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**Control of Fiber Differentiation form Cotton Ovule
Epidermis: An *In Vitro* Study**

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ABSTRACT

The distribution of fuzz and lint fibers on the surface of cotton ovules is genetically determined. Theoretically, with the enlargement of the ovule after fertilization, room for additional fibers is available. Our assumption has been that previous in vitro studies concerning fiber development focused on events occurring on or after the day of anthesis and because of this the cell elongation stage of fiber development was studied. The aim of this study was to gain an understanding of the fiber initiation process by investigation of pre-anthesis stages in ovule development, in vivo and in vitro. Using surface microscopical observations, and in vitro manipulations, the approximate time fiber initiation occurred was determined. The pattern of in situ fiber initiation of the ovule surface was analyzed for pattern and cell lineage. The interaction of IAA and GA on fiber elongation was studied in a naked-seeded variety. The effects of exogenously added plant growth substances on ovules isolated during different stages of development was studied in vitro. The natural pattern of ovule development within the ovary was determined as was the pattern of the endogenous levels of ABA within developing ovules. Production of fiber-like cells from internal tissues of ovules has been achieved in vitro.

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INTRODUCTION

The distribution of fuzz and lint fibers on the surface of cotton ovules is genetically determined. Theoretically, with enlargement of the ovule after fertilization, room for additional fibers is available. An understanding of the initiation process as well as the controlling factors in determination of lint and fuzz fibers could be important in developing the production of more commercially useable fibers per ovule.

Extensive research by Beasley and colleagues (1973a, 1973b, 1974, 1977) defined the in vitro parameters for fiber development from isolated cotton ovules. Our hypothesis has been that differentiation of the cotton fiber cell from the ovule epidermal layer involves at least two phases: initiation and elongation. In the view of this hypothesis, the medium developed for fiber growth on cotton ovules by Beasley and Ting (1974) was designed to influence the elongation phase of development. Initiated fiber cells are present on ovules the day of anthesis (da=0). Because this stage (d=0) was used exclusively in the development of the media to promote fiber growth, promotion of elongation was studied.

Previous in vitro attempts to induce fibers from pre-anthesis ovules (Beasley & Ting, 1973a; Hsu & Stewart, 1977) were not successful and, probably because of this, the parameters tested were not reported.

The aim of this study was to gain a better understanding of the initiation process by investigation of pre-anthesis stages in ovule development. The outline of the work was to: examine the pattern of fiber initiation on the ovule surface using microscopical observations of the ovule surface; determine the time(s) of fiber initiation using microscopical observations and in vitro culture of pre-anthesis ovules; attempt to force premature fiber initiation on pre-anthesis ovules by manipulation of the culture media; study an Acala naked-seeded relative in vitro to determine controlling elements in suppression of fiber initiation; and characterize some physiological events occurring in ovules during the time of initiation, i.e., extant proteins and endogenous levels of the plant growth regulators (PGRs).

Summary of Major Findings

(1) The pattern of in situ fiber initiation on the ovule surface appears to be random although a cell lineage may be involved.

(2) From in vitro trials of pre-anthesis ovules, fiber initiation appears first in the chalazal region of ovules, located in the base of individual ovaries, 4 days prior to anthesis (da-4) and does not appear in ovules in the stylar region of the ovary until 3 days prior to anthesis (da-3) or da-2. A major change in proteins appears da-3 and da-2 for the majority of ovules within an ovary, but probably occurs earlier in basal ovules. By da-1 most of the fiber cells on all ovule surfaces within an ovary have initiated.

(4) Endogenous levels of ABA in ovules, appear to follow the same pattern as fiber development, increasing with fiber development and elongation. This aspect raises questions as to the role of ABA in fiber initiation, elongation and in the later pattern of lint/fuzz fiber development, as well as the naked-seeded condition.

(5) Endogenous cytokinin may be high relative to IAA, up to da-1. This was estimated from the ability of functional chloroplasts to develop in the epidermal layer in response to blue light. Measurement of endogenous cytokinins are planned. In vitro, 24-hour-pulse trials using pre-anthesis ovules, suggested that cytokinins promoted fiber development in da-4 and da-3 stages.

(6) Endogenous levels of IAA appear to be low during the initiation period, increasing only slightly by da-1 and da=0.

(7) Masses of elongated fiber-like cells formed from internal ovular tissues, da+10 through da+14, on a medium containing a high concentration of cytokinin relative to auxin and including ABA and GA. These very preliminary results suggest that cytokinin and ABA, as well as IAA and GA, may also be involved in fiber initiation.

METHODS

A. Determination of the Sequence of Initiation In Vitro From the Culture of Pre-anthesis Ovules

Plants of G. hirsutum var. Acala SJ-2 were greenhouse grown according to the procedure of Beasley (1974). Approximately 50 plants were started every 2 weeks. Plants were retained for 8 months. At any given time, the greenhouse contained approximately 500 plants. Daytime temperature was maintained between 98 and 90°C; night temperatures maintained between 75 and 70°C. Ovules harvested from pre-anthesis ovaries were cultured according to Beasley & Ting (1974). Ovary stage was estimated from measurements of bud length and ovary length and correlation of these measurements with data from tagged buds. For individual experiments, flower buds were harvested from 6-month-old plants of the same planting date. Ovules were isolated and cultured according to the method of Beasley & Ting (1973b, 1974). The growth regulator composition of the medium was that of Beasley & Egli (1977). Two g/l Gel-rite (Kelco, San Diego) was used to solidify media, when semisolid media was employed. For culture of ovule halves, ovules of different pre-anthesis stages were bisected at right angles to the longitudinal axis of the ovule, roughly separating chalazal and micropylar regions and cultured. Cultures were evaluated 2 weeks after culture. Fiber length was minimal on the majority of these ovules, therefore, ovules were evaluated for presence or absence of fibers only.

B. Differential Response of Ovules to Light

Ovules in various pre-anthesis stages were cultured in media which included or excluded the B&T formulation of plant growth regulators. Cultures were maintained at 27°C, under low intensity, continuous, fluorescent or blue light.

