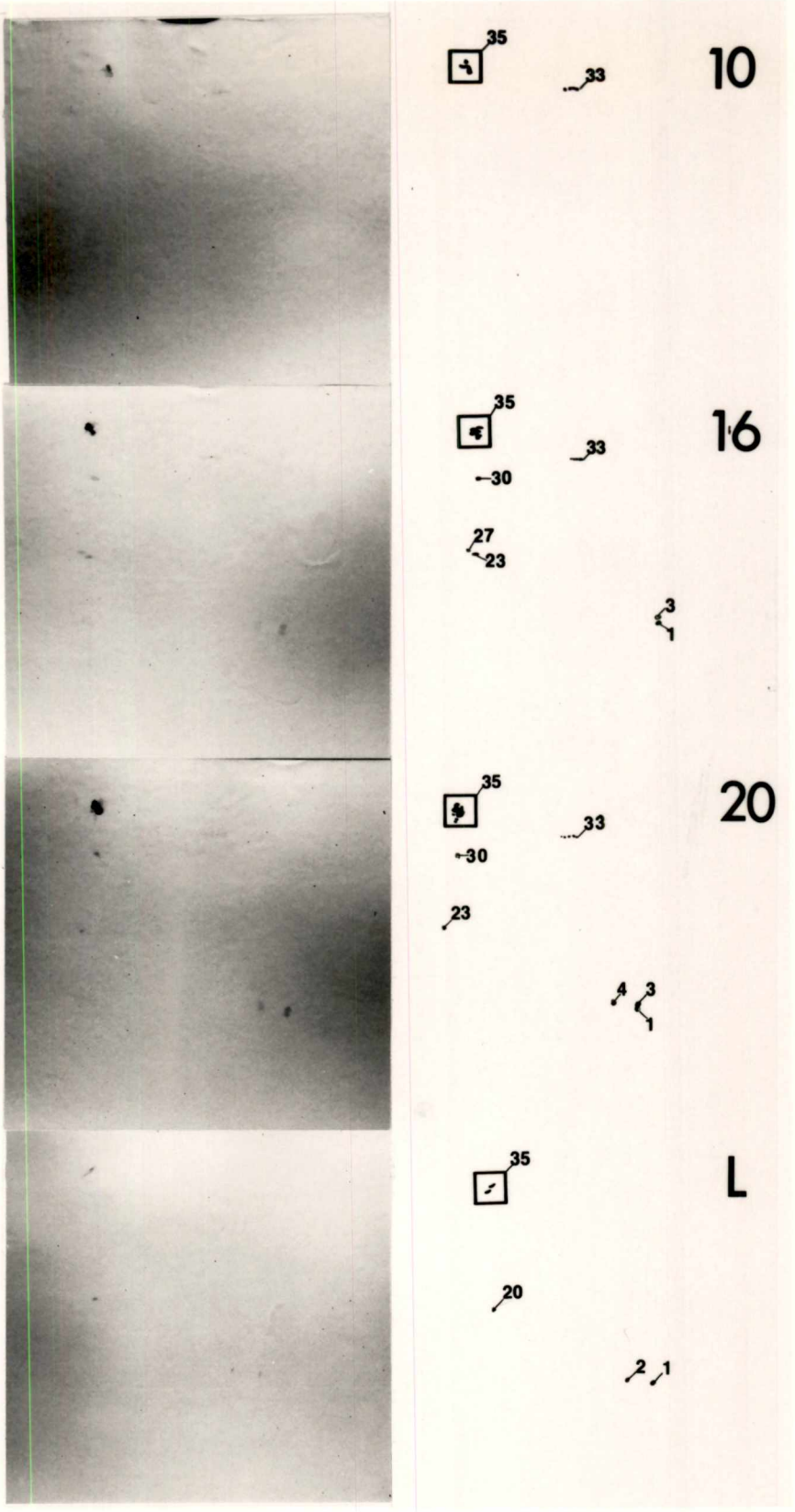
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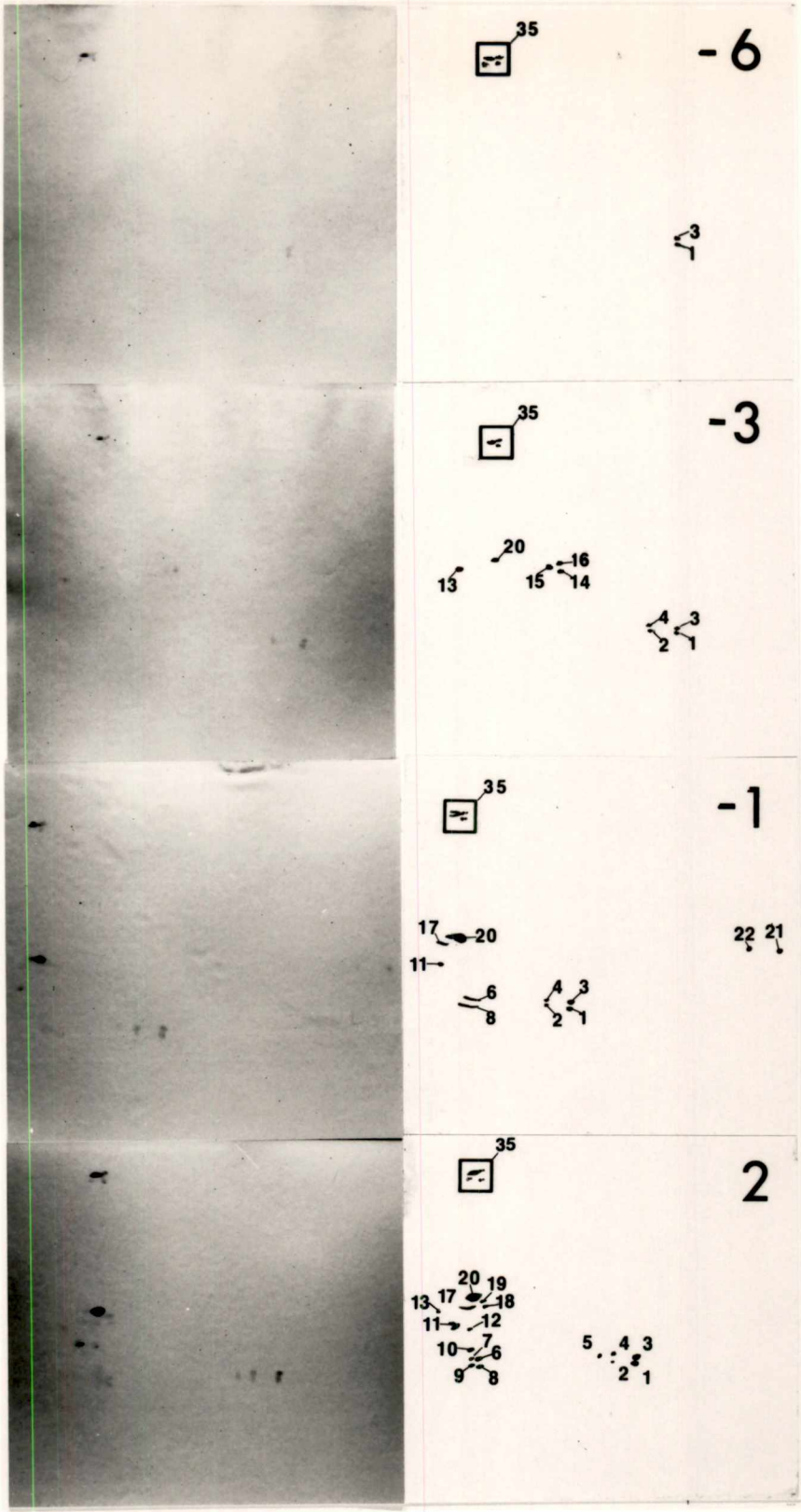
Fig. III.5. Immunoblots of DPL-61 intact ovules (-6 through 6 dpa) and isolated fibers (10 through 20 dpa). The age of each tissue is given in the right top corner of each sketch. Numbered protein spots are cross-referenced with proteins on all blots.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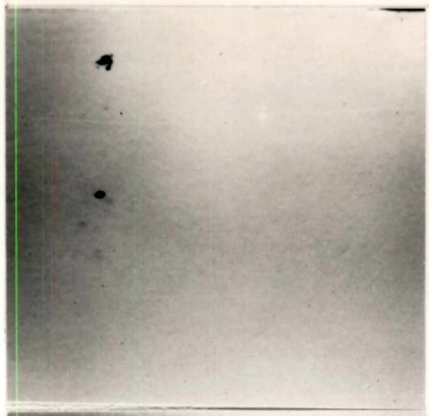

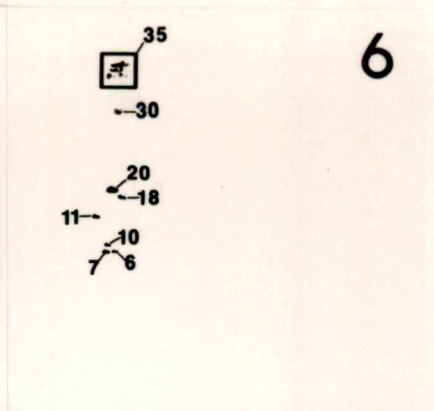
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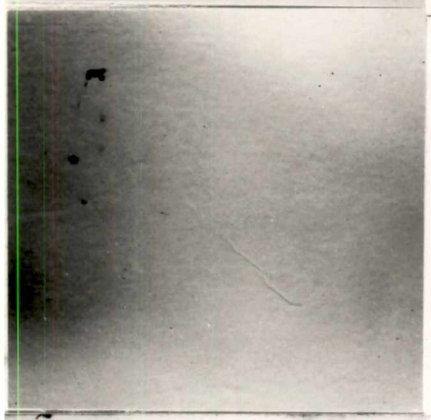

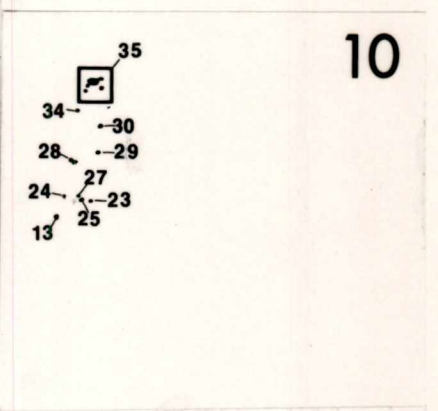
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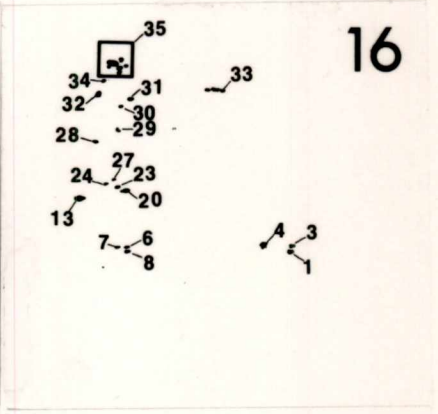
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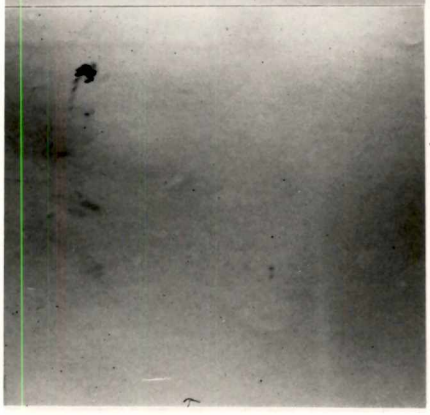

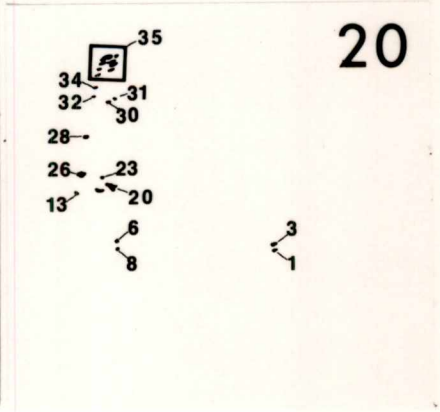
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those proteins that stained with greater intensity on blots of intact DPL-61 ovules and fibers compared to other blots or those proteins that were detected for a longer period of time in intact DPL-61 ovules and fibers than in depilated DPL-61 ovules. Fiber-specific proteins were those seen only on blots of intact DPL-61 ovules or fibers (Fig. III.5). Fiber-specific proteins were not detected on blots shown in Figs. III.3 and 4. Numerical designation, tissue specificity, molecular weight and the expression regime of each detected protein are given in Tables III.1, 2 and 3.

When the expression pattern of each protein was examined, three general classes were apparent. Table III.1 lists early proteins that were only expressed before 6 dpa; Table III.2 contains late proteins that were expressed at 6 dpa or later; and Table III.3 shows proteins that were expressed both before and after 6 dpa.

Of the 14 early proteins (Table III.1) none were expressed in detectable amounts prior to -3 dpa. At -3 dpa four early proteins were detected, and three were apparently fiber-specific. By -1 dpa six early proteins were detected, but only two were fiber-specific. Nine of the 14 early proteins were being expressed at 2 dpa. One was fiber-specific, one was fiber-enriched, one had undetermined tissue identity and the other six were common. By 6 dpa only three common early proteins were detectable.

Twelve late proteins were detected (Table III.2). Only two, #30 and #33, were expressed prior to 10 dpa. All but two late proteins were being expressed at a detectable level by 10 dpa. These

Table III.1. Catalog of immunodetected early proteins.

Number[a]	Type[b]	MW[c]	Occurrence[d]							
			-6	-3	-1	2	6	10	16	20
2	C	46	-	+	+	+	-	-	-	-
5	FS	48	-	-	-	+	-	-	-	-
9	FE	46	-	-	±	+	-	-	-	-
10	C	49	-	-	-	+	+	-	-	-
11	C	56	-	-	+	+	+	-	-	-
12	C	56	-	-	-	+	-	-	-	-
14	FS	62	-	+	-	-	-	-	-	-
15	FS	63	-	+	-	-	-	-	-	-
16	FS	64	-	+	-	-	-	-	-	-
17	*	62	-	-	+	+	-	-	-	-
18	C	63	-	-	-	+	+	-	-	-
19	C	64	-	-	-	+	-	-	-	-
21	FS	60	-	-	+	-	-	-	-	-
22	FS	60	-	-	+	-	-	-	-	-

[a]Number refers to the numerical designation of each unique protein spot on the blots. Numbers are cross-referenced to Fig. III.3 (page 40), Fig. III.4 (page 42) and Fig. III.5 (page 45).

[b]Type indicates tissue location of each protein. C = common, a protein common to DPL ovules, fibers and the fiberless mutant. FE = fiber-enriched, a protein common to DPL ovules, fibers and/or the fiberless mutant and expressed in the fiber for a longer time or at greater concentration. FS = fiber-specific, a protein which occurs only in the fiber. * = proteins whose type cannot be determined by comparison of blots.

[c]MW = apparent molecular weight x 10^{-3} (see Appendix, Section 4).

[d]Occurrence indicates the time points each protein was detected.

Table III.2. Catalog of immunodetected late proteins.[a]

Number	Type	MW	Occurrence							
			-6	-3	-1	2	6	10	16	20
23	FE	70	-	-	-	-	-	+	+	+
24	*	70	-	-	-	-	-	+	+	-
25	*	70	-	-	-	-	-	+	-	-
26	FS	71	-	-	-	-	-	+	-	+
27	FE	72	-	-	-	-	-	+	+	-
28	FE	90	-	-	-	-	-	+	+	+
29	FS	95	-	-	-	-	-	+	+	-
30	*	117	-	-	-	-	+	+	+	+
31	FS	127	-	-	-	-	-	-	+	+
32	FS	128	-	-	-	-	-	-	-	+
33	C	145	-	-	-	-	+	-	+	-
34	*	145	-	-	-	-	-	+	+	+

[a]Table headings are the same as in Table III.1, page 48.

Table III.3. Catalog of immunodetected proteins that are present early and late.[a]

Number	Type	MW	Occurrence							
			-6	-3	-1	2	6	10	16	20
1	C	46	+	+	+	+	-	-	+	+
3	C	48	+	+	+	+	-	-	+	+
4	C	48	-	+	+	+	-	-	+	-
6	FE	48	-	-	±	+	+	-	+	+
7	FE	46	-	-	±	+	-	-	+	+
8	FE	46	-	-	±	+	-	-	+	+
13	FE	61	-	+	+	+	-	+	+	+
20	FE	65	-	+	+	+	+	-	+	+
35	Ccl[b]	190-160	+	+	+	+	+	+	+	+

[a]Table headings same as in Table III.1, page 48.

