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THE EFFECT OF BRASSINOSTEROID ON COTTON FIBER DEVELOPMENT

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ABSTRACT

The current understanding of the role of phytohormones in the development of cotton fibers is derived largely from an amenable culture system in which cotton ovules, collected on the day of anthesis, are floated on liquid media. Under these conditions, supplemental auxin and gibberellin were found to promote fiber initiation and elongation. More recently, addition of low concentrations of the brassinosteroid brassinolide (BL) were also found to promote fiber elongation while a brassinosteroid biosynthesis inhibitor brassinazole2001 (Brz) inhibited fiber development.

This present results confirm that exogenous BL promotes fiber elongation while treatment with Brz inhibits it. Furthermore, treatment of cotton floral buds with Brz results in the complete absence of fiber differentiation, indicating that BR is required for fiber initiation as well as elongation. Expression of fiber genes associated with cell elongation increased in ovules treated with BL and was suppressed by Brz treatment , establishing a correlation between brassinosteroid - regulated gene expression and fiber elongation.

These results establish a clear connection between brassinosteroid and fiber development and open the door for genetic analysis of cotton development through direct modification of the brassinosteroid signal transduction pathway.

Keywords: Brassinazole , Brassinolide , Brassinosteroid , Cell elongation , Cotton fiber , *Gossypium hirsutum* , Ovule culture.

INTRODUCTION

Cotton fibers are single specialized, highly elongated cells that grow from the epidermal cells of seed integument. At maturity, these trichomes have thickened secondary cell walls that account for their economic value. Fiber initials undergo a developmental program that includes cell fate determination, initiation, elongation, specialization, and finally, programmed cell death. Fiber cells are distinguished by their ability to grow to 6 cm in length in a relatively short time and then to synthesize large amounts of cellulose in subsequent stages. The rapid, semi-synchronous growth and maturation of fiber growth makes them an amenable model system to investigate cell elongation independently of cell division. Among the factors that regulate fiber cell elongation are plant hormones. Several independents investigators reported that exogenously applied indole-3-acetic acid (IAA) and gibberellic acid (GA) enhanced the differentiation of fibers and promote their elongation, while abscisic acid (ABA) has an inhibitory effect on fiber growth (Beasly and Ting,1973). Ovule epidermal cells differentiate into fiber initials before anthesis, and initiation of fiber elongation requires exogenous auxin. While addition of brassinosteroids (BR) is not required for fiber initiation, it does promote fiber elongation (Ashcraft 1996).Brassinolide promotes cotton fiber development . Cotton ovule culture provides a useful system to

investigate the effects of plant hormones or other chemicals on fiber development (Kim and Triplett 2001). Furthermore, BR along with auxins and GA, these phytohormones play a critical role in promoting cotton fiber initiation and elongation . Application of exogenous BR to cultured ovules or intact bolls increased fiber length and over-expression of a BR-regulated xyloglucosyl endotransglycosyltransferase (XET) in transgenic cotton plants also resulted in the production of longer mature fibers. Importantly, treatment of cultured ovules with the BR biosynthesis inhibitor Brassinozole (BRZ) strongly inhibits fiber initiation and elongation indicating that BR produced by the developing ovule is required for fiber development.

This study aimed to elucidate the role of brassinosteroid in cotton fiber development ; further, the experiment has performed a more detailed analysis of the effects of these chemicals on cultured cotton ovules.

MATERIALS AND METHODS

Plant growth conditions :