C. In Vitro Trials of Plant Growth Regulators

Ovules from individual pre-anthesis ovaries, were divided between a control culture containing regular B&T medium and a test culture containing various levels of GA, IAA, kinetin or ABA. The growth regulators were tested singly, in media containing the standard levels of those growth regulators not being tested.

D. Naked-Seeded and Conditional-Fiberless Mutant

The Acala naked-seeded variety was cultured on a basal B&T medium, and the concentration of IAA and GA adjusted to allow fiber production in vitro. The temperature-sensitive, conditional-fiberless mutant, SMA4, reported by Beasley & Egli (1977), would have been an excellent subject for growth regulator analysis due to the conditional nature of fiber production; however, seed for this variety could not be located until only recently (2/87). We are obtaining this seed to determine if the variety still carries the temperature-sensitive characteristics.

E. Production of Fiber-like Cells from Culture of Post-Anthesis Ovules

Fibers were removed from ovules 14 days post-anthesis. The ovules were dissected into chalazal and micropylar sections and cultured on a medium containing 1 mg/l 2iP, 0.1 mg/l IAA and containing GA or ABA or both GA and ABA.

F. Proteins

Two-dimensional polyacrylamide gel electrophoresis of proteins (O'Farrell, 1975) extracted from pre-anthesis ovules.

G. Measurement of Endogenous ABA, IAA, and Cytokinin

ABA was measured in intact ovules da-4 through da+2; in chalazal and micropylar halves and in apical and basal ovules in pre-anthesis ovaries. Tissue was extracted in 80% methanol containing 10 mg/100 ml BHT and processed according to Weiler (1984). ABA was measured using polyclonal antibodies raised by M. Harris of this laboratory (Harris and Dugger, 1986) in a solid-phase enzyme-immunoassay, ELISA, according to the procedure of Weiler (1982a) and later using a radio-immunoassay, RIA (procedure developed at Monsanto, communicated by E. Bray), employing monoclonal antibodies developed by Wieler, a gift of E. Bray. IAA was measured in intact ovules da-4 through da+2 using an enzyme-linked solid-phase immunoassay, ELISA, supplied by IDTECK (San Bruno CA). Tissue was prepared according to Weiler (1982b).

RESULTS AND DISCUSSION

A. Culture of Pre-Anthesis Ovules

1. The Time of Fiber Initiation. By repeating previous work involving culture of pre-anthesis ovules (Beasley and Ting, 1973), we have concluded that the stage in ovule development when fibers will elongate from ovules cultured in a modified Beasley and Ting (B&T) medium occurs between 3 and 4 days before anthesis (da-3 and da-4); however this timing appears to be variable and can be

advanced or delayed with changes in environmental conditions and water status of the plant. Fibers cannot be forced from the majority of da-4 ovules, using combinations of IAA and GA. From this data, it appears that fiber cells begin to differentiate from the epidermis approximately 4 days before anthesis. From the culture of pre-anthesis ovules, the pattern or gradient of fiber development on the ovule surface and within the ovary also became observable (Fig 1.). The vagueness in the estimation of the time of fiber initiation from this data reflects a later discovered gradient of ovule development within individual ovaries. This is discussed in section C, below, concerning the gradient of fiber development within ovaries.

2. Sequence of Fiber Initiation on Individual Ovules. The day of anthesis, da=0, fiber initials can be seen in the chalazal region of ovules, but not in the micropylar region (Stewart, 1973). When these ovules are cultured in B&T medium, fibers elongate rapidly from both regions. The day before anthesis, da-1, fiber initials cannot be seen in either region of the ovule surface, however, fiber cells will rapidly elongate in both chalazal and micropylar regions in response to culture in B&T medium. Fibers develop only in the chalazal region of most da-2 and some da-3 stages of ovules cultured in B&T medium.

The PGRs, IAA and GA, appear to have no effect on the epidermal cells in the micropylar region, da-2, da-3, or entire ovules of da-4 and da-5 stages. The response to this treatment is division and elongation of cells beneath stomata in the cortical layer, producing outgrowths of tissue. The epidermal cells in these regions, continue to divide covering the increased surface caused by the outgrowths (Fig. 2).

Therefore, the levels of IAA and GA in B&T medium have different effects on ovular tissue depending on the developmental state of the epidermal cells. The ability for epidermal cells to elongate as fibers, in response to IAA and GA, is acquired in the chalazal region between 3 and 4 days pre-anthesis (da-3 and da-4). Fibers will elongate in this region; however, cortical outgrowths occur in the micropylar region. This differential effect can also be observed in bisected da-2 ovules in culture, the chalazal half producing fibers and the micropylar region producing cortical outgrowths. This graphically demonstrates the different states of epidermal cell differentiation on the surface of a single ovule. Furthermore, absolute fiber elongation in B&T medium increases as anthesis approaches, suggesting increased availability of RNA, protein, and/or substrate after the initiation event(s).

In summary, the results from this series of experiments indicate: (1) Da=0, although fiber initials are not visible in the micropylar region of the ovule surface, fiber cells have initiated. (2) Da-1, fiber cells in both regions, although not visible on the ovule surface, have initiated, as evidenced by the rapid production of fibers in response to culture. (3) Da-2, cells in the chalazal regions of ovules will elongate in culture, indicating that only the cells in this region have initiated, while those in the micropylar region have not. Therefore, the pattern of fiber initials, observed da=0, probably reflects the pattern of initiation which occurred two to three days earlier.

3. Sequence of Fiber Initiation within the Ovary. Superimposed on the gradient of fiber initiation on the epidermis of individual ovules, is a gradient of development within an ovary from the base of the ovary to the apex. This phenomena was suggested previously as a percentage of pre-anthesis ovules which would not form fibers in culture (Beasley & Ting, 1973a). Upon culture of pre-anthesis ovules on solidified B&T medium to retain the relative positions of ovules within an ovary, it was observed that ovules isolated from the basal regions of ovaries produced fibers in culture 1 to 2 days earlier than ovules isolated from the apical, or stylar region (Fig. 3). Therefore, within a single da-2 or da-3 stage ovary, ovules in many phases of epidermal cell development are present.