[b]Ccl = common cluster, this high MW cluster of proteins was not sufficiently resolved to allow cataloguing of individual proteins.

two proteins, #31 and #32, were first detected at 16 and 20 dpa, respectively, and both were fiber-specific. The increase in silver stain intensity of several protein spots at 16 dpa (Chapter II, Fig. II.3, page 18) was not reflected by an obvious increase in spot intensity on the corresponding blot, but at 16 dpa more common and late proteins were detected than at any other late point. Nine late protein spots were detected at 16 dpa with only two being fiber-specific. Late protein #33 consisted of a family of at least four proteins that appeared to be coordinately regulated.

Table III.3 lists proteins that were detected both early and late. All of these proteins were present at -1, 2 and 16 dpa. Only three proteins, including an unresolved cluster of proteins (spot #35), were present at -6 dpa. At 6 and 10 dpa the expression of many of these proteins fell below the detection limit and then increased to detectable levels later. Five of the nine proteins in this group were fiber-enriched, and no fiber-specific proteins were observed.

The cluster of high molecular weight proteins (#35) consisted of several proteins that appeared developmentally regulated. This cluster contained peptides with similar pI's and apparent molecular weights of 190k to 160k. The gel system did not clearly resolve this constellation of proteins; therefore, the cluster was treated as one protein. Although the collective cluster was designated a common protein, further analysis will likely prove this generalization incorrect.

Discussion

Enzyme immunoassays are sensitive and specific; however, limitations do exist and they must be considered when evaluating results. Large MW proteins generally have more antigenic determinants than smaller proteins, and some proteins stimulate the immune system better than others. Thus, the intensity of the reaction seen on blots is influenced not only by the protein concentration but also by the ability of the antibody to recognize and bind a particular protein, and by the amount of antibody bound. Comparison of an individual band or spot during a time series can give an estimate of changes in protein concentration, if the protein is transferred efficiently from the gel during blotting. Quantitative analysis of known or purified proteins can be performed with immunoblots, but blots of crude protein preparations containing unknown proteins present numerous uncertainties; hence, quantification was not attempted in this work.

The use of the fiberless mutant in the production of the fiber-specific serum invoked the following assumptions: 1) The mutant produces no fiber-specific proteins, since it has no fibers. 2) The mutant produces ovules which contain a complement of proteins identical to that produced by normal ovules. 3) Although the fiberless mutant is in a different G. hirsutum cultivar, its proteins will be antigenically similar to those in DPL-61; therefore, the fiber-specific antibodies against DPL-61 proteins can be affinity purified using

common or ovular proteins from the mutant to remove from solution antibodies to nonfiber-specific proteins.

These assumptions appear generally valid based on the detection of fiber-specific proteins, and the inability of the fiber-specific serum to bind to many of the major proteins present in 2D gels of total proteins. However, to fully verify these assumptions, an alternative approach must be considered, and the results compared. The approach which would provide the clearest verification would be to use quantitative 2D gel electrophoresis (14) to catalog every protein in the fiber and ovule and examine its tissue specificity when compared with fiberless mutant ovules or depilated ovules.

The manner in which the crude anti-ovule/fiber serum was elicited in rabbits must also be considered. Total protein extracts of ovules, 0 to 8 dpa, and isolated fibers and outer integuments, 10 to 20 dpa, were injected into rabbits. The antiserum was prepared early in the work because of the time required to generate a high titer serum. Also, only 0 dpa and postanthesis ovules were used because the original plan called for the analysis of 0 to 20 dpa ovules and fibers. Subsequent 2D gels indicated that the initially proposed temporal starting point (0 dpa) was not early enough, so the study was extended to -6 dpa. However, the original serum was used throughout the study. This means that only proteins which were present between 0 and 20 dpa were detected. Any fiber-specific proteins which may be unique to the period from -6 to anthesis would not be detected using this serum. That some of the early proteins

appear to be expressed only before anthesis was explained by the fact that the rabbits were exposed to repeated doses of total protein, and that the resultant antiserum contained antibodies to minor constituents of the total protein population. The proteins which appear and disappear before anthesis are expressed at a concentration that exceeds the threshold of detection and then they fall below that threshold.

Severe overloading of the 2D gels would allow some of these minor proteins to be detected at anthesis or later. Alternatively, immunoprecipitation of these proteins would allow selective enrichment of the proteins so that their concentration relative to other proteins would be greatly increased. Regardless of the approach considered, the threshold of detection is always a limiting factor. In spite of potential shortcomings, this method provided sufficient information to stimulate new ideas concerning the developmental scheme of fibers.

The expression patterns of immunodetected proteins generally agreed with those of total protein revealed on silver-stained 2D gels. An early group and a late group of proteins were detected, and 6 dpa represented the time when the transition from early to late protein expression occurred.

Late protein expression seemed to peak at 16 dpa. This observation is consistent with the fact that the fiber has primarily dedicated itself to cellulose synthesis and secondary wall formation (1,30,44). Sixteen dpa marks a sharp increase in the rate of cellulose synthesis (30), thus, the late proteins are probably

associated with secondary wall synthesis. However, the detection of several late proteins in the 10 dpa blot supports the findings in Chapter II which implied that some of the proteins necessary for secondary wall synthesis were being expressed as early as 10 dpa.

An interesting difference was observed between the trends noted on silver-stained gels and the early immunodetected proteins. Examination of the total protein population led to the hypothesis that most of the early proteins were expressed by -3 dpa. Immunoblots revealed that at -3 dpa only four early proteins were expressed; whereas, by 2 dpa nine early proteins were being expressed. Of the four early proteins seen at -3 dpa, three were fiber-specific, and these three were not detected at 2 dpa. The only early protein common to -3 and 2 dpa was a "common" protein. This suggested that substantial qualitative or quantitative differences in gene expression exist between -3 and 2 dpa. The expression patterns seen with immunodetected proteins may extend to other proteins that were not detected by this assay. Also, these patterns may represent only a small component in the schedule of protein expression in developing ovules. Protein expression patterns and the role of protein expression groups and individual proteins is an area for further research.

Two dpa seems to be an important time in fiber development. The complexity of the early protein group increased from -6 to 2 dpa with 2 dpa being the time when the most early proteins were present. The early proteins disappeared by 6 dpa. No critical events associated with fiber development have been shown to occur

at 2 dpa (2,44). These proteins may be associated with elongation or the regulation of elongation, even though the maximum rate of elongation does not occur until 10 dpa, long after these proteins are no longer detectable. If the 2 dpa proteins are associated with fuzz initiation, a pattern similar to that at -3 dpa would be expected, assuming -3 dpa is to lint as 2 dpa is to fuzz. Such a similarity was not apparent.

Based on silver-stained 2D gels, epidermal cells appeared to be potentiated or to acquire the ability to become fibers at -3 dpa. Furthermore, an "initiation plateau" was suggested to exist from -3 to 2 dpa during which time prefiber cells, epidermal cells capable of becoming fibers, were in a static mode awaiting the hormonal stimulus to initiate. Immunoanalysis suggests that the initiation plateau concept may be too simplistic. These data may be interpreted to suggest that an important event, perhaps epidermal cell potentiation, occurs at -3 dpa as fiber-specific proteins are being expressed. However, by 2 dpa the protein population had changed dramatically, indicating that fiber initiation was more dynamic than originally predicted, or that other developmentally regulated events associated with fiber-integument differentiation were occurring. Further investigation is needed to understand the meaning of the protein expression patterns around anthesis.

When blots were compared to the total protein gels, the immunodetected proteins were found to be minor constituents of the total protein population. This observation was supported by the

fact that 250 µg of total protein was required to provide enough protein for immunodetection. That these proteins were minor constituents suggested two hypotheses regarding fiber development. 1) Most or all biochemical functions carried out in fibers are performed by enzymes and structural proteins that are common to other nonfiber cells. 2) The fiber-specific and/or fiber-enriched proteins may act as regulators to direct, by unknown mechanisms, the function or expression of common proteins so that a fiber rather than some other cell is formed. Any functions that fiber-specific and fiber-enriched proteins have in the development of cotton fibers is currently unknown.

CHAPTER IV

EVALUATION OF THE INITIATION PLATEAU HYPOTHESIS USING *IN VITRO* OVULE CULTURE

The Hypothesis and Experimental Approach

Results presented in Chapters II and III describe an interesting period of protein expression from -3 to 2 dpa. These data were integrated with existing information, and a hypothesis was formulated which predicts some of the events concerning the initiation of fibers. The hypothesis is called the initiation plateau hypothesis, and it contains the following tenets:

1. Fiber cells are potentiated, set apart from other epidermal cells, by -3 dpa. Supporting data include the temporal presence of the early population of proteins presented in Chapter II, Figs. II.1 and 2 (pages 13 and 16, respectively), and the appearance of fiber-specific proteins by -3 dpa presented in Chapter III, Fig. III.5 (page 44) and Table III.1 (page 48). These observations suggest that a significant event in the development of fibers occurs between -6 and -3 dpa.

2. The potentiated fiber cells, prefibers, are initiated by a phytohormonal stimulus. This stimulus normally occurs at anthesis, but the prefiber cells are capable of responding to hormonal stimulation at any time following potentiation at -3 dpa to an undetermined time thereafter. *In vivo* and *in vitro* evidence from several laboratories has confirmed the importance of IAA and GA in fiber initiation and subsequent growth (2,44).

3. The initiation plateau probably extends to cell lineages other than fibers, and as such, it affords the floral bud the ability to synchronize diverse developmental schemes. It may also indirectly provide the plant with the capacity to control the time when the bud will bloom. Temporal control is probably limited to a few hours, but that is long enough for substantial changes in environmental conditions.

The initiation plateau hypothesis provides a provocative model of early fiber development. However, it may conflict with other hypotheses regarding fiber initiation and growth. Some potential conflicts are addressed.

1. According to the initiation plateau hypothesis, two possibilities exist to explain the distribution of fibers and nonfiber cells on cotton ovules. First, all epidermal cells are potentiated and are capable of becoming fibers but the first ones to initiate inhibit the initiation of adjacent cells as the Stewart and Gross model of "nearest neighbor inhibition" suggests (unpublished). The adjacent inhibited cells are then committed to function as nonfiber epidermal cells. This inhibition would occur during the initiation phase of fiber development. Alternatively, only a subset of the epidermal cells may be potentiated to prefiber cells. Under this scenario "nearest neighbor inhibition" must be associated with the potentiation event rather than initiation.

2. Stewart (43) showed that fibers initiate in a wave-like fashion. The wave is apparently caused by the transport of

phytohormones through the funiculus and vascular system of the young ovules to the chalaza where the phytohormones then diffuse through the epidermis toward the micropyle (43). If all the prefiber cells are set to initiate before anthesis, as the initiation plateau hypothesis suggests, then the wave would be explained by the movement of phytohormones from the plant through the vascular system of the ovule and finally by diffusion through the epidermis starting at the chalaza. If initiation is preceded by a potentiation event, then it may be possible to provide a uniform hormonal stimulus and thus cause the fiber cells to initiate at the same time.

3. Fibers are reported to initiate until about 6 dpa. The potentiation and initiation of postanthesis fibers (fuzz) is possibly associated with epidermal cell division which stops at 6 dpa. This latter set of fibers may arise from new cells that are released from "nearest neighbor inhibition." These fibers fall outside the predictions of the initiation plateau hypothesis since they probably do not exist when the first potentiation event occurs. Currently, insufficient evidence is available to make any predictions about the potentiation and initiation of fuzz fibers.

The preceding models have not been tested; therefore, their validity is subject to question. Using <u>in vitro</u> ovule culture (see Appendix, Section 12, for technical details), the first two tenets of the initiation plateau hypothesis were challenged. Several culture experiments were performed with each experiment being replicated four times. Each individual culture contained a minimum of 10 ovules

taken from 5 different ovaries. Excised ovules were floated on the surface of 20 ml of liquid medium and cultured at 32° C in 5% CO_2. [CO_2 enrichment was necessary for growth of fibers on preanthesis ovules (L. L. Marden, personal communication).] Fiber growth was quantitated by individually staining 10 ovules from each culture with a modified version of Beasley's fiber staining technique (3) (see Appendix, Section 13, for details). To avoid artifactually skewing the mean staining response of cultured ovules, only ovules which gave a "typical" growth response were stained. "Typical" growth refers to the response made by the majority of the cultured ovules in a treatment. "Typical" may mean fibered, fiberless or callused ovules depending upon the treatment. Most cultures contained a few (<20%) aberrant ovules that either made no noticeable response to culture or callused profusely. These abnormal ovules were not selected for staining. The percentage of ovules which produced fibers in each culture was determined by examining all ovules for the presence of visible fibers. The presence of any visible fiber constituted a positive response.

Results and Discussion

Using CO_2 enriched cultures, several parameters of fiber initiation and growth were investigated. Fig. IV.1 illustrates the experimental designs used to test the initiation plateau hypothesis.

Experiment A (Fig. IV.1) was conducted by culturing 0 dpa ovules for the indicated time, and then staining and destaining the

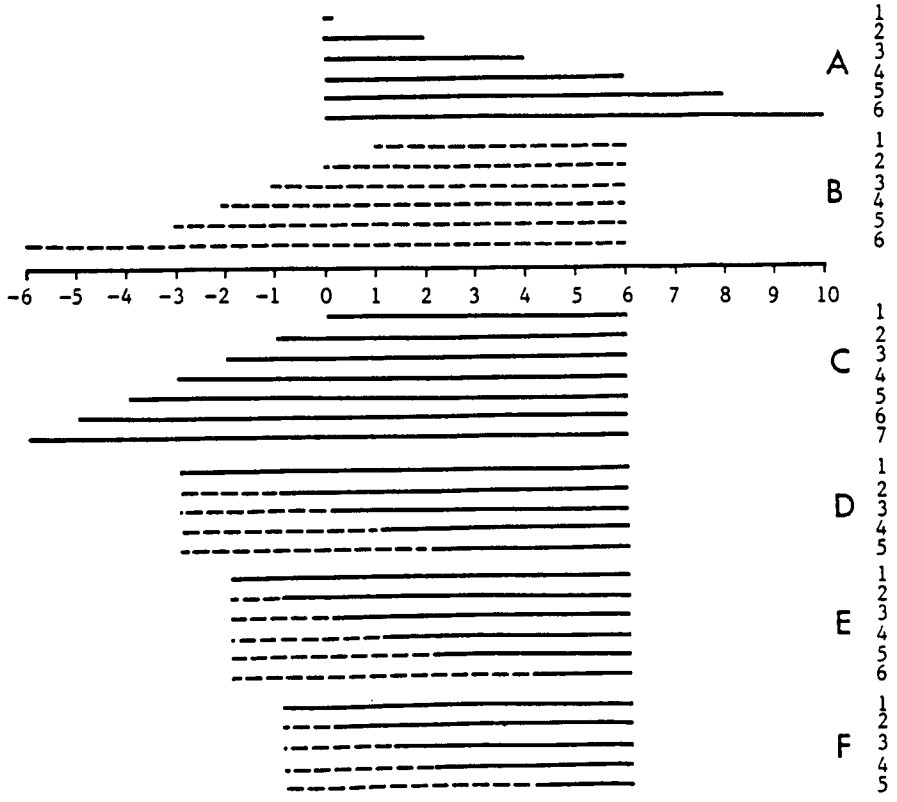


Fig. IV.1. Experimental design to test the initiation plateau hypothesis. Capital letters indicate individual experiments with the accompanying numbers representing treatments within an experiment. The time line between Exp. B and C indicates days relative to anthesis (0). The lines indicate time in culture with the left end showing the age of the ovules when the treatments started and the right end showing when the treatments ended. Solid lines indicate culture in 1.0 µM IAA and 10 µM GA. Broken lines indicate hormone-free culture. The change from broken lines to solid lines in Exp. D, E and F show when IAA and GA were added.

ovules. The A_{640} of the destain solution was plotted against time in culture to give the standard growth curve in Fig. IV.2. Nearly 100% of the ovules responded to culture by producing fibers.

Experiment B (Fig. IV.1) tests the ability of 1 and 0 dpa and preanthesis ovules to produce fibers in the absence of phytohormones in the medium. Fig. IV.3 shows that the staining characteristics of the ovules deviate from the standard growth curve. One dpa ovules stain like 0 dpa ovules grown on hormones for 1.3 days. This response was due to the fact that the ovules initiated fibers and grew for 1 day under *in vivo* conditions before fiber growth was arrested by harvest and culture on hormone-free medium. Anthesis and preanthesis ovules displayed greater staining than expected for ovules that grew no fibers. However, these ovules had enlarged and had often produced some callus. Increased size and callus cells were responsible for the increased staining response. None of the ovules cultured in hormone-free medium produced fibers.

Experiment C (Fig. IV.1) involved the culture of -6 to 0 dpa ovules in medium plus IAA and GA. The staining response and the percentage of ovules which grew fibers indicated that the outer epidermis had developed the capacity for fiber initiation by -2 dpa (Fig. IV.4). The ovules were grown to the equivalent of 6 dpa; therefore, the -2 dpa ovules were in culture for 8 days, the -1 dpa ovules for 7 days and 0 dpa ovules for 6 days. The fiber staining response was consistent with the hypothesis that these ovules began to grow fibers soon after they were placed in culture, i.e., the

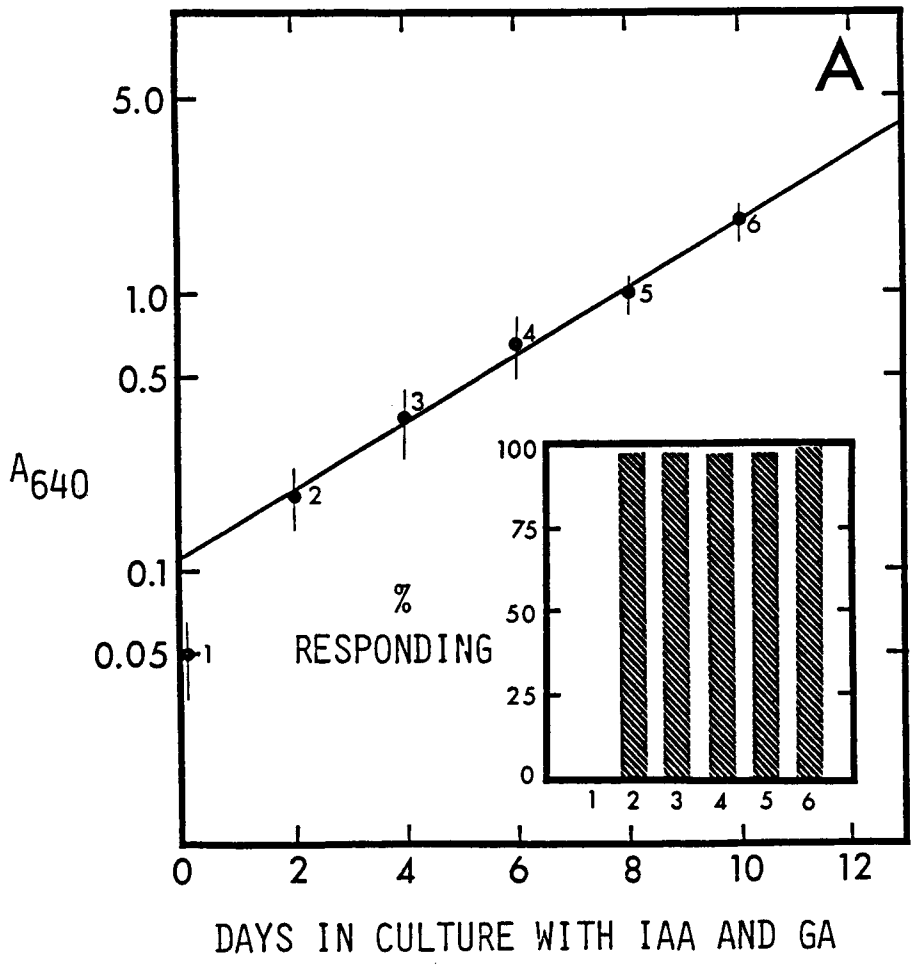


Fig. IV.2. Experiment A, standard growth curve for <u>in vitro</u> cultured fibers/ovules. Numbered points correspond to the numbers in Fig. IV-1A, page 62. The standard curve was constructed from fiber staining data using the least squares method with point 1 excluded. The inset shows the percentage of ovules which produced fibers at each point. The standard curve was superimposed on subsequent figures.

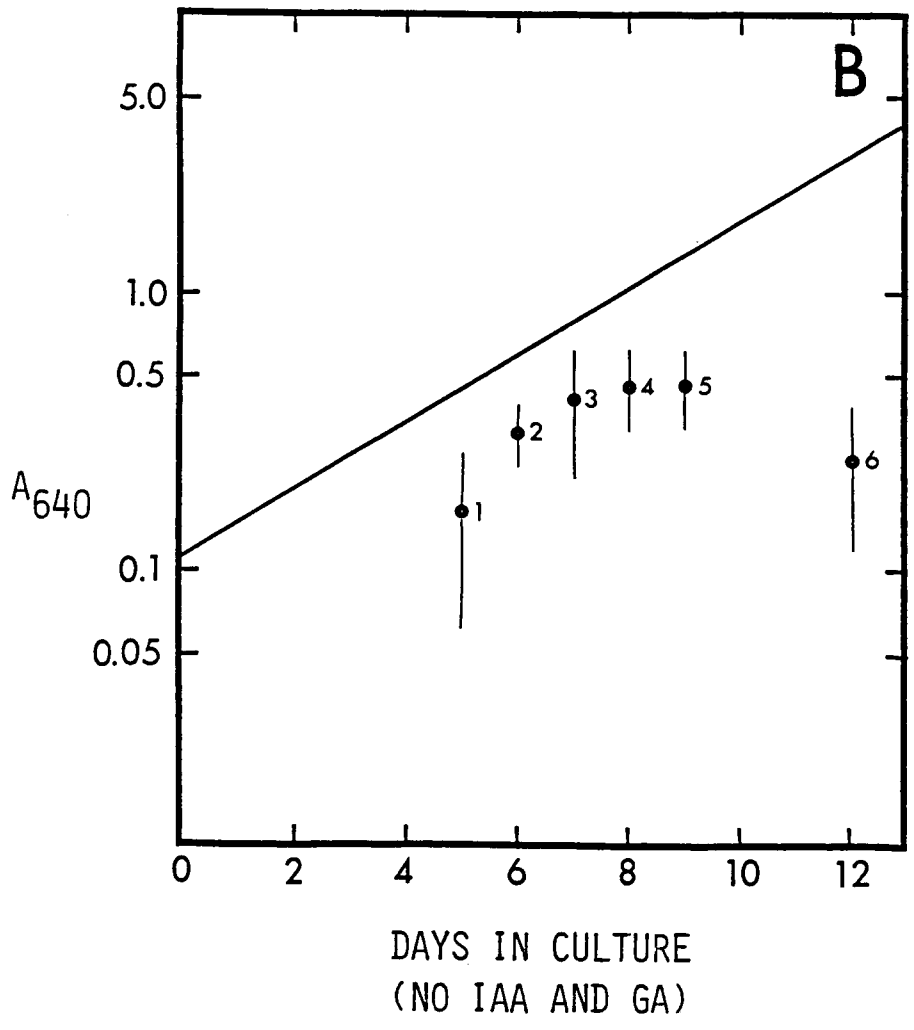


Fig. IV.3. Experiment B, the staining response of ovules after hormone-free culture. Numbered points indicate treatments shown in Fig. IV.1, page 62, Exp. B. The standard curve from Fig. IV.2, page 64, is superimposed.

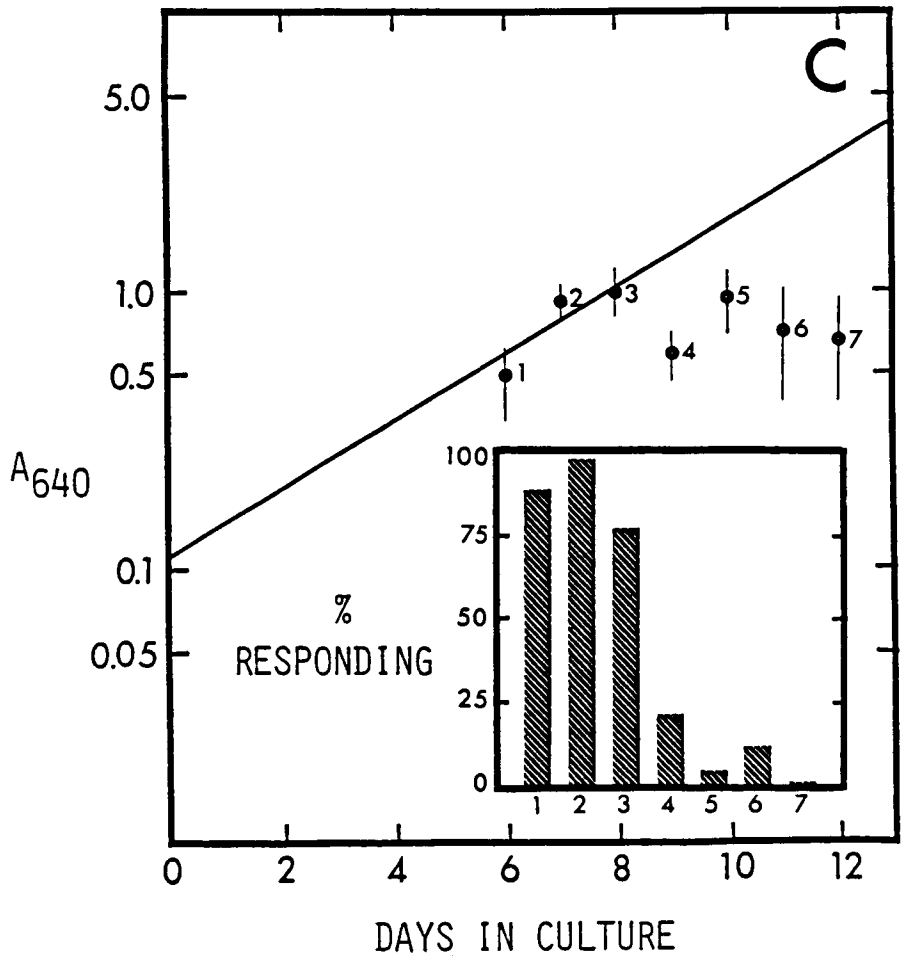


Fig. IV.4. Experiment C, fiber production on preanthesis ovules. Numbered points indicate treatments shown in Fig. IV.1, page 62, Exp. C. The standard curve from Fig. IV.2, page 64, is superimposed. The inset shows the percentage of ovules which produced fibers in each treatment.

-2 dpa ovules stained like 0 dpa ovules grown for 8 dpa, the -1 dpa ovules stained like 7 dpa ovules and the 0 dpa ovules stained like 6 dpa ovules. For each of these ages a high percentage of the ovules produced fibers. Ovules younger than -2 dpa did not stain like ovules cultured for 9 to 12 days and the percentage of ovules which produced fibers declined for -3 dpa and younger ovules. In general, when ovules were cultured in the presence of IAA and GA they either produced fibers or they produced callus. The two distinct types of response overlapped infrequently.

These results point out that the outer epidermis of the cotton ovule is capable of producing fibers by -2 dpa and that the potentiation of epidermal cells is apparently complete at this time. In vivo, prefiber cells do not initiate until anthesis. The stasis appears to result from the lack of a proper hormonal environment since the block is overcome in vitro by the addition of IAA and GA to the culture medium. This result strongly suggests that fiber cells are "preprogrammed"; however, the prefiber cells still require a transcriptional event to initiate enlargement. RNA synthesis inhibitors block fiber initiation on 0 dpa ovules (L. L. Marden, personal communication); therefore, the "preprogramming" which occurs between -3 and -2 dpa is not complete.

"Preprogramming" suggests that the cells are held in a static or inactive state until the hormonal stimulus occurs. Experiments D, E and F test this prediction. In Exp. E, -2 dpa ovules were cultured without phytohormones for the times indicated by the broken

lines in Fig. IV.1, page 62. IAA and GA were added to the cultures as indicated in Fig. IV.1, and the ovules were grown to the equivalent of 6 dpa.

All ovules stained as if they grew the predicted amount of fiber for the time that they were cultured in the presence of IAA and GA (Fig. IV.5). However, the percentage of ovules which grew fibers clearly shows that after 2 days in hormone-free culture, the ability of the ovules to grow fibers was substantially reduced. The aberrant staining response was explained by the observation that ovules held for 3, 4 and 6 days without hormones produced callus upon the addition of IAA and GA to the medium. The staining response of the callus was very similar to the staining response for fibered ovules 2, 4 and 5 dpa.

That the -2 dpa ovules were capable of producing fibers up to and through the in vivo scheduled day of anthesis suggested that fiber initiation was linked to the in vivo scheduled time of anthesis. Alternatively, this response could be due to degeneration of ovules after 2 days in hormone free culture. This issue was resolved with Exp. F and D (Fig. IV.1, page 62).

One day preanthesis (-1 dpa) ovules were cultured for the indicated periods without phytohormones, then IAA and GA were added and incubation was continued to the equivalent of 6 dpa. If the -2 dpa ovules in Exp. E were competent for 2 days degenerating thereafter, then the -1 dpa ovules should also remain competent to produce fibers for 2 days in hormone-free culture. On the other hand, if

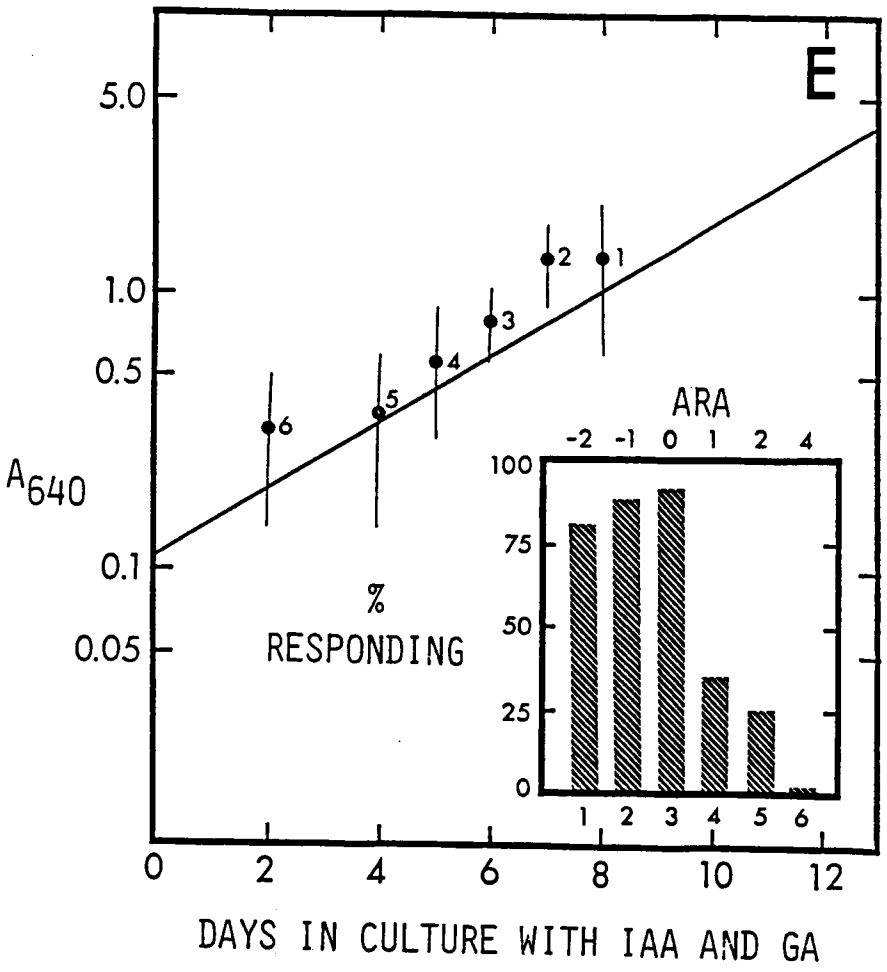


Fig. IV.5. Experiment E, delayed fiber production on -2 dpa ovules. The inset shows the percentage of ovules in each treatment which produced fibers. ARA, the age of ovules relative to anthesis when IAA and GA were added to the cultures. Numbered points indicate treatments shown in Fig. IV.1, page 62, Exp. E. The standard curve from Fig. IV.2, page 64, is superimposed.

the ability to grow fibers was linked to anthesis, then the -1 dpa ovules should be able to produce fibers in response to IAA and GA after 1 day in hormone-free culture but not after 2 days. The data in Fig. IV.6 support the latter case. The percentage of fiber producing ovules declined markedly by 2 days in hormone-free culture, similarly, the staining response of -1 dpa ovules was near normal when hormones were added immediately or after 1 day in hormone-free culture but not after 2 days.