4. Observations, Epidermal Cell Differentiation, and Ovule Vasculature. From observations made during the in vitro trials of pre-anthesis ovules, we believe that the gradient of fiber elongation within the ovary and along the axis of individual ovules, described above, must be influenced by the developing vascular system of the ovary and ovule, respectively. This view suggests that growth regulators which promote initiation, are transported into the ovule by the vascular system, or are synthesized by tissues associated with the vascular system. In above-ground organs of cotton, epidermal trichomes are associated with vascular tissue, suggesting a correlation of epidermal cell differentiation with vascular tissues throughout the plant.

The vascular system in an ovule is uncomplex. Vessel elements can be traced from the raphis, analogous to the petiole of a leaf, into the chalazal region of the ovule, and then toward the micropylar region by da+5. The developing bundles lie just beneath the epidermis and do not penetrate into the ovule. This arrangement is typical of ovular vasculature in general (Bouman, 1984). The extent of vascular development at the time of fiber initiation, da-4 through da-2, is not known and transport may occur primarily via the symplast from the raphis. Although the micropylar region of the ovule appears to be closest to placental attachment, due to the recurved, or anatropus configuration of the ovule, the micropylar region is actually the most distant from this vascular connection. Therefore, as in the developing ovary, fiber development on the ovule surface is from the basal or chalazal region, to the apical, micropylar region.

If the developing cotton ovule is a sink for photosynthate and growth regulators, as ovules and seeds of other species appear to be (Brenner, 1981), these materials transported into the ovary will encounter the basal ovules before those in the apical region. Within individual ovules, these materials will encounter the epidermal and cortical layers of the chalazal region before diffusing to the bulk of the ovule and the micropylar region. Because of the chalazal to micropylar development of the vascular system, it is expected that chalazal and micropylar halves will contain different concentrations of these regulators. The gradient of fiber elongation occurring in vivo could be attributed to a gradient of photosynthate; however, the gradient along the ovule is also expressed in vitro where IAA, GA, sugars, vitamins, and inorganic nutrients are not limiting. This phenomenon also suggests the involvement of plant growth regulators other than IAA or GA.

5. Role of Developing Egg Sac. A role for the developing egg sac can be proposed to account for the gradient of fiber-forming ability on the ovule surface, specifically, the retardation of fiber development in the micropylar

region. Upon air drying of free-hand sections of ovules, the outer and inner integuments readily separate from each other at the interface of the internal epidermal layers. This suggests little symplastic connection between these cells. The tissue remains joined in the chalazal region. A suberized layer may exist between these epidermal layers of the integuments (visualized with Auramine O and fluorescence microscopy as a thin band of yellow fluorescence). Because of this arrangement, it is suspected that the majority of transport, from the vascular system to the developing egg sac occurs via the chalazal region. Putative PGRs synthesized by the egg sac may also be carried through the chalazal connection. These would be promotive of fiber development because fiber development occurs first in the chalazal region. Putative PGRs carried laterally, across the nucellar layers from the egg sac to the micropylar epidermal layer by the symplast or apoplast, would be proposed to be inhibitory to fiber development. Our observations suggest that this possibility may be unlikely.

Although egg sac development in cotton has been described by Pollack & Jensen (1964) the day-to-day sequence of egg sac development was not reported.

B. Differential Response of Ovules to Light

We have observed a change in the effect of light on plastid development in the epidermal layer which is coincident with elongation of epidermal cells in response to IAA and GA in B&T medium. Cotton ovules are normally white and do not contain mature chloroplasts. However, ovules harvested from da-2 and younger ovaries developed chloroplasts when cultured in B&T medium without added PGRs and exposed to blue light (Fig. 4). Ovules from da=0 and da-1 ovaries did not. Light does not appear to alter the time or the pattern of fiber elongation on ovules cultured on complete B&T medium. This loss of ability to 'green' suggests a decrease in the endogenous levels of cytokinin, which promotes chloroplast development, or more likely, an increase in endogenous IAA, which retards chloroplast development (Horne and Widholm, 1985). This apparent change in endogenous levels of IAA relative to cytokinins, da-1 and da=0, coincides with enlargement of initiated fiber cells in the chalazal region. This information also suggests a higher proportion of cytokinins relative to auxin during the period of fiber initiation.

C. In Vitro Trials of Plant Growth Regulators

(1) In vitro trials of pre-anthesis ovules, da-5, da-4, da-3, da-2, cultured from 1 to 2 weeks on B&T medium supplemented with all possible combinations of IAA & GA did not enhance or influence fiber initiation.

(2) Upon evaluation of these results, a different approach was taken. Pre-anthesis ovules were cultured on pulse treatments of IAA, GA, ABA and kinetin which were tested singly, not in combination, in a basal B&T medium. The pulse treatment lasted from 24 to 48 hrs and, if effective, should 'induce' initiation. These treatments were followed by reculture onto regular B&T medium to allow elongation of initiated cells.

Results from this series of experiments indicated that only pulse treatments of kinetin, appeared to enhance fiber production in any way, i.e., more ovules per ovary produced fibers in culture. Interestingly, this effect was age and concentration dependent. For example, kinetin pulse treatments affected fiber

production in da-4 and da-5 ovules only; the optimum kinetin concentration in da-4 ovules was $0.1 \mu\text{M}$, that for da-5 ovules was $0.5 \mu\text{M}$ (Fig. 5). However, these results were not dramatic, increases were slight and, in general, only chalazal portions of ovules produced fibers. Results were variable from one trial to the next indicating that the system was not optimized and that some other factor(s) were involved.

Difficulties with trials involving pre-anthesis ovules include estimation of developmental age by bud and ovary size. To circumvent this problem, the contents of one ovary were divided between a control, consisting of regular B&T medium, and a single-pulse treatment. These results were reported at the 1986 annual meeting of the American Association of Plant Physiologists.