Experiment D was conducted with -3 dpa ovules. Although only 20% of the ovules produced fibers, this age was included to address possible error in trying to accurately define responses that were only one day different. The ability of -3 dpa ovules to grow fibers should degenerate before the scheduled time of anthesis, or, if fiber production is linked to anthesis, the ovules should remain competent to produce fibers for 3 days, 2 days longer than -1 dpa ovules.

The results of Exp. D are shown in Fig. IV.7. Due to the low percentage of fiber producing ovules and the reduced density of fibers typical of -3 dpa ovules, the staining values did not approach the standard curve. Apparently the potentiation process was underway but not complete so that only a fraction of the prefiber cells were competent to initiate. The percentage of responding ovules indicated that after 3 days in hormone-free culture 20% of the ovules were still capable of initiating fibers upon the addition of hormones. Nevertheless, after 3 days in hormone-free culture, the ability of the -3 dpa ovules to product fibers was approximately the same as

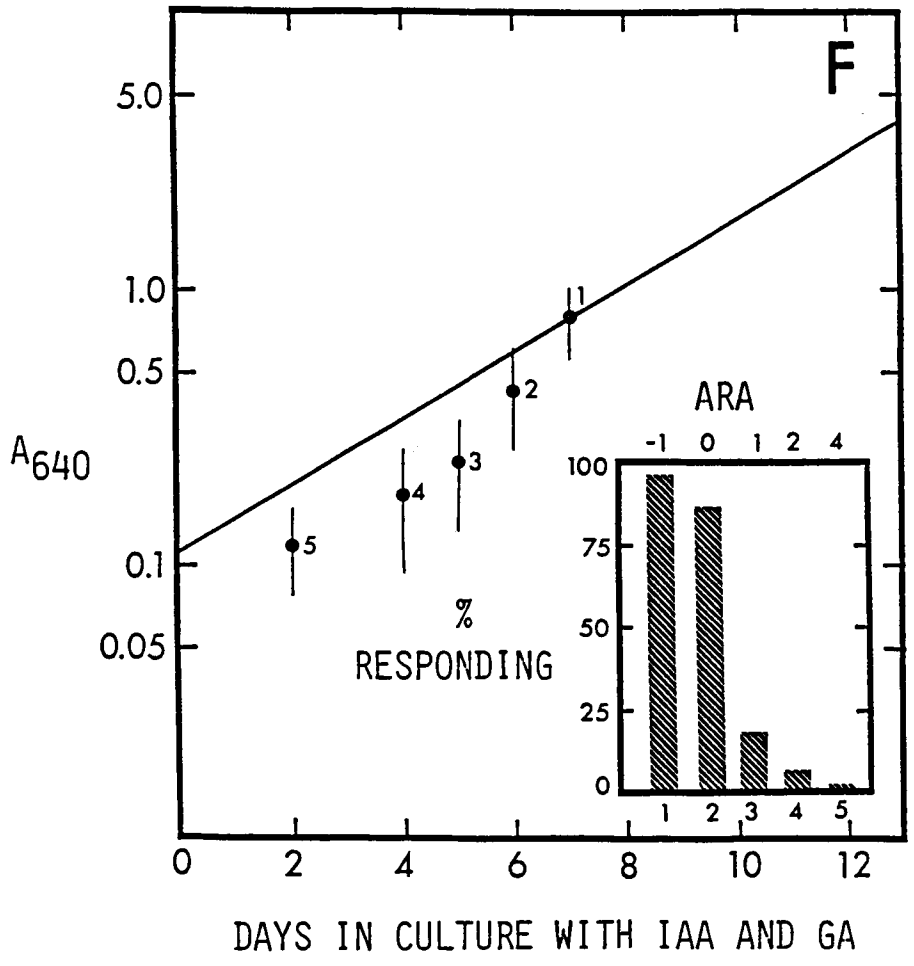


Fig. IV.6. Experiment F, delayed fiber production on -1 dpa ovules. The inset shows the percentage of ovules that produced fibers in each treatment. ARA, the age of ovules relative to anthesis when IAA and GA were added to the cultures. Numbered points indicate treatments shown in Fig. IV.1, page 62, Exp. F. The standard curve from Fig. IV.2, page 64, is superimposed.

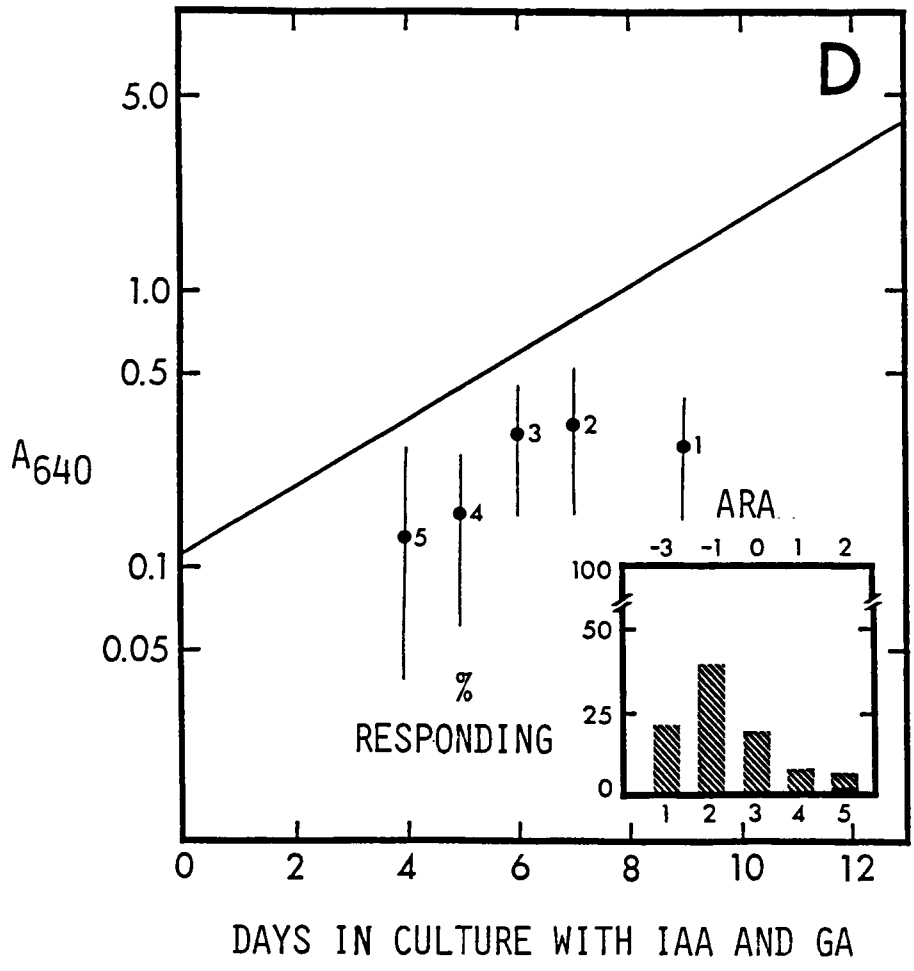


Fig. IV.7. Experiment D, delayed fiber production on -3 dpa ovules. The inset shows the percentage of ovules producing fibers in each treatment. ARA, the age of ovules relative to anthesis when IAA and GA were added to the cultures. Numbered points indicate treatments shown in Fig. IV.1, page 62, Exp. D. The standard curve from Fig. IV.2, page 64, is superimposed.

-3 dpa ovules placed immediately in culture with phytohormones. At least some of the epidermal cells seemed to be potentiating to prefiber cells by -3 dpa, and the process was apparently dependent on the mother plant as culture interrupted the process, i.e., potentiation did not approach maximum once -3 dpa ovules were excised and cultured.

Based on Exp. D, E and F, fiber initiation appears to be linked to the in vivo scheduled time of anthesis. The link is not broken by in vitro culture. The data support the concept that prefiber cells are held in a static state until the enabling hormonal stimulus arrives. In vitro grown ovules appear to keep track of their scheduled time of anthesis. Perhaps potentiated prefiber cells have a given lifespan that begins at the potentiation event and ends 2 to 3 days later. In vitro manipulations do not alter the internal clockwork of prefiber cells.

The results of these experiments provide information which supports the initiation plateau hypothesis. 1) Fibers with near normal growth characteristics and population density may be initiated on ovules as young as 2 days preanthesis. 2) Initiation requires a hormonal stimulus and elevated CO_2. 3) Epidermal cells respond quickly to hormonal stimulation. 4) Protein expression patterns and the ability of preanthesis ovules to grow fibers soon after culture in the presence of IAA and GA suggest that all or some epidermal cells are set apart or potentiated to become fibers between -3 and -2 dpa. 5) Prefiber cells remain competent until anthesis. Fiber

initiation is linked to anthesis as fibers may be initiated on *in vitro* grown ovules prior to and on the *in vivo* scheduled day of anthesis but not afterwards. 6) A major developmental shift from callus formation to fiber production occurs between -3 and -2 dpa.

These results provide information for a developmental scheme in which differentiating epidermal cells are preprogrammed to become a particular terminal cell type. The scope of this preprogramming may extend beyond the fibers to the rest of the young ovule. The preprogramming of certain cell lines may facilitate the commencement of the multitude of events which occur during anthesis. The competency period prior to initiation may afford the plant the ability to delay or expedite initiation and anthesis according to environmental conditions. Such a control mechanism would likely function over only a few hours but that is long enough for substantial changes in temperature, light intensity and moisture to occur.

The molecular events involved in the "preprogramming" scheme and the ramifications for individual cotton bolls and the whole plant are areas for further research.

CHAPTER V

COMPARISON OF THE PROTEIN PROFILE OF NORMAL AND MUTANT FIBERS

Rationale and Experimental Approach

The study of numerous mutants has made possible the understanding of a great variety of biological phenomena. The fiber mutants mentioned in Chapter I lend themselves to the study of fiber development. A survey of the protein constituency of three fiber mutants was conducted with the anticipation that particular proteins, groups of proteins or protein expression patterns could be associated with the mutant phenotypes. Comparison of normal protein profiles with mutant protein profiles may indicate proteins or groups of proteins that are involved with particular functions or developmental events. However, the mutants proved to be more complex than anticipated; they displayed major changes in the developmentally regulated progression of protein expression.

The three mutants used for this part of the study were Pilose (41), Ligon lintless (24) and Immature fiber (24) (Fig. V.1). Pilose, caused by a single incomplete dominant mutation, is characterized by having slightly shorter and thicker fibers than normal (41). Physical measurements of length and dry weight increase indicate that Pilose elongates normally at first but then elongation ceases

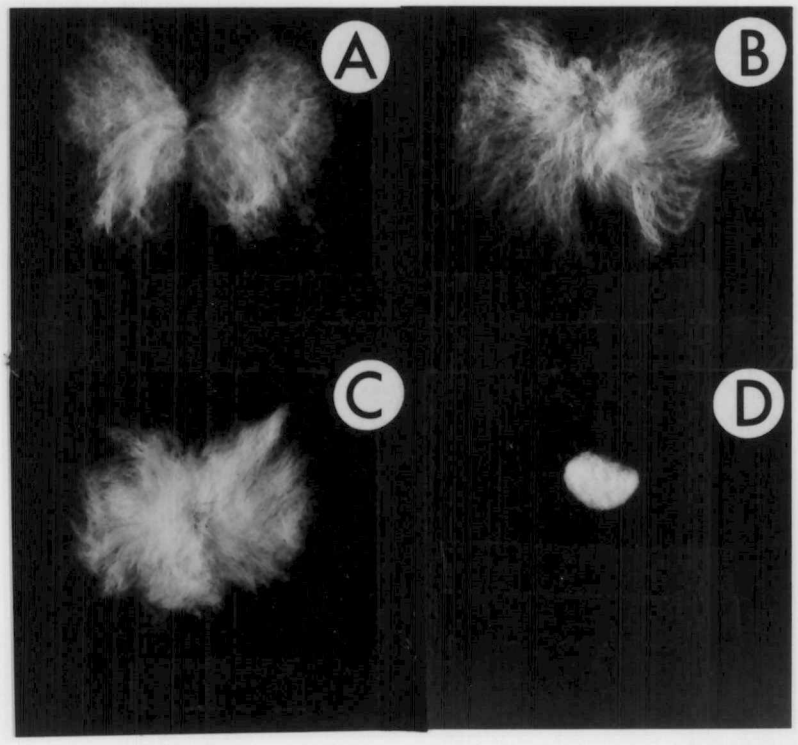
Fig. V.1. Mature normal and mutant ovules.

A. DPL-61.

B. Immature fiber.

C. Pilose.

D. Ligon lintless.


A
B
C
D

prematurely (24). Dry weight accumulates faster and continues longer than normal (24).

Ligon lintless is characterized by having very short fibers (0.5 cm), twisted stems and contorted leaves (24). This mutant is caused by an incomplete dominant mutation. The fibers do not elongate normally but dry weight increases at a rate similar to that seen in normal fibers (24). The result is short fibers with very thick secondary cell walls.

Immature fiber is characterized, as the name implies, by immature fibers. The fibers apparently have a defect in their ability to synthesize a normal secondary cell wall. Elongation is normal in Immature fiber. Dry weight accumulation is nearly normal at first, but accumulation ceases prematurely (24). The result is a weak fiber due to a decreased amount of cellulose in the secondary cell wall. There is no apparent pleiotropy associated with this mutant.

These mutants were not in isogenic lines. Immature fiber and Ligon lintless were in the genetic standard for upland cotton, Texas Marker-1 (25). Texas Marker-1 was derived from Deltapine 14 (DPL-14) by repeated backcrossing. DPL-61 and DPL-14 are related cultivars developed by the Delta and Pineland Co. Texas Marker-1 was not used as the source of normal tissue because it performed poorly and was unproductive at Knoxville, probably due to severe inbreeding. Pilose was in the obsolete cultivar, Empire. Empire is not related to the Deltapine lines; therefore, variation due to the distance between DPL-61 and Empire was quite possible. However,

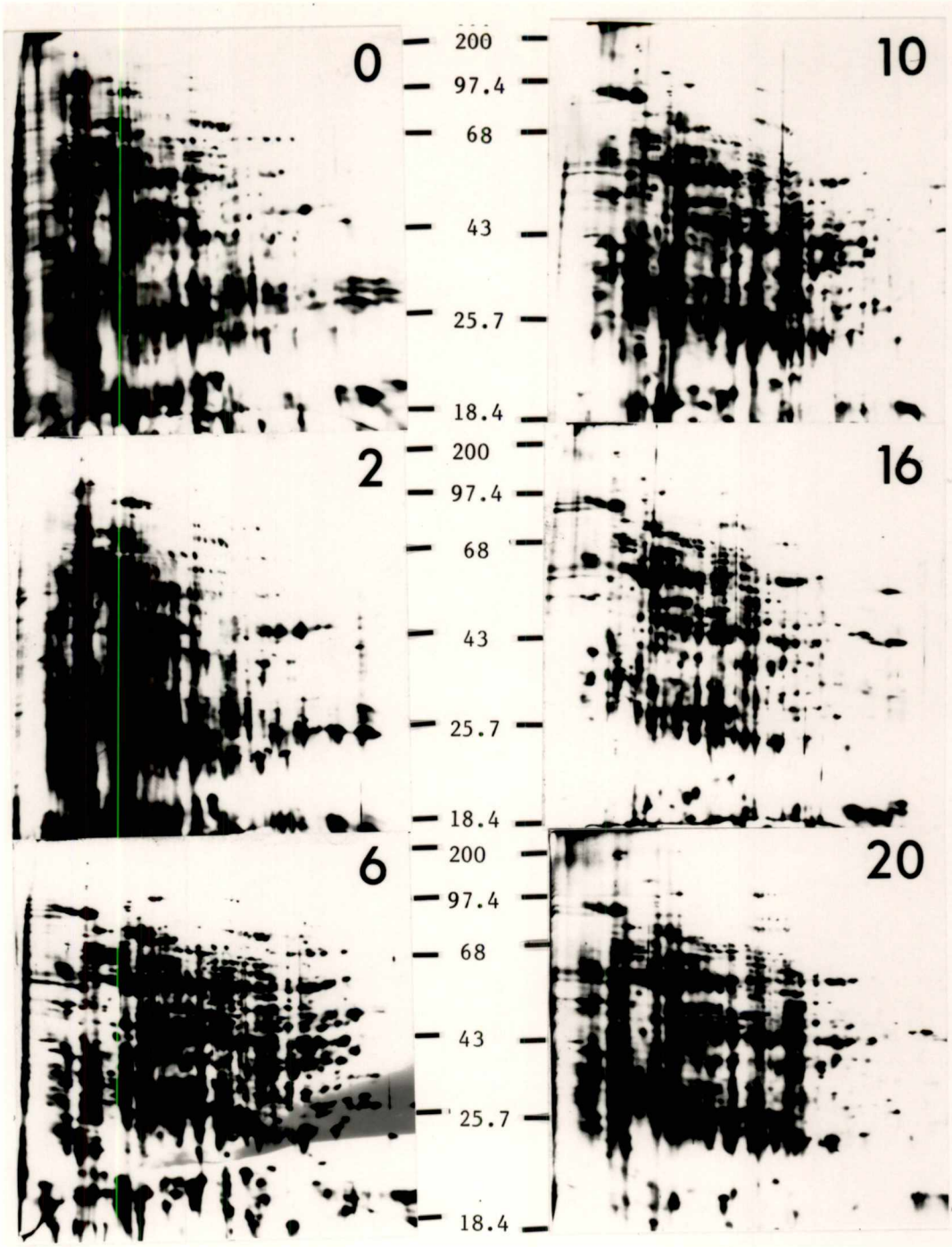
upland cotton in the United States apparently originated from a small population of wild G. hirsutum (39). Early breeding programs were simply selections of more productive plants with no introduction of foreign genes (39). Even today the upland cotton gene pool is generally closed with very few introductions of exotic genes. For these reasons and the time required to generate isogenic lines, the mutants were used in their different genetic backgrounds. Possible variation due to varietal differences was generally ignored in this study. As more detailed information is generated, such differences will likely become more prominent and significant.