D. Assays for Endogenous Levels of Plant Growth Regulators

1. ABA. Ovules from da-2 ovaries will develop fibers only in the chalazal region in culture. Observations presented above suggest the existence of initiation promotor(s) which are transported into the developing ovule. However, a role for an inhibitor in the micropylar region, possibly synthesized by the egg sac, can also be proposed to account for the gradient on the ovule. ABA was reported to inhibit fiber elongation in vitro (Beasley and Ting, 1973b, 1974; Dindsa, et al., 1977), and ABA is critical in later embryo development in vivo (Ihule and Dure, 1972).

In our hands, these preliminary studies revealed reproducible differences in the endogenous levels of ABA between samples; however, the absolute amount of ABA in these samples is still uncertain at this point.

(a) Changes in ABA Concentration with Development. ABA concentrations in intact pre-anthesis ovules increased from a low level (for example: 0.5 ng/mg tissue fresh weight) in da-3 ovules, to 1.0 ng/mg da=0 to a higher level (3 to 10 ng/mg) in da+2 ovules (Fig. 6), and appeared to increase exponentially thereafter. This increase in endogenous ABA coincides with fertilization and subsequent embryo development. The increase also occurs during a time of rapid fiber elongation. Therefore, fiber elongation may not be as sensitive to ABA as was previously suspected (Beasley & Ting, 1974; Dindsa et al, 1977). Endogenous levels of IAA and GA may increase during this time, allowing fiber elongation to continue (also see 'Naked Seeded Variety' section).

(b) Interaction of ABA with GA and IAA. The possibility that increased IAA and GA concentrations could overcome an increased level of ABA, da=0 ovules were cultured in 1 and $5 \mu\text{M}$ ABA with 1, 3, 10, 30, 50 and 100X. IAA & GA of the B&T formulation. In general, the 50 & 100X treatments were toxic; however, the 10X and 30X treatments improved fiber growth over 1X controls. Higher concentrations of ABA will be tested. It appears that increased IAA and GA can overcome the elongation retarding effects of low concentrations of ABA (Table 1).

It is interesting to speculate that increasing concentrations of ABA within the developing seed coat, da+2 and later, may account for the shorter 'fuzz' fibers which initiate 3 to 8 days after fertilization. The fact that these secondary waves of fiber initiation occur despite increasing levels of ABA in the seed, argues that the initiation may not be inhibited by moderate levels of ABA.

(c) Basal and Apical Ovules. ABA was measured in basal and apical ovules within ovaries of da-1, da-2, da-3, and da-4 stages. ABA concentrations were higher in ovules developing at the base of ovaries until da-1 when the concentration of ABA in apical and basal ovules appeared to be the same (Fig. 7). This association of ABA with the gradient of fiber-forming ability, indicates that low amounts of ABA apparently do not inhibit fiber initiation and does not rule out the possibility that ABA may be involved in fiber initiation.

(d) Chalazal and Micropylar Ovule Halves. ABA was measured in chalazal and micropylar halves of ovules harvested da-3, da-2, da-1, da=0, da+1. Concentrations of ABA appear to be equal in both halves; however, these measurements were taken from all the ovules in individual ovaries (Table 2). These measurements are difficult to assess because of the small size of the samples (6 to 10 mg tissue) which increases the error in estimation of fresh weight. Measurements from chalazal and micropylar halves of only basal ovules will be performed in future assays. Because the ABA concentration is apparently higher in ovules taken from the base of ovaries than those from the apical regions, we anticipated that ABA will be higher in the chalazal halves of ovules and lower in the micropylar regions. However, we have not been able to get unequivocal results for this possibility. The ABA concentration in the epidermal layer itself was not measured.

(e) Discussion-ABA. ABA appears to increase in developing ovules along with fiber initiation. ABA also increases dramatically in developing seeds during the time of rapid fiber elongation. This situation raises several questions concerning the role of ABA in fiber initiation and in later fiber development. Is there a relationship between the ABA content in the seed coat and the formation of fuzz as opposed to lint fibers? Is the genetically determined pattern of lint to fuzz based on a pattern of ABA levels or tissue sensitivities to ABA? Can ABA be involved in the change from primary to secondary wall mode of metabolism, induction of cellulose synthesis, or the senescence of the mature cotton fiber? Is there a relationship between the fiberless state and ABA content of the seed coat or is this an interaction of another growth regulator such as IAA?

2. IAA. Preliminary results suggest very low levels of IAA in ovular tissue until da-1 and that IAA levels increase dramatically after fertilization as predicted by Beasley. These results are preliminary only and are based on the loss of greening ability of the epidermal layer and on loss of IAA dependency after fertilization. The IAA assays that were completed also indicated low IAA in pre-anthesis tissues. The measurements concerning IAA will have to be continued with the aid of an established investigator in the field. Our estimation at this time; however, is that IAA is not as critical in the fiber initiation process as other PGRs.

E. Proteins

Distinct changes occurring between da-3 stage, when fibers will not elongate in culture, and da-2 stage, when fibers will elongate in the chalazal region. This data, indicating an upstream event occurring da-4 or da-5, supports the view that a major change, possibly involving fiber initiation, occurs in the ovules between da-3 and da-2. This data was generated from all ovules within single ovaries and, therefore, represents a collection of all the developmental stages within the ovary. Study of initiation specific ovule proteins

and possible antibody production will be continued only after the plant growth regulator associations have been well characterized.

F. Naked-Seeded Variety and Conditional Fiberless Mutant.

1. We have attempted to locate seeds of the temperature-sensitive, conditional-fiberless mutant SMA 4, of *G. aboreum* studied by Beasley and Egli (1977); however, only very recently (2/87) have located material bearing this identification. Under the conditions reported by Beasley (1977), this mutant was fiberless when cultured on B&T medium at 28°C, but produced fibers when cultured at 38°C.