The protein constituency of the mutants was investigated using 2D gel electrophoresis as described in Chapter II and the Appendix, Sections 4 and 5.

Results

Figure V.2A, B and C show 2D gels of 0, 2 and 6 dpa intact ovules and 10, 16 and 20 dpa fibers from Immature fiber, Pilose and Ligon lintless, respectively. Examination of mutant ovules for changes in individual proteins did not seem to be the most appropriate means to evaluate the protein population of the mutants since the lines were not isogenic. The 2D gels of mutant proteins were analyzed from the perspective of major developmental changes in the total protein population. Results in Chapter II (see Fig. II.2, page 16) indicated the expression of early and late populations of proteins. Assuming this is a common characteristic of normally developing cotton

Fig. V.2. 2D gels of Immature fiber (A), Pilose (B) and Ligon lintless (C). Gels are designated by the age of the tissue. Gels 0, 2 and 6 contain proteins from intact ovules. Gels 10, 16 and 20 contain proteins from isolated fibers. MW is indicated as 10^{-3}. Top left of each gel is acidic and high MW.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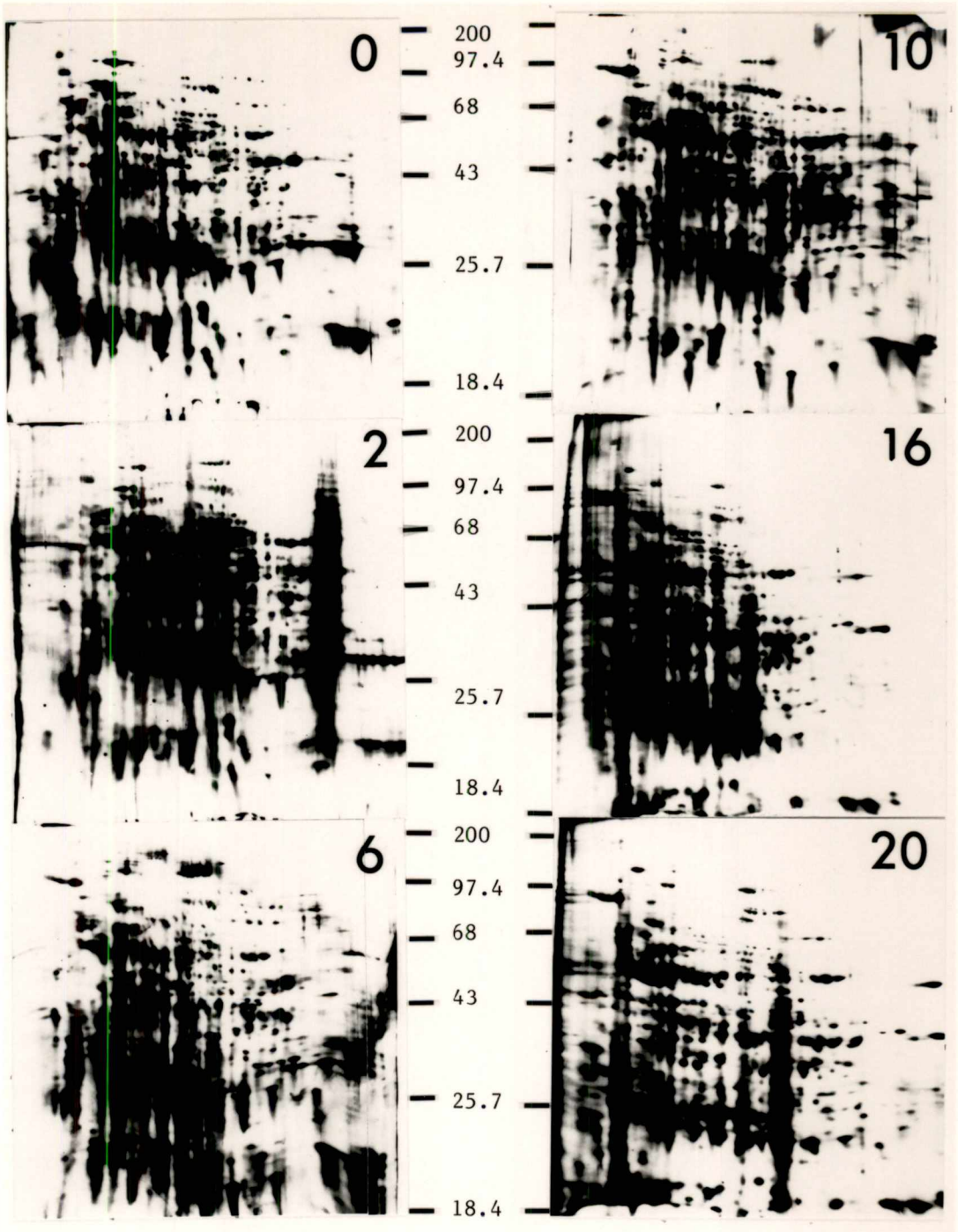
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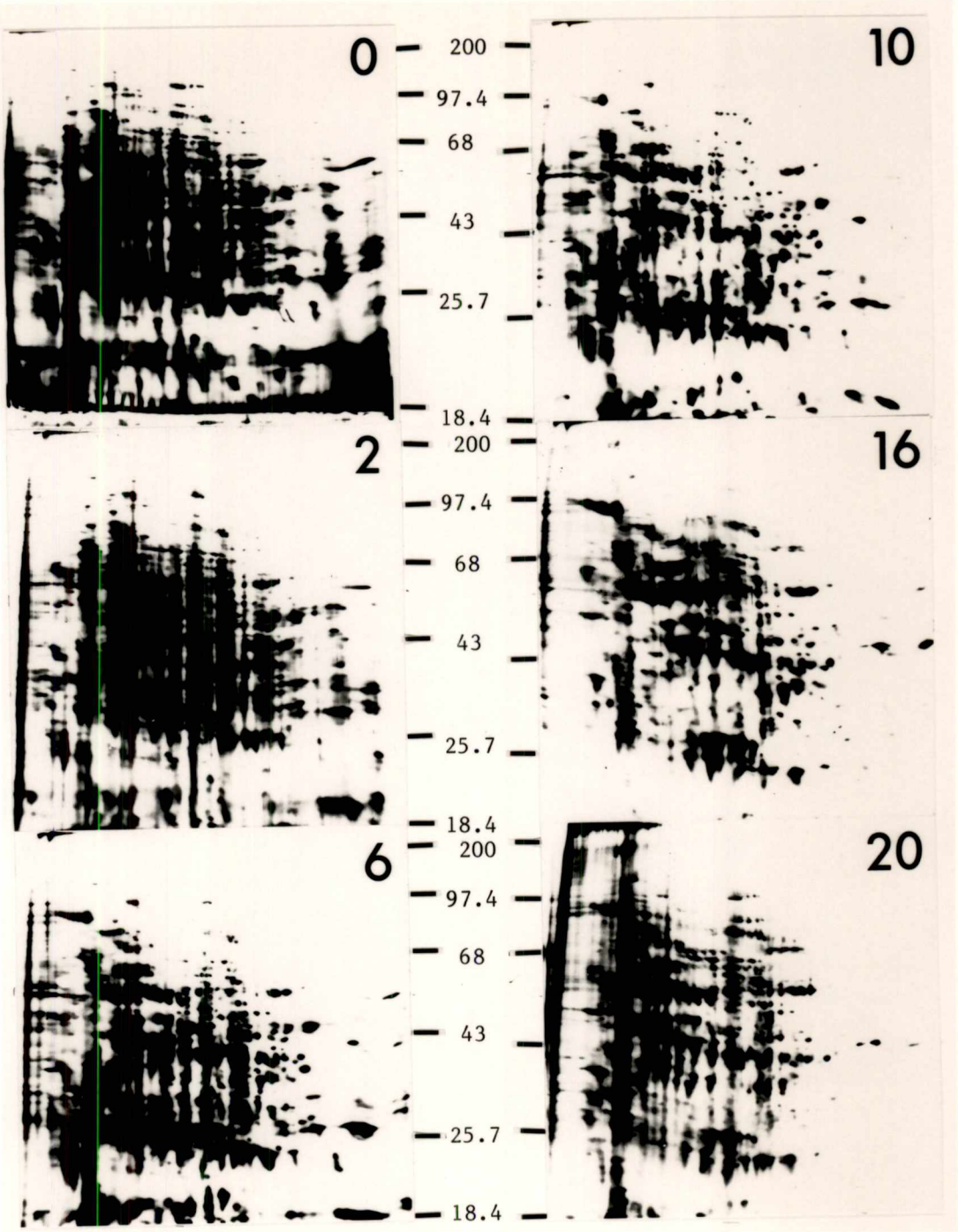
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fibers irrespective of the cultivar, the mutants were examined to see how well they fit this pattern of expression.

Immature fiber (Fig. V.1A) seemed to follow the expected pattern of protein expression. However, at 16 dpa the increase in the expression of several proteins seen in DPL-61 was not apparent, suggesting that Immature fiber was defective in its ability to regulate the expression of certain proteins putatively involved with secondary cell wall synthesis.

Pilose and Ligon lintless (Fig. V.2B and C) did not fit the expected patterns of protein expression. Late protein expression appeared to occur earlier than expected in both mutants. No clear demarcation between early and late protein expression was seen in the protein profiles of these mutants.

Discussion

The spot patterns on 2D gels of each mutant were complex, and these patterns were not easily matched with spots on gels containing DPL-61 proteins or proteins from the other mutants. However, general trends were examined without matching individual protein spots from different sources.

Immature fiber apparently has a defect in its ability to synthesize or deposit cellulose in the secondary cell wall as the fibers are weak and the weight per unit length ratio is lower than for normal cotton fibers (24). Results from 2D gels suggest normal protein expression during the elongation phase. The absence of an

increase in the staining intensity of several protein spots at 16 dpa may indicate that Immature fiber lacks the ability to regulate the expression of proteins involved in cellulose synthesis or secondary wall deposition. The exact nature of the defect can not be ascertained from this work; however, immature fiber should be useful in studies of the regulation of secondary cell wall synthesis. The mutant may also be useful for identifying proteins that are involved in the complex process of cell wall formation.

Pilose and Ligon lintless deviated from the typical early-transition-late pattern of protein expression characterizing DPL-61 ovules and fibers. Although both mutants display shorter than normal fibers and greater than normal dry weight per unit length ratios, any association of these phenotypes with altered patterns of protein spots on 2D gels is speculative. The two mutants are so different in both fiber phenotype and associated pleiotropies that the apparent similarity in trends of protein expression seen on 2D gels is probably due to insufficient resolution of protein expression. Other experimental approaches should be used to investigate protein and gene expression in Pilose and Ligon lintless.

These mutants provide a largely untapped resource for the study of cotton fiber development. This cursory examination of the protein profiles of each provides some suggestions on which to build future experiments.

CHAPTER VI

SUMMARY AND PROSPECTUS

This report addresses the biology of cotton fiber development from -6 to 20 dpa. Major findings are summarized:

1. The total protein species composition of developing ovules and fibers was composed of at least three distinct expression groups. An early population of proteins was expressed from -3 dpa to 2 dpa. A late population was detected from 6 to 10 dpa through 20 dpa. A constitutively expressed group of proteins was present at all ages.

2. *In toto*, 35 proteins were immunodetected using a fiber-specific serum. These proteins displayed the same early, late and constitutive expression patterns seen with the total protein population.

3. Nine fiber-specific proteins were discovered.

4. Nine fiber-enriched proteins were detected.

5. *In vitro* ovule culture confirmed that prefiber cells are potentiated between -3 and -2 dpa.

6. Prefiber cells were initiated on preanthesis ovules by phytohormonal stimulation following the potentiation event through the day of anthesis.

7. Preanthesis ovules maintain the ability to produce fibers in response to phytohormones after culture in hormone-free medium through the *in vivo* scheduled time of anthesis.

8. Immature fiber did not show an increase in the staining intensity of protein spots on 2D gels of 16 dpa fibers; whereas, an increase in the staining intensity of proteins on 2D gels of normal 16 dpa fibers was seen. Other than lacking this characteristic of normal fibers, immature fiber followed the general protein expression patterns seen with normal fibers and ovules.

9. Pilose and Ligon lintless did not follow the protein expression patterns seen with normal ovules and fibers. Late protein expression appeared to begin earlier than normal.

Based on this information and data from the literature, some conclusions were drawn concerning fiber development. Expansion of those conclusions led to the formulation of hypothetical models describing some aspects of fiber differentiation. Although many of the ideas presented in these models are speculative, they take into account published information and the data presented here.

The potentiation of epidermal cells occurs at -3 to -2 dpa and this is the earliest time that fiber cells are distinguishable from epidermal cells. Since not all epidermal cells become fibers, the question arises of when the event occurs that distinguishes which cells become fibers and which do not. This is an important issue because defining the time when the event occurs will indicate a developmental period that merits further investigation. If the mechanism that determines which cells become fibers and which remain epidermal cells can be discovered, it may suggest ways of altering the process so that more cells become fibers.

At least three schemes for the distinguishing event seem feasible. In the first scheme all epidermal cells become prefiber cells at -2 dpa. These prefiber cells continue to divide but the division of prefiber cells is unequal resulting in one epidermal cell and one prefiber cell. (Unequal divisions are common in embryonic and other differentiating tissues (33).) Another possibility is that all epidermal cells are potentiated at -2 dpa, and when initiation occurs, the cells that first respond are able to inhibit adjacent cells so that they cannot become fibers. The third alternative suggests that by some unknown mechanism only certain epidermal cells are potentiated. This effect could be mediated by unequal cell division resulting in the terminal, nondividing fiber cell. The same result could occur if potentiated cells inhibit the potentiation of neighboring cells.

The timing of this process can be determined immunocytochemically by labeling thin sections of the epidermis with a labeled IgG which recognizes a fiber-specific protein. The distribution of cells in the epidermis which can bind the labeled IgG will indicate which model is most likely correct. Further experimentation based on the results of immunocytochemistry will be needed to define the biochemical and molecular basis of the determining event.

The period between -2 dpa and anthesis seems to be an important time in the development of the fiber. The apparent "preprogramming" of potential fiber cells is not understood. Detailed study of this period should be conducted at both mRNA and protein levels to

determine the concentration and activity of these macromolecules. That prefiber cells are initiated on preanthesis ovules by simply providing CO_2 and IAA and GA and that transcription inhibitors can block fiber initiation on 0 dpa and -2 dpa ovules (L. L. Marden, personal communication) indicate that these phytohormones stimulate gene expression. The preanthesis cotton ovule, thus, becomes a useful model for studying phytohormone mediated regulation of gene expression. The differences noted in the immunodetected proteins on 2D protein blots of -1 and 2 dpa ovules may be related to phytohormone mediated gene expression.