2. The Mexican Naked-Seeded variety of Acala normally produces lint fibers only in the chalazal region of the ovule; it does not produce the short fuzz fibers. In vitro trials using ovules of this variety were performed to determine the exogenous PGR requirements for fiber elongation. Trials using the da=0 stage of this variety showed that high, but apparently non-toxic, concentrations of GA (30 mg/l) and IAA (100 mg/l) were required to allow elongation of fibers in the chalazal region, producing essentially the same pattern of fiber production in vitro as in vivo (Table 3). Because of the high levels of IAA and GA required for in vitro fiber elongation, it is suspected that this variety contains high levels of ABA or other inhibitory compounds, which produce the naked-seeded condition. However, fiber elongation as well as initiation may be affected in this variety, making it difficult to evaluate in vitro, without a prior investigation into the ABA content of the ovule.

As mentioned above, experiments involving the regular Acala SJ-2 variety, indicated that increased levels of IAA and GA in the culture medium can overcome the inhibition of fiber elongation caused by low to moderate levels of ABA.

G. Production of Fiber-Like Cells from Culture of Post-Anthesis Ovules

Because of our interest in the 'waves' of initiation of fuzz fibers which occur on the ovule surface after anthesis, a preliminary test using da+10 to da+14 ovules and ovule slices was performed. The basal media tested was 'high' in cytokinin relative to auxin. This combination was tested because of previous results with pre-anthesis ovules discussed above. Also tested were GA and ABA. When GA or ABA were absent from the medium, the tissue turned reddish-brown and did not grow. On media containing either GA or ABA, elongated, fiber-like cells 'exploded' from the interior of the ovule within a week of culture. Elongated cells on GA treatments were septate or formed chains of cells due to enhanced cell division. Cells on the ABA treatment were considerably longer than those on the GA treatment and more fiber-like. These experiments have been repeated with the same result. When these tissues were recultured onto regular B&T medium, to enhance elongation, these fiber-like cells did elongate but not to the extent expected (Figs. 8a and b).

The origin of the elongated cells appears to be almost all internal ovule cell types: the cortex of the outer integument, the epidermal layer of the integuments, even cells of the original epidermal layer which were not previously fiber cells. The origin of elongated cells may be treatment dependent, for example: with ABA, the cells appear to elongate from the epidermal layers of the integuments; with GA treatment, elongation and division appears to occur in the cells within the integuments.

These results have just been recently obtained and have not been optimized with respect to the PGR levels. The results suggest, as do other results described above, that a cytokinin and ABA interaction may be involved along with IAA and GA in fiber initiation in cotton. What is also unknown at this time are the endogenous levels of IAA, GA, cytokinin and ABA in this post-anthesis tissue, and how this relates to the normal initiation process which occurs many days before anthesis.

The tentative organization of topics of future publications concerning this research is: the pattern of fiber initiation on the ovule surface and within the developing ovary based on microscopical analysis and culture of pre-anthesis ovules; the estimation of the time of fiber initiation and in vitro trials of exogenously supplied PGRs using pre-anthesis ovules; reports on analysis of endogenous ABA and the effect of exogenous ABA on these tissues in vitro.

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Table 1. Effect of IAA & GA on ABA-induced inhibition of cotton fiber elongation in vitro. Kinetin maintained at 0.5 μ M. *G. hirsutum*, var. Acala SJ-2. Data expressed as visual scoring and approximate fresh weight of fibers from 20 ovules in mg.

	1x 5:0.5	3x 15:1.5	10x 50:5	30x 150:15	50x 250:25	100x B&T 500:50
[ABA]						
0 μ M	+++	++++	+++	++		
	297	352	275	84		
[ABA]						
1 μ M	++	++	+++	++++		+
	193	211	271	424		90
[ABA]						
5 μ M	+	++	+++	+++	++	
	90	150	225	256	137	

Table 2. Estimations of ABA in chalazal and micropylar regions of cotton ovules, harvested 3, 2, and 1 day prior to anthesis (d-3, d-2, d-1); the day of anthesis (d=0), and 1 day after anthesis (d+1). *G. hirsutum*, var. Acala SJ-2.

	[ABA] in ng/mg fresh weight tissue*				
	D-3	D-2	D-1	D=0	D+1
Expt. 1					
Chalazal	0.66	0.50			
Micropyle	0.56	0.51			
Expt. 2					
Chalazal		6.0*	7.2*	8.0*	6.0
Micropyle		6.0*	7.6*	7.5*	6.2
Expt. 3					
Chalazal			3.3	4.5	8.0
Micropyle			4.2	4.7	8.0
Expt. 4					
Chalazal			6.5	7.5	9.0
Micropyle			8.5	7.0	8.0
Expt. 5					
Chalazal			6.5	8.0	
Micropyle			8.0	8.5	
Expt. 6					
Chalazal	1.72		5.2		
Micropyle	1.65		4.0		

* Value are: ng/mg tissue dry weight.

Table 3. Determination of the optimum concentrations of IAA and GA for normal pattern of fiber elongation in vitro. G. hirsutum var. Acala, naked-seeded. Values represent percent (%) ovules producing fibers*.

Expt. 1&2		[IAA in mg/l]			
[GA] mg/l	0	1	3	10	
0.3	0	11.5(+.03)	16.0(+.05)	18.3(+.10)	%
1.0				18.0(+.02)	
3.0				31.3(+.06)	
10.0				45.9(+.05)	
30.0				10.1(+.03)	
Expt. 3 IAA = 5		10	15	30 mg/l	
[GA]=10 mg/l					
	11.1(+.01)	20.3(+.08)	21.1(+.07)	46.7(+.1)	%
Expt. 4 IAA = 20		30	40	50 mg/l	
[GA]=15 mg/l					
	26.4(+.1)	24.1(+.05)	30.0(+.07)	61.0(+.03)	
Expt. 5 IAA = 20		25	30	40	50 mg/l
[GA]=20 mg/l					
	36.5(+.06)	22.0(+.06)	33.6(+.2)	31.2(+.1)	17.3(+.06)

FIGURE LEGENDS

Figure 1a. The effect of B&T medium on da-3 & da-4 ovules. Ovules isolated from ovaries of different maturity and cultured on Beasley and Ting medium. Clockwise from left: Motes, or aborted ovules, from da-3 ovary (note fiber proliferation); upper culture, normal ovules isolated from same da-3 ovary as motes (note fibers limited to tip of chalazal region on two ovules only); lower right, ovules from an ovary da-2 stage (notice the chalazal region of some ovules covered with fibers).