The elongation phase of fiber development was not clearly elucidated in this study; however, an interesting result was seen in the 2D gels of Pilose and Ligon lintless ovules and fibers. These mutants have abnormally short fibers and the expression of late proteins occurs prematurely in both. This observation suggested that late protein expression may be linked to premature cessation of fiber elongation. The switch from expression of early proteins to the expression of late proteins may be coordinated so that whenever expression of proteins involved with elongation stops, expression of proteins associated with secondary wall formation begins, assuming that some early proteins function in elongation and that some late proteins function in secondary cell wall synthesis. This hypothesis can be tested by carefully mapping the temporal expression of early and late fiber-specific proteins using <u>in vitro</u> translated total mRNA or by northern blot analysis of early and late mRNA's using

fiber-specific cDNA clones. If this link exists then Ligon lintless should show the earliest shift from early to late protein or gene expression followed by Pilose and then normal cotton. The coordination of these events is central to understanding the progressive programming of a developing cell line. Understanding the relationship between elongation and secondary cell wall synthesis may suggest a mechanism for genetically adjusting the length and micronaire of cotton fibers.

Late protein expression occurs at about the same time that secondary cell wall synthesis first becomes evident. To assume that some of the late proteins are directly involved in cellulose synthesis and deposition is tempting but this work has produced no evidence to substantiate that assumption. The most pertinent investigation of this protein expression group is to determine the general and then the specific functions of late proteins. Fine control of protein expression and function are important topics for later studies.

Another important topic for further investigation is in determining the function of various proteins seen in the developing ovule and fiber. Determination of the exact function of a protein is a difficult task. However, the presence, absence or concentration of particular proteins can be positively associated with various fiber characteristics. Much variation in fiber properties is seen in the G. hirsutum germplasm. These variations are often difficult to incorporate into a breeding program due to the lack of an early and efficient selection scheme. If a protein is directly and

consistently associated with an interesting trait, it can become the basis for a selection scheme. Using monoclonal antibodies to several proteins that are positively associated with certain traits, the presence or absence of selected proteins can be determined with relative ease and accuracy. This approach would give the plant breeder the ability to screen hybrids for a particular protein constitution. The impact of such an approach to cotton breeding will not be seen for several years as pertinent protein markers have not been determined. However, the detection of fiber-specific proteins in this work suggests that this approach is feasible given the time to carefully analyze the differences in the protein constituency of fibers with different physical characteristics.

The results presented in this work provide an impetus for a variety of research areas involving fiber development and the development of terminal angiosperm cell lines in general. Although more questions were raised than were answered, this research has fulfilled the initial goal of providing a foundation of data on which hypotheses can be built and tested. Future research can be more efficiently focused to individual problems in the developmental process.

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APPENDIX

APPENDIX

Section 1

CULTIVATION, COLLECTION AND STORAGE OF PLANT MATERIAL

1. Gossypium hirsutum L. c.v. "Deltapine 61" (Delta and Pineland Co., Scott, MS) was treated with a fungicide and planted in a field plot in mid to late May depending on climatic conditions.

2. Weed control was performed manually.

3. Insect control was conducted by the field station personnel (Sevin was the primary insecticide used).

4. Peak blooming occurred in August.

5. Flowers were tagged daily and after the appropriate time the bolls (ovaries) were collected manually.

6. Anthesis (0 dpa) bolls were collected as fresh flowers.

7. Preanthesis bolls (-6, -3 and -1 dpa) were collected as follows (29):
   A. -1 dpa ovaries were collected as expanded but unopened buds in the "candle" stage.
   B. -3 dpa ovaries were collected by picking the proximal bud or "square" one branch up from a branch containing a fresh flower at the first node.
   C. -6 dpa ovaries were collected by picking the next square distal to and on the same branch as a fresh flower.

8. Ovules were hand picked from ovaries using a dissecting needle and a small spatula and placed in clean baby food jars.

9. Ovules were frozen at -20° C as soon as possible after collection.

10. Ovules were next lyophilized in a Virtis model USM-15 freeze dryer until completely dry.

11. The dry ovules were sealed in the baby food jars by wrapping the lids with several wraps of parafilm.

12. The ovules were stored at -20° C until use.

13. Ovules were allowed to warm to room temperature in a dessicator before the seal of the container was broken.

14. For protein extraction an appropriate amount of -6 to 6 dpa ovules was weighed and extracted. For older ovules the locules were cracked open and the intact ovules were removed. This allowed pure fiber and outer integument to be used for protein extractions. Estimated contamination of the fibers with non-fiber epidermal cells was less than 5% w/w.

15. Ovules from the fiber mutants, the fiberless mutant and DPL-61 leaves were treated exactly as described above.

Section 2

PROTEIN EXTRACTION

This procedure is derived from that of Dure and Chlan (11).

1. Weigh lyophilized tissue (0.02 g minimum) and place into a sterile 40 ml Oak Ridge style centrifuge tube.

2. Add 5 parts insoluble polyvinylpyrrolidone (Polyclar AT) (w/w).

3. Add 15 ml of extraction buffer/0.1 g tissue and grind the mixture with a Polytron (Brinkman Instruments, generator PTA 10TS, power setting 7.5, 15 sec).

   Extraction Buffer: 50 mM TRIS-HCl pH 8.6
   2% 2-mercaptoethanol
   2% SDS

4. Heat homogenized tissue to 100° C for 5 minutes.

5. Centrifuge at 12,000 xg for 10 min to pellet debris.

6. Decant supernatant into a clean sterile centrifuge tube.

7. Add 10 volumes of acetone at -20° C, mix and incubate at -20° C for 2 hrs to overnight.

8. Collect precipitate by centrifugation at 12,000 xg for 10 min.

9. Decant acetone and air dry pellet.

10. Dissolve pellet in an appropriate buffer and centrifuge to remove any undissolved material before use.

Section 3

PROTEIN QUANTITATION (12)

Staining solution: 10% acetic acid, 25% isopropanol, 65% ddH_2O, 0.1% Coomassie Brilliant Blue (CBB) R-250. Stir solution until all CBB dissolves, filter and store in glass. Stain may be reused.

Destain solution: 1% SDS in dd H_2O.

PROCEDURE:

1. Cut Whatman #1 chromatography paper into an appropriate sized strip. Paper must be clean.
2. Label paper with pencil to designate samples and their location.
3. Spot 1 µl aliquots of each protein sample onto the paper strip.[a]
4. Spot standards on paper--0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0 and 7.5 µg/µl are good when using BSA.
5. Allow spots to air dry.[b]
6. Put paper in stain for 15 min with shaking.
7. Decant stain.
8. Rinse paper in running dH_2O for 30 sec.
9. Wash paper for 3 min in ddH_2O with shaking.
10. Decant water.

---

[a]Proteins may be dissolved in many common buffers. Some buffers may cause artifactual staining. Buffers should be tested by spotting and staining, compare against water. Background staining due to the buffer may be obviated by making the standards in the buffer being used. The 9 M urea sample buffer used in 2-D gels is best diluted 2X with water and measured against a standard curve generated using BSA in 0.5 X sample buffer.

[b]May hasten drying by using a hair dryer or by hanging the paper in a warm place.

11. Hang wet paper to air dry.[b]

12. To quantitate remove stained spots by punching them out with a paper punch. A 1 µl spot is slightly smaller than the diameter of the hole punch.

13. Place paper circle in individual 1 ml aliquots of 1% SDS and shake until all the CBB has diffused out of the paper.[c]

14. For a blank destain a paper circle punched from the stained paper where no protein was added.

15. Read absorbance at 600 nm. (Fig. A.1 shows a typical standard curve.)

16. For semiquantitative analysis visually compare unknown spots with standard spots.

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[c]Destaining is facilitated by heating to 50°-70° C.

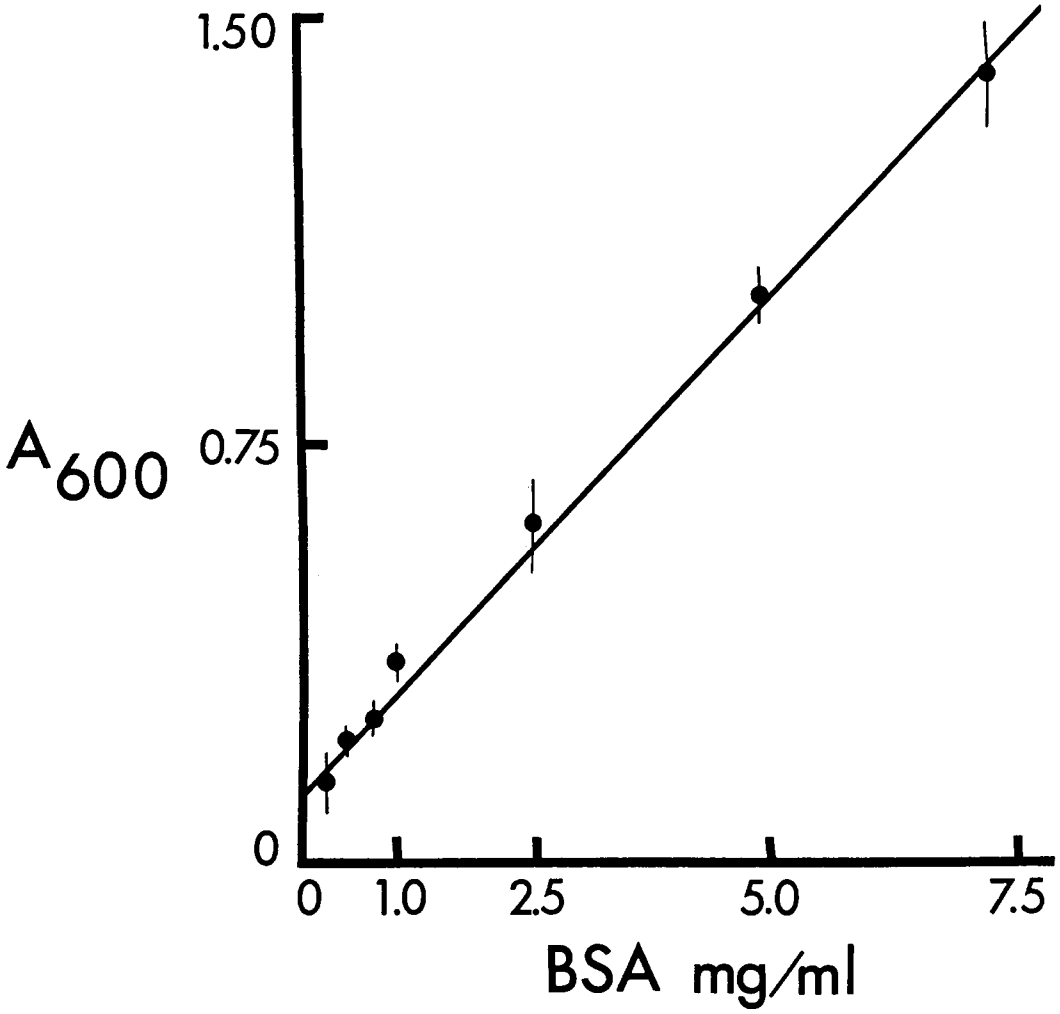

Fig. A.1. Standard curve for protein quantitation. Curve was generated using BSA dissolved in extraction buffer. Points represent the mean and standard deviation of three independent measurements.

Section 4

SDS-PAGE

The procedure used for SDS-PAGE was based on the protocol of Laemmli (27). All slab gels were 0.75 mm thick and run in a Bio-Rad Protean I dual slab gel apparatus (equivalent to Hoefer SE600). The apparatus was assembled and operated according to Bio-Rad instructions and electrophoresis was conducted at 12.5 mA constant current/gel with cooling until the dye front came within 1 cm of the bottom of the gel. Slabs were poured with a 15 ml resolving gel bed (17 ml for 2D gels).

STOCK SOLUTIONS:

1. Acrylamide--30% T, 2.7% C
   30 g acrylamide
   0.8 g bisacrylamide
   dissolved in ddH_2O to 100 ml

2. Stacking gel buffer, 10X, pH 6.8

component	amt/100 ml	1X conc
TRIS-HCl	15.14 g	0.125 M
SDS	1.0 g	0.1%

3. Resolving gel buffer, 2X, pH 8.8

component	amt/500 ml	1X conc
TRIS-HCl	40.35 g	0.375 M
SDS	1.0 g	0.1%

4. Electrode buffer, 10X, pH 8.3

component	amt/1	1X conc
TRIS	30.25 g	0.025 M
Glycine	144 g	0.192 M
SDS	10 g	0.1%

MONOMER SOLUTIONS:

30 ml of 10% T resolving gel solution

stock solution	amt for 30 ml total vol.
Acrylamide	10 ml
2X buffer	15 ml
ddH_2O	5 ml
10% Ammonium persulfate	0.330 ml (make fresh daily)
TEMED	25 µl

15 ml of 3% T stacking gel solution

stock solution	amt for 15 ml total vol
Acrylamide	2.55 ml
10X buffer	1.5 ml
ddH_2O	10.95 ml
10% Ammonium persulfate	0.250 ml
TEMED	25 µl

HINTS:

Degas monomer solutions before adding APS and TEMED.

Overlay resolving gel with ddH_2O. Allow 30 min for polymerization.

Remove ddH_2O from top of resolving gel, wash surface with ddH_2O, drain, insert comb and fill gel assembly with stacking gel solution. Allow 20 min for polymerization. Remove comb and wash each well with ddH_2O. Finally, fill each well with electrode buffer.

Molecular weight determinations were made using Bethesda Research Laboratories (BRL) high molecular weight protein standards.

Section 5

2D GEL ELECTROPHORESIS

This procedure is essentially that of O'Farrell (33). It came to me via Wayne Hughes in Glenn Galau's lab at Georgia (19). Some modifications of their procedure were made; in its current state it gives fairly good results but I am not satisfied with the pH gradient. My literature review has led me to believe, however, that very few researchers have made "perfect" pH gradients especially when focusing large amounts of protein for CBB or even silver staining. For IEF--always use the purest reagents possible. Acrylic acid is one of the greatest culprits. It can be minimized by using high quality acrylamide. Acrylic acid may be removed with Rexalyn mixed bed resin.

SOLUTIONS for IEF

Gel monomer stocks

Acrylamide: (30% T, 5.4% C), 28.4 g Bio-Rad acrylamide + 1.62 g Bis acrylamide to 10 ml with ddH_2O, store at 4° C

20% Triton X-100

Ampholytes: Servalyts 3-10 Isodalt Grade for 2-D gels. Servalyts come as a 40% conc. 2% servalyts in the gel solution works best. LKB Ampholines, Bio-Rad Biolytes, Pharmacia's Pharmalytes and Isolab's Isolytes will probably work just as well although electrode solutions or ampholyte concentration may need to be altered. 3-10 Isodalt Grade was used exclusively. The gradient may be stretched by adding narrow range ampholytes with the total conc. being about 2%. Amino acids may be used to make the gradient more linear.

Sample overlay buffer: (separates sample buffer from the electrolyte)

Stock	Vol/100 ml	Final conc.
Solid Urea	48.05 g	8 M
40% Servalyts	2 ml	2%
20% Triton X-100	10 ml	2%
ddH_2O	to 100 ml	

Freeze solution in small aliquots or only make small batches and store at 4°. Use only Ultrapure Urea! (Bethesda Research Laboratories)

Sample buffer: (used to solubilize proteins for IEF)

Stock	Vol/100 ml	Final conc.
Urea	55.00 g	9M
Servalyts	2 ml	2%
2-mercaptoethanol	5 ml	5%
20% Triton X-100	10 ml	2%
0.5% Pyronin Y	0.5 ml	0.0025%
ddH_2O	to 100 ml	

Freeze in small aliquots or make small batches and store at 4° C. Pyronin Y described below.

Electrolytes: Anolyte--30 mM phosphoric acid, 2.1 ml of 85% acid/liter. Catholyte--0.1 N NaOH, 4 g/l.

Equilibration buffers: (to prepare IEF tubes for 2D)

BME+	
0.250 M Tris HCl	30.3 g Tris pH + 5.7 ml conc. HCl
3% SDS	30 g clean SDS
5% 2-mercaptoethanol	50 ml
ddH_2O	to 1 liter

BME- same as BME+ but without mercaptoethanol.

Dye Stocks: 0.5% Pyronin Y in ddH_2O. 0.5% Brom phenol blue in ddH_2O.