Figure 1b. Representation of relationship between floral bud length and ovary length to developmental stage of the ovary. Error bars indicate standard deviation.

Figure 2. Maturity differences and tissue response. Ovules from two different ovaries of different maturity (upper group, da-2 ovary; lower group, da-3 ovary), cultured on Beasley and Ting medium for 2 weeks. Note outgrowths on ovules of the lower group, fibers as well as outgrowths on ovules of the upper group.

Figure 3. Positional differences. Ovules isolated from a single da-2 ovary, cultured on solidified Beasley and Ting medium for 2 weeks. Ovules arranged on the culture medium to reflect the original position of each ovule within the ovary. The top of the figure contains ovules from the apex of the ovary nearest to the style. Ovules at the bottom of the figure were isolated from the ovary base. Notice the size differences in the ovules and the extent of fiber production of the stylar ovules compared to the basal ovules.

Figure 4. Effect of light. Ovules on the left were harvested da-3, split into chalazal and micropylar sections and cultured on Beasley and Ting medium in blue light for 3 weeks. Note: 2 chalazal sections are dark green, the micropylar section is white, one intact ovule is green and white. The green areas also contain nongreen fibers. Ovules on the right were harvested da-1 and cultured in the dark on Beasley and Ting medium, 1 week. They were transferred to medium containing increased cytokinin and exposed to blue light for 2 weeks. Fibers formed normally and are nongreen. Green callus formed in chalazal region; however, the majority of the ovular tissue remained white.

Figure 5. Plant growth substance trials. Graphical representation of the results of in vitro trials using various treatments of GA, IAA, and kinetin with ovules from different stages in maturity, da-5 through da=0. Only in the kinetin treatments was a beneficial effect detected and only in the da-4 and da-5 stages.

Figure 6. Change in ovule ABA content with development. Representative result of analysis of the ABA content of cotton ovules from different stages in maturity, da-4 through da+2.

Figure 7. ABA content of stylar vs. basal ovules. Representative sample of analysis of the ABA content of ovules isolated from only the stylar and basal regions of an ovary. Each maturity date represents the ABA content of ovule sets from single ovaries.

Figure 8a. Fiber-like cells developing from ovule tissues. Ovule cultured on medium containing cytokinin, ABA and GA.

Figure 8b. Ovule from same ovary and cultured on the same medium as above, but lacking ABA and GA.

Research conducted in Israel

"Pattern and Density of Cotton Fibers"

INTRODUCTION

The Questions of Hair Spacing and Density

Only one of every three or four epidermal cells of the cotton ovule develops as a fiber (Stewart, 1975; Ramsey & Berlin, 1976). This raises the question of the nature of the processes that determine the difference in the fate of neighboring epidermal cells. Major possibilities are: (a) the cells of the epidermis have differentiated into two types, (b) correlative influences of the forming fibers prevent additional cells from expressing their developmental potential, and (c) there is no innate difference between the cells; the difference in their fate depending only on chance.

Not much is known concerning comparable patterning and density controls of cotton fibers. Ramsey & Berlin (1976) stated that cotton fiber differentiation from epidermal cells appeared to occur randomly. Two or more adjacent elongating fibers were often found. Careful studies concerning the cellular sequences that lead to fiber differentiation have not been done. Nor have there been studies of the relations between the differentiation events in neighboring parts of the ovule epidermis.

One approach to this question is to consider the determination of other similar specialized single cell types and to compare the development of fiber cells to the development of these cell types. There are many specialized cells, generally referred to as 'idioblasts,' that differentiate relatively late in tissue ontogeny, after the general shape of an organ has already been determined. Common examples are stomata, root hairs, and leaf sclereids. The first observable stage of most idioblasts is an unequal cell division. This division separates a small, cytoplasm-rich, meristemoid cell (Bunning, 1948). Idioblasts are formed from these cells after additional divisions and are, therefore, the products of a complex differentiation process involving a number of cells.

The idioblast distribution that has received most attention is that of the stomata (Bunning, 1948; 1965; Korn, 1972; Sachs, 1978). The distribution of stomata appears to be 'spaced.' The distances between neighboring stomata are regular and few stomata are in direct contact and departure from a random distribution has been statistically confirmed. The most commonly held hypothesis is that a forming stomata effects the immediate neighbor cells, preventing differentiation of these cells into stomata. However, observations of the development of stomata suggest a different patterning mechanism. Each stoma is formed together with its immediate neighbors as part of the same determinate process. This produces a minimal distance between neighboring stomata, and simple statistical tests show that this distance accounts for the departures of stomata distribution from the expected results of random events. This patterning mechanism does not account for the density of stomata. Different densities are possible with the same minimal distance between neighbor stomata (Marx & Sachs, 1977).

A possible approach to the question of the determination of the density of cotton fibers would be to study the distribution of fibers statistically using methods that have been found to be useful in the case of stomata. Such statistical work could indicate the relative role of controls of the distances between individual fibers as compared with direct controls of their density over relatively large, multicellular regions. It could also be useful to follow individual hair development, to search for evidence for repeatable cellular sequences. Such sequences or cell lineages could lead to minimal distances between fibers and could be factors in the determination of fiber density. Any orderly cell lineage could also be evidence of the time at which the fate of a cell to form a fiber is determined.

Statistical Studies

The distribution of fibers was studied in ovules of the Acala variety. The plants were grown in commercial fields in the coastal plain of Israel, near Petah Tikva. For microscope studies, the surfaces of ovules were copied using commercial nail polish diluted with equal amounts of ethylacetate. The nail polish that was peeled from the ovules could be kept indefinitely and could be studied directly. Replicas of the ovule surfaces were made as soon as the potential fiber cells could be observed as bulges. Copies of later stages were unclear because of interference of elongated fibers.

The statistical study employed the method developed for study of stomata distribution (Sachs, 1974, 1978; Marx & Sachs, 1977). The purpose of this study was to determine if the occurrence of a fiber was correlated with the presence of a similar fiber nearby and how this correlation changed with distance. For this purpose the distribution pattern of many microscope fields were superimposed and regular or repeated aspects were noted. In every case a fiber chosen at random was placed in the center of the field of a Reichert projection microscope. The location of all additional fibers in the vicinity of the central fiber was recorded on transparent paper. The number was counted as a function of distance from the central fiber, and the frequency for a comparable unit area was calculated.

The results of preliminary statistical studies were confusing and showed a high variability. After various attempts, it was found that it was essential to separate the fields in accordance with the total number of fibers contained. As discussed below, this fiber density probably reflected the developmental stage of the region at the time it was copied with nail polish. The statistical results are presented in the following table:

FIBER FREQUENCY AS A FUNCTION OF DISTANCE FROM A CENTRAL FIBER, CHOSEN AT RANDOM

Distance from center, μm	0.0- 6.1	6.1- 12.2	12.2- 18.3	18.3- 24.4	24.4- 30.5	30.5- 36.6	36.6- 42.7
Low density 9-21	100	6	41	33	32	34	34
Medium density 21-25	100	28	55	49	45	46	51
High density 27-38	100	47	67	64	62	61	63

Numbers of hairs, $\times 100$, in a unit area of $117 \mu\text{m}^2$ within the distances indicated. Therefore, they can be considered percent chances of finding a fiber within the area indicated. The numbers are averages taken from 33 fields for every density group, the area of each field being $5725 \mu\text{m}^2$.

The results suggest the following conclusions: (a) Between 6 and $12 \mu\text{m}$, there was a significant reduction in the chance of the occurrence of an additional fiber. The presence of a fiber is thus correlated with the presence of a region that is relatively fiber free in its immediate vicinity. This correlation decreases as fiber density increases, an increase which presumably reflects the maturation of the epidermis. The meaning of this concerning hair pattern and density is considered further below. (b) Up to $6 \mu\text{m}$, there was always one fiber within the region studied. This is not a result, but rather a consequence of the fact a fiber placed in the center of the field under study occupied the $117 \mu\text{m}^2$ area. (c) At distances of 12 to $18 \mu\text{m}$ from a given fiber, the frequency of additional fibers increase; these are greater than the random frequencies found at large distances and low densities and the correlation could be causal. A similar correlation was also found in studies of stomata distribution (Sachs, 1978). (d) At distances greater than $18 \mu\text{m}$, there is no significant correlation between the occurrence of a fiber and the chance of the occurrence of additional fibers. Therefore, there is no reason to consider correlative mechanisms at these distances.

Microscopy Studies of Fiber Initiation

The comparison of cotton fibers to idioblasts suggested that the pattern of fiber distribution, and possible involvement of a determinate cell lineage could have an important role in determining fiber density. Direct observation of the epidermis of living, untreated tissue using a WILD epi-illumination microscope (400X) was complemented with observations using a copy of the surface details of the ovule surface. The epidermal replicas were either supplied from California or prepared in Israel.

The appropriate time for these studies was anthesis, when the fiber cells first appear. Ovules taken from different flowers apparently in the same developmental stage were not found to be at the same stage from the point of view of fiber development. The variability might be related to the position of the ovules within the ovaries, but this possibility has not yet been studied carefully.

In immature ovules, there was a gradient of fiber density from the base towards the tip. This gradient was not present, or not obvious, when older ovules were observed. Two conclusions follow: (a) there is a wave of epidermal maturation that proceeds downward from the tip, and (b) the initiation of fibers in any given region does not occur all at once: there is a gradual increase in the density of the fibers.

An attempt was made to find repeated patterns of fiber distribution. As expected from the statistical results presented above, a spacing pattern was noticeable in immature ovules in the basal region. Once the density of fibers increased, there were many groups of two or three fibers in direct contact. The orientation of these groups was either along or at right angles to the long axis of the ovule. Much effort was spent trying to find rules that govern the occurrence of these groupings; however, no consistent rules could be determined. In immature ovules there were also patches of epidermis that were relatively

free of fibers. These were not present in mature ovules again indicating that epidermal maturation was not synchronous. A similar unsynchronous development is known for stomata (Sachs, 1978).

It is conceivable that the occurrence of fibers is influenced by other ovular structures. One such structure could be the veins and these could be recognized in living, untreated tissues with low magnification and epi-illumination. The vein pattern was on a much larger scale than that of the fibers, so it could not be an important determinant of fiber localization or distribution. Furthermore, no relation was noticed between the location or density of the fibers and veins. The only other structure observed on ovules were scattered stomata. These were formed before the fibers and surrounded by a distinct fiber-free zone. However, the number of stomata were very small and their combined effect on fiber distribution virtually negligible.

Finally, the major purpose of the anatomical studies was the search for cellular patterns that could indicate a consistent lineage leading to fiber initiation. No repeated cellular indication of future fiber formation could be found in ovules taken from flowers a day or longer from anthesis. The most common aspect of idioblast formation are unequal divisions (Bunning, 1948; 1965). Such divisions were found in the fiber forming epidermis as well. At low fiber densities, there was a repeated occurrence of fibers that appeared to be the product of two divisions, at right angles to one another. This sequence formed a complex of three cells, only one of which developed as a fiber. Such a cell lineage could have accounted for the density of the fibers on the ovules, but there was no evidence that it was required for the formation of all or even most of the fibers. It was clear that as the density of the fibers increased, there were no consistent restrictions on the lineages that led to fiber formation. As mentioned above, new fibers were often initiated from cells that were in direct contact with older fibers.