PROCEDURE

1. Glass tubes 3 mm i.d. x 14 cm are cut from long glass tubing. Only segments with square ends are used and these are lightly fire polished. Tubes are soaked in conc. Chromerge overnight, rinsed in dH_2O, siliconized and dried in the autoclave or oven. After the first cleaning, soaking tubes in Alconox, rinsing and drying is usually sufficient for four or five uses then the tubes must be acid cleaned and siliconized again.

2. Bottom ends of tubes are sealed with at least two wraps of parafilm.

3. Prepare gel solution.

Stock	Amount
Acrylamide	0.126 x = 3.8% T
Urea	0.551 g/ml = 9 M
Servalyts	0.05 x = 2%
20% Triton X-100	0.1 x = 2%
ddH_2O	0.3 x

TEMED	0.001 x
10% Ammonium Persulfate	0.001 x

x = total volume

Prepare 10% APS fresh daily, 0.1 g APS in 1 ml ddH_2O.

4. Casting gels. Mix all non-catalysts. Do not heat solutions above 30° C. Degas solution after all urea has dissolved. Add TEMED and APS, swirl gently to mix and quickly pour solution in tubes. Mark tubes to the desired height (12.5 cm) and using a long pasteur pipet add solution to the mark. Trap no bubbles! Pour all gels, then overlay gels with water without mixing.

5. After polymerization (about 30 min), remove water and add sample buffer about 3 mm deep. Let gels stand for 2 hrs to overnight. Cover tubes.

6. Preparation for running. Cut parafilm from bottom of tube with scalpel or razor blade. Squares of washed, wet dialysis membrane are used to seal bottoms of tubes so gels do not slide out during the run. Sleeves of Tygon tubing are used to hold membranes in place. Use the glass tube with gel to align one piece of membrane (one layer thick) over a sleeve and push tube into sleeve. All this is done under water to avoid trapping air bubbles. (It is not as hard as it sounds!) After a membrane and sleeve is in place, remove the tube from the water and slide the sleeve up on the tube so that the tube extends beyond the sleeve. Repeat until all tubes are sealed. Tubes are put in electrophoresis apparatus.

Prefocusing

1. Old sample buffer is removed, 20 µl of fresh buffer is added.
2. Anolyte is carefully overlaid on sample buffer to top of tube.
3. Upper buffer chamber is filled with anolyte to cover tops of tubes.
4. Fill lower buffer chamber with catholyte. (Make buffer level equal in both chambers.)
5. Connect apparatus to power supply. - to catholyte, + to anolyte.
6. Set power to a maximum of 0.2 watts/tube.
7. Set voltage to a maximum of 500 volts.
8. Set current to maximum value of instrument.
9. Apply current. Volts start at about 205 v and reaches 500 v after about 2 hrs. This is very consistent experiment to experiment.

Buffers must be degassed!

Focusing

1. After voltage-power crossover remove anolyte from top chamber.
2. Remove anolyte from tubes.
3. Wash anolyte out of tubes by adding sample overlay buffer.
4. Remove sample overlay and add protein sample dissolved in sample buffer. 100 µl of sample may be added/tube with no deleterious effects.
5. 20 µl of sample overlay is added to top of sample.
6. Anolyte is added to top of tube.
7. Anolyte is added to chamber to cover tops of tubes.
8. Connect apparatus to power supply, set power to 0.1 watt/tube, set voltage at 500 v. Focus for 17 hrs (7500-10,000 vhrs)

Removal of gels from tubes. Rim gels with water from a syringe and needle. Creatively attach tube to a 20 ml syringe and extrude gel using air pressure. Extrude gel into fixative or BME+. Be careful lest the gel shoots out of the tube with great velocity (bad for integrity of the gel). The orientation of the gel can be determined by observing swellings and distortions or by marking the gel with India ink or fine gauge wire.

Staining gels. Gels are extruded into fixative (30% MeOH, 7% Acetic acid) and fixed for at least 1 hr with 2 changes of fixative. Gels are stained for at least 1 hr in 0.1% CBB R-250 in fixative. Gels are destained in multiple changes of fixative.

Equilibration of gels for 2nd dimension electrophoresis. Gels either stained or unstained are put in BME+ for 15 min. The gels are transferred to BME- for 15 min. The buffer is removed and gels are stored at -20° C or used immediately.

Analysis of pH Gradient. Designate one gel for pH determination. Use a scanning pH meter with a surface probe or cut the gel into 0.5 or 1 cm segments. Place each segment into 1 ml of degassed water in a microfuge tube and shake for 1 hr. Measure pH of water in each tube.

Second dimension separations are conducted by SDS-PAGE as described in Appendix, Section 4. Tube gels are used immediately after equilibration or completely thawed if stored frozen. A tube gel is placed onto parafilm and slid onto the top of a slab gel. The tube gel is sealed in place with agarose sealing buffer, being careful to trap no bubbles and to keep the IEF gel in close contact with the slab gel.

Agarose sealing buffer:
3.63 g/l Tris pH 8.5 1 = (0.030 M)
5 ml/l BPB stock
Agarose--0.5 to 0.8% added to an appropriate aliquot, melt and temper to 55° C.

Section 6

SILVER STAIN (31)

1. Fix gel in 50% methanol, 12% acetic acid for a minimum of 20 min for a 0.75 mm gel. Polyphenolics were washed from gels by fixing for 18 hrs at room temperature.

1a. The gel may be treated with 10% glutaraldehyde to cross link the proteins to the gel.

2. Rinse the gel in 10% ethanol, 5% acetic acid for 10 min, repeat 2 or 3 times, use about 200 ml per rinse. (This step removes SDS, delete this step if gel does not contain SDS.)

3. Soak the gel in 200 ml of 3.4 mM potassium dichromate, 3.2 mM nitric acid for 5 min.

4. Wash the gel in several changes of deionized water until the gel is nearly colorless.

5. Soak the gel in 12 mM $AgNO_3$ for 30 min. (200 ml per gel).

6. Rinse twice in deionized water.

7. Rinse the gel rapidly with two 300 ml volumes of 0.28 M Na carbonate, 0.5 ml/l formaldehyde (developer).

8. Soak the gel in 300 ml of developer until the desired staining intensity is reached.

9. Stop development by soaking the gel in 100 ml of 1% acetic acid for 5 min.

10. Wash gel twice in 200 ml of water.

11. Store in water or dry on paper or cellophane.

Recipes and tips:

3.4 mM K dichromate = 1 g/l

3.2 mM Nitric acid = 0.3 ml/l

12 mM $AgNO_3$ = 0.4 g/200 ml

0.28 M $NaCO_3$ = 29.7 g/l (do not forget to add formaldehyde)

Use the best quality water possible; never use tap water for any step!

The K dichromate, nitric acid may be stored for about 1.5 to 2 months, if background staining becomes a problem discard this solution and make a fresh supply.

$AgNO_3$ may be made as a 10X concentrate and diluted before use or made fresh each time.

The developer should be made just prior to its use each time.

Section 7

PREPARATION OF ANTISERUM TO TOTAL COTTON OVULE AND FIBER PROTEINS (20)

1. Extract protein from 0 to 8 dpa lyophilized ovules and from 10 to 20 dpa isolated fibers.

2. Quantitate protein. (See Appendix, Section 3.)

3. Mix extracted proteins 1:3 with Freund's incomplete adjuvant.

4. Collect preimmune serum from all rabbits.

5. Inject 4 young New Zealand White rabbits with approximately 1 mg of total protein per rabbit. Inject each rabbit at 4 different sites, subcutaneously.

6. Give rabbits booster injections of 0.5 mg total protein in Freund's incomplete adjuvant at biweekly intervals.

7. Collect small serum samples at 7 days after each injection and titer serum by immunodiffusion (6).

8. When a sufficient titer is achieved (32 in this case), bleed rabbits on the ninth day postinjection. Collect about 50 ml of blood from the marginal ear vein of each rabbit.

9. Allow blood to clot at room temperature for 1 hr and to retract overnight at 4° C. Drain the serum away from the clot, centrifuge to clarify, filter sterilize, make 0.1% Na azide and store in 1 ml aliquots at -70° C.

Section 8

PROTEIN A-AGAROSE AFFINITY CHROMATOGRAPHY

Protocol is for the MAPS Kit from Bio-Rad (Technical Bulletin 1172 and ref. 16).

1. Assemble column, tubing, buffer reservoir, etc.

2. Affi-Gel Protein A comes as a suspension, add up to one volume of PBS-azide to make a loose slurry.

3. Degas Affi-Gel.

4. Pour desired amount of Affi-Gel into column. (1 ml of gel will adsorb the IgG from 1 ml of serum.)

5. Allow Affi-Gel to settle briefly. Connect column to buffer reservoir and pack column with several volumes of PBS-azide. Pack with a flow rate of 1 to 1.5 ml/min.

6. After packing equilibrate the column with 5 bed volumes of Binding Buffer.

7. Dilute the whole serum 1:1 with Binding Buffer.

8. Apply the sample to the column.

9. Wash the column with 10-15 bed volumes of Binding Buffer. If A_{280} is being monitored, a large peak will elute immediately after washing begins. Washing should continue for about 1.5 to 2 times the number of fractions that were in the A_{280} absorbing peak and until A_{280} is consistently near 0.

10. Elute IgG by washing the column with 10-15 bed volumes of Elution Buffer. An A_{280} absorbing peak should be detectable soon after adding Elution Buffer, continue to elute until A_{280} returns to near 0 and for at least one times the number of fractions were were in the peak.

11. Wash the column with 5 bed volumes of Regeneration Buffer.

12. Equilibrate the column with 5 bed volumes of Binding Buffer for the next cycle or with PBS-azide for storage.

Recipes and Hints:

1. PBS-azide (Phosphate buffered saline with 0.05% Na azide, pH 7.4, 0.15 M NaCl)
   solution A - 0.067 M KH_2PO_4 = 9.073 g/l
   solution B - 0.067 M $Na_2HPO_4 \cdot 2H_2O$ = 11.87 g/l

   1 liter of pH 7.4 PB = 197 ml A + 803 ml B.
   0.15 M NaCl = 8.77 g/l
   0.05% Na azide = 0.5 g/l

2. Binding Buffer has a pH of 8.7.

3. Elution Buffer has a pH of 3.65 - to prevent possible acid denaturation elute IgG into 1M Tris pH 9. (350 µl/2.5 ml fractions gives a pH of 7-7.5.)

4. Regeneration Buffer has a pH of 6.85.

5. If collecting by drops, fraction volume may vary with buffer due to differences in density and drop size.

6. Prepare, run and store column at 4° C.

7. Use short columns regardless of sample size, flow rates are better and changing buffers is facilitated.

8. See Fig. A.2 for a typical elution profile.

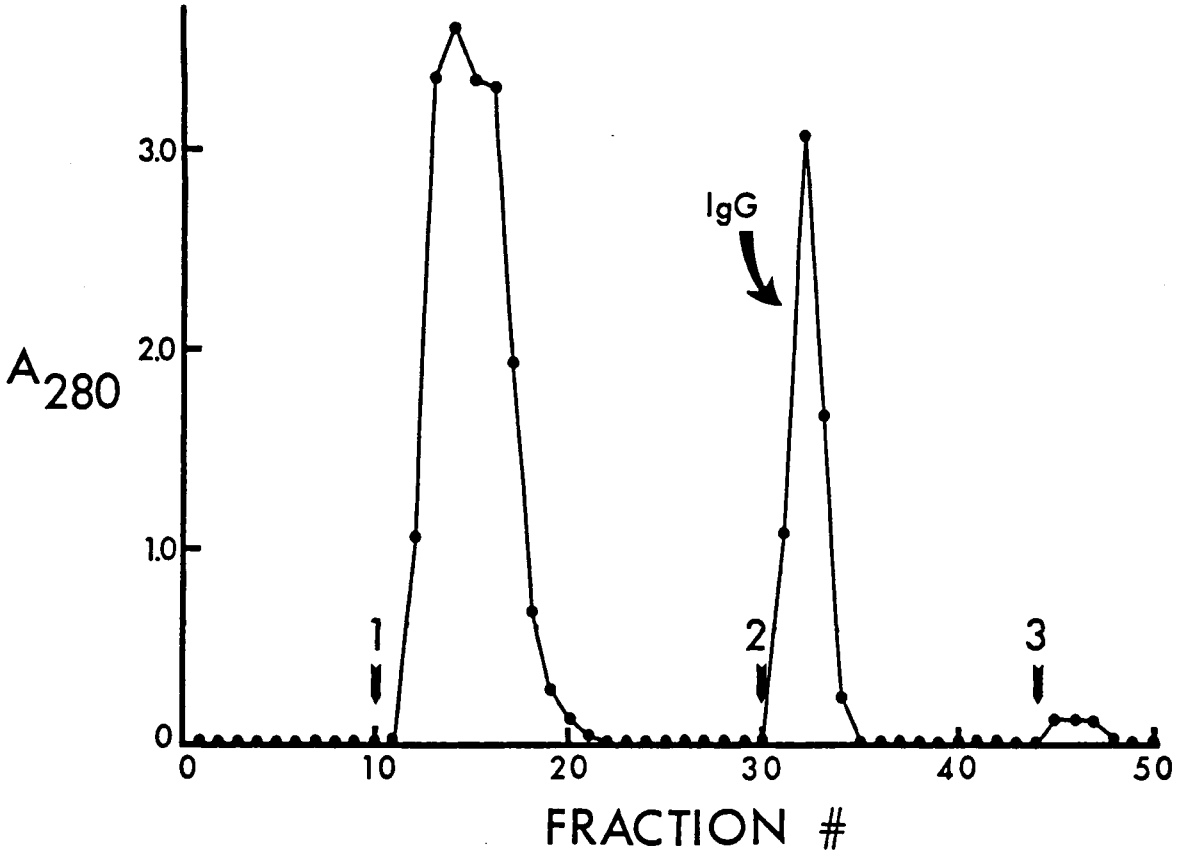

Fig. A.2. Typical elution profile of serum through the Protein A-agarose column. Fraction size was 50 drops. Events are marked with arrows. 1. Serum added, step 7. 2. Elution buffer, step 10. 3. Regeneration buffer, step 11.

Section 9

AFFINITY PURIFICATION OF FIBER-SPECIFIC IgG's

SUPPORT MATRIX:

Affigel 10 and Affigel 15--activated sepharose with spacer arms. Bio-Rad Laboratories. (For detailed description, see Bio-Rad Technical Bulletin #1085.)

BUFFERS:

Protein Extraction Buffer:
0.05 M TRIS, 2% SDS, 2% 2-mercaptoethanol, pH 8.6

Binding Buffer
0.1 M Hepes, 2% Triton X-100, 1% SDS pH 8.2

Phosphate Buffered Saline plus Na Azide (PBS-azide) pH 7.4
803 ml 0.066 M KH_2PO_4, 197 ml 0.066 M K_2HPO_4, 8.5 g NaCl

Washing Buffers:
A. 0.05 M TRIS, 0.5 M NaCl pH 8.2
B. 0.05 M Na Acetate, 0.5 M NaCl pH 4.1

Blocking Solution
1 M ethanolamine

Regeneration Buffer
2.5 M Na thiocyanate

PROCEDURE:

1. Prepare all buffers and solutions.
2. Remove Affigel 10 and 15 from -20° C freezer--it is stored in isopropanol and does not freeze.
3. Shake vials to thoroughly mix gel which will have settled.
4. Remove desired amount of gel from each vial using a syringe with a large bore needle (16 1/2 gauge).
5. Mix the two gels or place directly onto Whatman #1 filter paper in a Buchner funnel.

6. Drain the gel with light vacuum.

7. Wash the gel with ddH_2O until the isopropanol is removed (3 to 5 washes with three volumes of ddH_2O each time).

8. Remove the gel cake from the funnel.

9. Mix the gel with protein solution.[a,b] (Steps 2 to 9 should be completed within 20 min.)

10. Incubate with shaking at 4° C for 3 hrs.

11. Incubate with shaking at room temperature for 1 hr.

12. Add 0.1 ml/ml total reaction volume of Blocking solution.[c]

13. Incubate at room temperature with shaking for 30 min.

14. Pour gel into column, let gel settle by gravity for several hrs to overnight at 4° C. Keep the column at 4° C for the rest of the procedure.

15. Drain reaction solution (Binding buffer and protein) from the column. (Drain to the top of the gel; do not drain the gel or let it become dry!)

16. Wash the column with PBS-azide until the A_{280} of the eluate is 0.

17. Wash the column with Washing buffer B until the A_{280} is 0 or for three bed volumes if the A_{280} does not increase upon washing.

18. Wash the column with Washing buffer A until the A_{280} is 0 or for three bed volumes if the A_{280} does not increase upon washing.

---

[a]Proteins are extracted in extraction buffer, acetone precipitated and resuspended in binding buffer. TRIS and 2-mercaptoethanol will interfere with the binding reaction and cannot be used in the coupling reaction.

[b]The protein solution consists of proteins dissolved in binding buffer. The protein concentration should be 15 to 20 mg/ml of gel with a vol/vol ratio of 2 to 4.5 ml of binding buffer/ml of gel.

[c]Blocking solution saturates any unreacted coupling sites on Affigel.

19. Repeat steps 17 and 18 until no change in A_{280} occurs when buffers are changed.

20. Equilibrate the column with 3 bed volumes of PBS-azide.

21. Load IgG in PBS-10% glycerol.[d]

22. Wash IgG into the column and allow to sit for several hrs.

23. Wash IgG through the column with PBS-Azide.

24. Collect all fractions which absorb at A_{280}.

25. Wash column with PBS until the A_{280} is 0.

26. Regenerate column by washing with regeneration buffer until the A_{280} is 0.[e]

27. Equilibrate and store column in PBS-azide at 4° C.

COLLECTION OF AFFINITY PURIFIED FIBER-SPECIFIC IgG.

1. All A_{280} absorbing fractions from step 24 above are pooled.

2. Volume is determined.

3. 1 to 2 mg of BSA is added per ml. BSA serves as a carrier protein to protect and aid in precipitation of the IgG.

4. 50% w/v ammonium sulfate is added to the pooled fraction.

5. After 30 min to 1 hr the precipitated protein is pelleted by centrifugation at 20,000 xg for 20 min.

6. The supernatant is decanted.

7. The pellet is resuspended in PBS-azide to the original volume that was loaded onto the column. Store at 4° C.

Fig. A.3 shows the elution profile of the column during construction and use.

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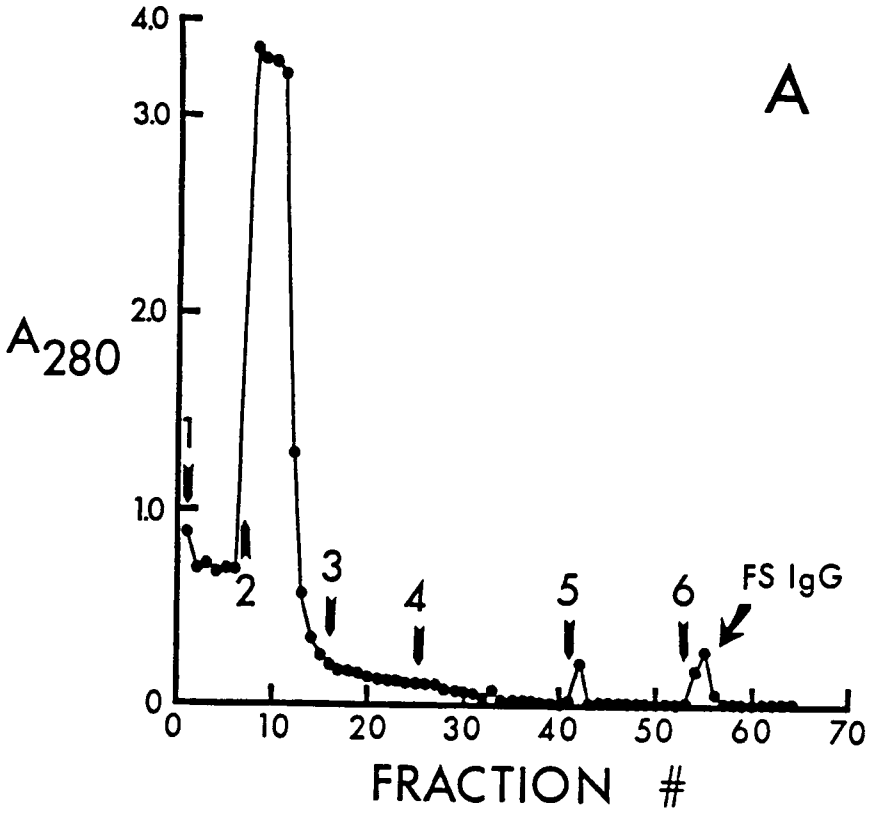
[d]The binding capacity of the column has to be determined empirically or add only a small amount of IgG so that saturation is not achieved or approached.

[e]Urea, acidic pH (1 to 2) and guanidinium HCl may be used but Na thiocyanate is more complete and less harsh than the others.

Fig. A.3. Elution profile of the affinity column. Arrows mark events.

A. Elution profile during construction and first passage of purified IgG. 1. Start, step 15. 2. PBS-azide, step 16. 3. Washing buffer B, step 17. 4. Washing buffer A, step 18. 5. PBS-azide, step 20. 6. IgG loaded and followed by PBS-azide, steps 21 through 23.

B. Elution profile of affinity column during second passage of IgG. 1. Regeneration buffer, step 26. 2. PBS-azide, step 20. 3. IgG + BSA loaded and washed with PBS-azide, steps 20 through 23.


A
A_{280}
4.0
3.0
2.0
1.0
0
1
2
3
4
5
6
FS IgG
0
10
20
30
40
50
60
70
FRACTION #

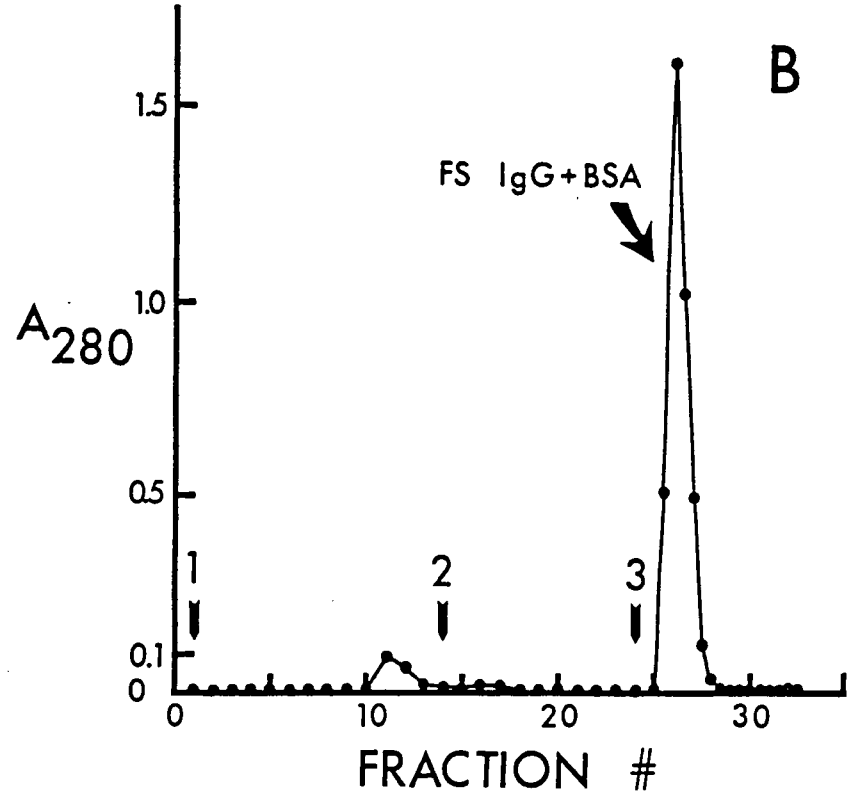

B
A_{280}
1.5
1.0
0.5
0.1
0
FS IgG+BSA
1
2
3
0
10
20
30
FRACTION #

Fig. A.3

Section 10

ELECTROPHORETIC TRANSFER OF PROTEINS TO NITROCELLULOSE

(WESTERN BLOTTING) (15,47)

1. Run SDS-PAGE according to Laemmli (27) (Appendix, Section 4).

2. Make 6 l of transfer buffer - 20% MeOH = 1200 ml
   25 mM TRIS = 18 gm pH 8.3
   192 mM Glycine = 86.4 gm
   (must degas)

3. Prepare nitrocellulose (NC) (Bio-Rad transfer medium, 15 X 15 cm). Soak NC in transfer buffer for several hours to overnight. To achieve thorough wetting put one edge of NC in contact with buffer and gradually lay the sheet on the surface of the buffer. HANDLE NC WITH GLOVED HANDS AND FORCEPS!

4. Wet sponges and 2 sheets of Whatman 3 MM paper cut to size in transfer buffer.

5. Cassette assembly. (Hoefer TE42 Transphor, TE51 power supply)
   a. In a glass or plastic baking disk place back of cassette.
   b. Place one sponge on top of cassette.
   c. Place one prewet sheet of 3 MM paper on top of sponge.
   d. Separate glass plates of gel sandwich. Carefully apply one dry sheet of 3 MM paper to gel being certain even and complete contact is made. Roll a clean test tube over the paper then gently lift the paper up starting at one edge. This peels the gel off the glass plate without tearing. Turn the paper over and apply a wet sheet of NC on top of the gel. Roll the NC to assure that no air bubbles are trapped! Mark the top of the resolving gel and the dye front with an ink pen (using two colors is convenient). Place 3 MM, gel and NC atop the assembly and cover all with a wet sheet of 3 MM. Roll to remove air.
   e. Place a wet sponge on top of the assembly.
   f. Put cover over entire stack and lock into place.

6. Add degassed transfer buffer to transfer tank. Precooling buffer is a good idea.

7. Insert cassette into tank.

8. Insert cooling coil.

9. Connect cooling coil with circulating water bath set at 4-6° C.

10. Connect electrodes to power supply. NC must be facing the anode (red or + lead) as proteins migrate from - to + poles.

11. Turn power on max. or no greater than 2 amps.

12. Transfer for 2.5 to 3 hrs/gel with cooling.

Section 11

ENZYME IMMUNOASSAY FOR FIBER ASSOCIATED PROTEINS IN COTTON [BIO-RAD IMMUNE-BLOT (GAR-HRP) KIT]

1. While proteins are blotting, make 1 liter of TRIS buffered saline (TBS)/blot.
   20 mM TRIS = 2.42 g/1 = 4.84 g/1 liters
   500 mM NaCl = 29.24 g/1 = 58.48 g/2 liters
   (pH 7.5 with HCl)

2. Make blocking solution. TBS - 100 ml/blot
   Carnation nonfat dry milk - 3%

3. Make antibody buffer. TBS - 100 ml/blot
   Carnation nonfat dry milk - 1%

4. Prepare primary antibody solution in 10 ml of antibody buffer/blot. Dilute serum 1:750. (Add 15 µl/10 ml of buffer.)

5. Dissemble gel cassette.

6. Put NC into 3% milk block and shake for a minimum of 30 min to 1 hr.

7. Transfer NC into a Dazey seal-a-meal bag which has been rinsed with antibody buffer. Add primary Ab and seal with as few bubbles as possible. Incubate at room temperature with shaking for 1.5 hrs or overnight at 4° C. 1.5 hr incubation followed by overnight incubation increases sensitivity and background.

8. Remove NC from bag by cutting three edges with scissors, briefly rinse with ddH_2O and rinse in TBS for 20 min with 2 changes.

9. Place NC in 1:2500 - 1:3000 dil of Horse radish peroxidase-goat anti-rabbit IgG (Bio-Rad, GAR-HRP) for 1 hr at room temp with shaking. (20 µl GAR-HRP in 60 ml of buffer)

10. Rinse briefly in ddH_2O.

11. Wash NC in TBS for 20 min with 2 changes and rinse in ddH_2O.

12. Prepare HRP color development immediately prior to use (18). Color development solution. Dissolve 60 mg of 4 chloronapthol in 20 ml MeOH. Add 60 µl of 30% H_2O_2 to 100 ml of TBS. Mix two solutions and pour onto NC in baking dish.

13. Develop for 15-30 min.

14. Stop reaction by rinsing in ddH_2O for 10 min.

15. Air dry, protect from light as blot will eventually fade (after months).

Section 12

IN VITRO OVULE CULTURE (3,45)

Basal medium for ovule culture.

Ingredient	Stock Solution (g/l)	Final Conc. (mg/l)	ml of stock/l
KNO_3	252.8	5055.5	20
$CaCl_2 \cdot 2H_2O$	44.1	441.0	10
$MgSO_4 \cdot 7H_2O$	49.3	493.0	10
KH_2PO_4	27.2	272.0	10
Na_2EDTA	1.12	11.2	10
+ $FeSO_4 \cdot 7H_2O$	0.83	8.3	
NH_4Cl	53.5	535.0	10
Micronutrients	See below	--	10
Vitamins	See below	--	1

Micronutrients.

Ingredient	Stock Solution (mg/l)	Final Conc. (mg/l)
H_3BO_3	620	6.2
$MnSO_4 \cdot H_2O$	1690	16.9
$ZnSO_4 \cdot 7H_2O$	860	8.6
$Na_2MoO_4 \cdot 2H_2O$	24.2	0.242
$CuSO_4 \cdot 5H_2O$	2.5	0.025
$CoCl_2 \cdot 6H_2O$	2.5	0.015
KI	83	8.3

Vitamins.

Ingredient	Stock Solution (mg/l)
Nicotinamide	25
Pyridoxin HCl	41
Thiamin HCl	67.5

Phytohormones:

Indole Acetic Acid (IAA) - 1 µM

Gibberellic Acid (GA_3) - 10 µM

Stock solutions:

1. 17.5 mg IAA in 1 ml of DMSO (0.1 M).
2. 34.6 mg GA_3 in 0.9 ml of DMSO (0.1 M).
3. Add 0.1 ml of IAA stock to GA_3 stock - final conc. = IAA 0.01 M, GA_3 0.1 M.
4. Store at -20° C.
5. Add 1 µl of IAA + GA_3 stock/10 ml of medium.

Add sucrose to 4% (40 g/l), adjust pH to 5.3 with 1N KOH.

Autoclave medium, cool and add phytohormones.

CULTURE PROCEDURES

1. Cotton ovaries are surface sterilized by dipping in 70% ethanol and flaming.
2. Ovules are aseptically removed from ovaries using sterile needles and floated on the surface of the medium.
3. Ovules are cultured at 32° C in 5% CO_2 in the dark with high humidity.

Section 13

QUANTITATION OF FIBER GROWTH BY STAINING

The procedure of Beasley and Ting (3) was modified to allow measurement of fiber growth on individual ovules.

Stock Solutions and Buffers:

A. 4.2% citric acid (monohydrate) in ddH_2O.
B. 5.9% Na citrate in ddH_2O.
C. Stain Solution.
    1. Combine 27.5 ml of A and 22.5 ml of B to prepare 0.2 M citric acid buffer.
    2. Adjust pH to 4.5.
    3. Add 0.2 g Toluidine Blue O.
    4. Add 2.21 g Na_2HPO_4.
    5. Bring to 1 liter with ddH_2O.
    6. Add 1 ml Triton X-100.

Procedure:

1. Remove ovules from culture and place in a petri dish or breaker. Add enough stain solution to cover all ovules.
2. Stain for 5 min with occasional swirling.
3. Remove stain and rinse ovules with ddH_2O.
4. Remove ddH_2O and repeat rinse.
5. Remove ddH_2O.
6. Place individual ovules in separate test tubes and add 50% ethanol to destain.
    a. For small ovules and ovules that do not take up much stain use 1 ml of 50% ethanol.
    b. For medium sized ovules use 2.5 ml.
    c. For large ovules use 5.0 ml.

    The destain solution becomes saturated at about 2.5 A_{640} units, hence the need to use different destaining volumes for different sized ovules. Adjust absorbance readings by an appropriate factor to make data comparable.
7. Destain ovules for at least 1 hr with shaking.
8. Decant solution into cuvettes and read absorbance at 640 nm.

VITA

Duane A. Graves was born in Memphis, Tennessee on December 8, 1957, and was reared in Grand Junction, Tennessee. He received his high school diploma from Fayette Academy in May, 1975, and entered The University of Tennessee, Martin the following September. In June, 1979, he received the Bachelor of Science degree in biology and assumed the position of research assistant in plant and soil science at Ames Plantation.

After one year at Ames Plantation, the author accepted a graduate teaching assistantship at The University of Tennessee, Knoxville, and entered the Master of Science program in September, 1980. He was awarded a Master's degree in Microbiology in June, 1983.

In September, 1983, the author accepted a predoctoral research assistantship and began work toward the Doctor of Philosophy degree in Life Sciences with specialization in Plant Physiology and Genetics. He was awarded the Ph.D. degree in August, 1986.