DISCUSSION, CONCLUSIONS AND RESEARCH POSSIBILITIES

The following picture of fiber initiation emerges. There appears to be no early differentiation of epidermal cells to ones that can and cannot initiate fibers. As the epidermis matures practically all its cells become competent to form hairs, this competence appearing first at the most apical parts of the ovule, extending gradually downward. The location of the first fibers might well be random, but there is some mechanism that reduces the initiation of fibers next to a fiber that is already developing. This mechanism might be partially due to a lineage leading to only one of three products of an original cell developing as a fiber. Initiation, however, continues for some time and whatever differentiation occurred in the cells that were adjacent to an existent fiber, competence could be regained. Thus, any spacing or other mechanism that influences the relative location of fibers has only a transient, relatively minor effect. The final density of the hairs is not determined by their precise location.

What could be the nature of global controls of density, ones that do not influence precise location? There are no proven cases of the operation of such controls, but the two following examples could indicate reasonable possibilities. The formation of fibers may be limited by the supply of a metabolite. Any fiber that is initiated would reduce its supply, but if this metabolite moves freely in the epidermis the effect would not be local. The total number

of fibers would then be controlled, but the precise location would be due to chance. Another possibility is that as each cell passes through the competent state, there is a certain chance of its undergoing the differentiation leading to fiber formation. The number of fibers would then be the product of the number of epidermal cells and the chance of their conversion. There need not be any active limitation of the number of fibers or their density.

What experimental or other studies could test or otherwise extend our knowledge concerning fiber differentiation? Statistical methods could be used to define the size of the area at which the number of fibers is least variable. This area would be indicative of the nature of the controls that determine final fiber density. The anatomical work is also incomplete. It would be desirable to follow the events that occur in specific regions rather than to reconstruct development on the basis of individual stages observed by destructive methods (Sachs, 1978). This could be done by epi-illumination microscopy of cultured ovules. Such direct studies of development would be especially interesting concerning the origin of the rows of fibers observed in the late stages of initiation. The development of fibers should also be compared with the development of other idioblasts of the cotton plant. Precisely timed heat or other shock treatments of developing ovules might supply information about distinct initiation processes that occur at different times. Finally, microsurgical damage of epidermal fibers would presumably lead to regenerative processes. The degree to which damaged fibers are replaced could be a test whether they interacted with the surrounding tissues before they were damaged.

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EVALUATION OF ACHIEVEMENT

1. One of the original goals of the project was to determine when cotton fiber initiation occurred. Previous research originally defined fiber initiation as occurring the day of anthesis; later research indicated that initiation occurred during the day before anthesis. Our thesis was that fiber initiation occurred much earlier. We have been able to obtain data from cultured pre-anthesis ovules which indicates that fiber initiation occurs between 5 and 4 days before anthesis.

2. One goal was to systematically examine the natural pattern of fiber initials occurring on the ovule surface in situ to determine if an underlying pattern or a cell lineage could be involved in generating the apparently random fiber initiation process. After extensive study, it appears that the pattern of initiation is indeed random; however, involvement of a cell lineage cannot be ruled out.

3. In vitro trials with the plant growth regulators focused on use of pre-anthesis material and a closely related naked-seeded variety. The trials with the growth regulators did not produce decisive data other than IAA and GA do not appear to be involved with the initiation process when tested separately or in combination. Kinetin appeared to be beneficial to fiber production when added in the early stages of development. Some other, as yet untested, combination of plant hormones may bring about initiation in uninitiated material. Research in this area did, however, allow understanding of the formation of the pattern of initiation on the ovule, occurring days before fiber initials could be detected, the gradient of development within the ovary, and the association of these related phenomena with the developing vascular system.

4. Metabolic events, such as the changing pattern of ovule proteins and/or analysis of the endogenous levels of plant growth regulators were proposed. This portion of the proposal was left vague because of the exploratory nature of the project, and it was unknown at the time of preparation what would be found in the in vitro trials. Effort was first put into determining if a change in ovule proteins could be detected prior to anthesis and if this avenue could be productive. Two-dimensional polyacrylamide gel electrophoresis was performed and differences detected; however, these differences were erratic and it was determined that this approach could be valuable after the growth regulator associations and exact pattern of development understood.

The endogenous levels of ABA in developing ovules was determined. Estimation of endogenous levels of IAA and cytokinins were based on response of tissue to light. Several preliminary analyses of endogenous levels of IAA indicate very low levels of IAA in pre-anthesis material. This information indicates that ABA may be involved in initiation as well as effecting later fiber quality. This line of research concerning ABA led to testing ABA in vitro and led to the production of fiber-like cells from ovular tissues.

5. In vitro work with the related naked-seeded variety indicated that only with greatly increased IAA and GA in the culture medium could the normal pattern of fibers be produced indicating presence of an inhibitor or an IAA and GA antagonist in the ovule which was responsible for the naked seeded condition.

DESCRIPTION OF RESULTS OF COOPERATION

The cooperation between Professor Sachs and individuals in the Department of Botany and Plant Sciences was very good.

CONTRIBUTION TO AGRICULTURE

In general, there are no direct or immediate contributions to agriculture of an exploratory project such as this. The project has established the time of fiber initiation, and it is much earlier than previously thought. The research has described the pattern of development within the developing boll which was previously unknown. The change in ABA levels with development has been described and indicates that ABA is present and can be important. The research indicates that IAA and GA, although important in fiber elongation, are not key factors in fiber initiation. Cytokinins and ABA, which were thought to be inhibitory of fiber elongation, may be important in fiber initiation. A promotive role of ABA in fiber initiation is possible, but unclear at this point; however, the role of ABA in varieties exhibiting the naked-seeded condition is a definite possibility. Also, the increasing levels of endogenous ABA within the ovule may be the agent which generates the lint and fuzz pattern of developing fibers. It is hoped that this investigation will open up research into this previously unstudied stage in ovule development and focus attention on growth regulators other than IAA and GA.

TOP
FIGURE 2

TOP
FIGURE 3

TOP

FIGURE 4

544

ABA CONTENT of COTTON OVULES

[ABA] ng/mg f.wt.

ABA CONTENT of COTTON OVULES
HARVESTED from BASAL or STYLAR REGIONS
of DEVELOPING OVARYS

TOP
FIGURE 8a

TOP
FIGURE 8b

TOP
FIGURE 8b

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