Seeds of Cotton plants (*Gossypium hirsutum* cv. Coker 312) were obtained from the Experimental Station of USDA (Texas Tech University).and grown in potting in the Agricultural Experiments Department of Biological Sciences, Texas Tech University, Texas USA . Seeds were sown on March 17th 2005 using a randomized complete block design with 3 repeats. Normal cultural practices were carried out as recommended. Observations were recorded on flowering buds collected on the day of anthesis, are floated on liquid media. Under conditions of supplemental auxin and gibberellin were found to promote fiber initiation and elongation. . Flowers were tagged on the day of anthesis. Exogenous application of Brz to flowers prior to anthesis was performed according to the method of Seagull and Giavalis (2004). Daily application of 250 µl of 2.5 µM Brz (RIKEN, Saitama, Japan) tithe developing squares was started at bud initiation and continued until the day of anthesis. Where necessary, the sepals were gently pulled back so that the solution could be applied directly to the bud. Brz was dissolved in 95% ethanol to make a 10 mM stock solution. Mock treatment consisted of deionized water adjusted with the amount of ethanol equivalent to that used to dissolve the Brz.. Statistical analysis following Snedecor and Cochran (1982)

Cotton ovule culture :

Flowers were harvested 1 d post-anthesis (DPA), and ovaries were surface sterilized by using 75% ethanol. Ovules were carefully dissected from the ovaries under sterile conditions, and immediately floated on liquid media supplemented with 5 µM NAA and 0.5 µM GA3 in 50 ml flasks (Beasley and Ting, 1973 and Ashcraft, 1996). The ovules were incubated at 34°C in the dark without agitation. Preliminary experiments indicated that addition of 0.1 µM Brassinolide (BL) (Sigma Chemical Co., St. Louis, MO, USA) and 10 µM Brz, along with standard concentrations of NAA and gibberellin, provided optimal effects. BL was dissolved in 95% ethanol to make a 1 mM stock solution. BL and Brz were added to the liquid media at the same time. Similar

levels of ethanol were used for the control treatment. Fiber length for all developed ovules was measured after 14 d of incubation. The cultured ovules were soaked in 95°C water for 5 min to relax the fibers. The fibers were then spread and measured with a ruler.

Light microscopy :

The cultured cotton ovule samples were fixed in FAA for 48 h, and dehydrated in an ethanol series (60, 70, 85 and 95%). The ovules were then embedded in Paraplast (Sigma Chemical Co., St. Louis, MO, USA). Tissue sections (15 µm thick) were cut with a Reichert- Jung 2050 microtome, mounted and stained with Safranine O and Light Green, and examined with a Zeiss Axiophot light microscope (LM) .

Scanning electron microscopy :

SEM of randomly selected cultured cotton ovules under different treatments at 3 DPA was performed according to Craig and Beaton (1996). Samples were fixed in 3% glutaraldehyde followed by postfixation in 1% osmium tetroxide. After dehydration in an ethanol series, the samples were transferred to amyl acetate and critical point dried. Dried samples were mounted on stubs using silver paint, and sputter coated. The specimens were viewed and photographed with a Hitachi S-570 SEM

RESULTS

Cultured ovules were grown on liquid media containing optimum concentrations of auxin [5 µM naphthalene-1-acetic acid (NAA)] and gibberellin (0.5 µM GA3). Some cultures were also supplemented with either 0.1 µM BL or 10 µM Brz, or both. Ovules removed from culture after 3 d were fixed and processed for scanning electron microscopy (SEM) and light microscopy (LM). As shown in Fig. 1,

cultured ovules grown with 0.1µM BL appear to have more extensive fiber growth than those grown without supplemental BL (compare panels labeled CK and BL). Ovules grown in the presence of 10 µM Brz have much shorter fibers than either control ovules or ovules grown with additional BL. Importantly; addition of 0.1 µM BL reverses most of the inhibitory effects of 10 µM Brz on fiber elongation. These qualitative results confirm those reported previously by Sun *et al*. (2004). Although the control ovule shown as an example in Fig. 1 is slightly smaller than the other ovules, no apparent differences in ovule development were seen under the conditions used. However, treatment with Brz at higher concentrations does inhibit ovule growth.

Control ovules (CK) were grown on liquid medium containing auxin and GA. Ovules treated with 0.1 µM brassinolide (BL) had more extensive fiber development while ovules treated with 10 µM brassinazole2001 (Brz) had reduced fiber length. Fiber on ovules treated with both Brz and BL (Brz + BL) had fibers similar to control ovules.

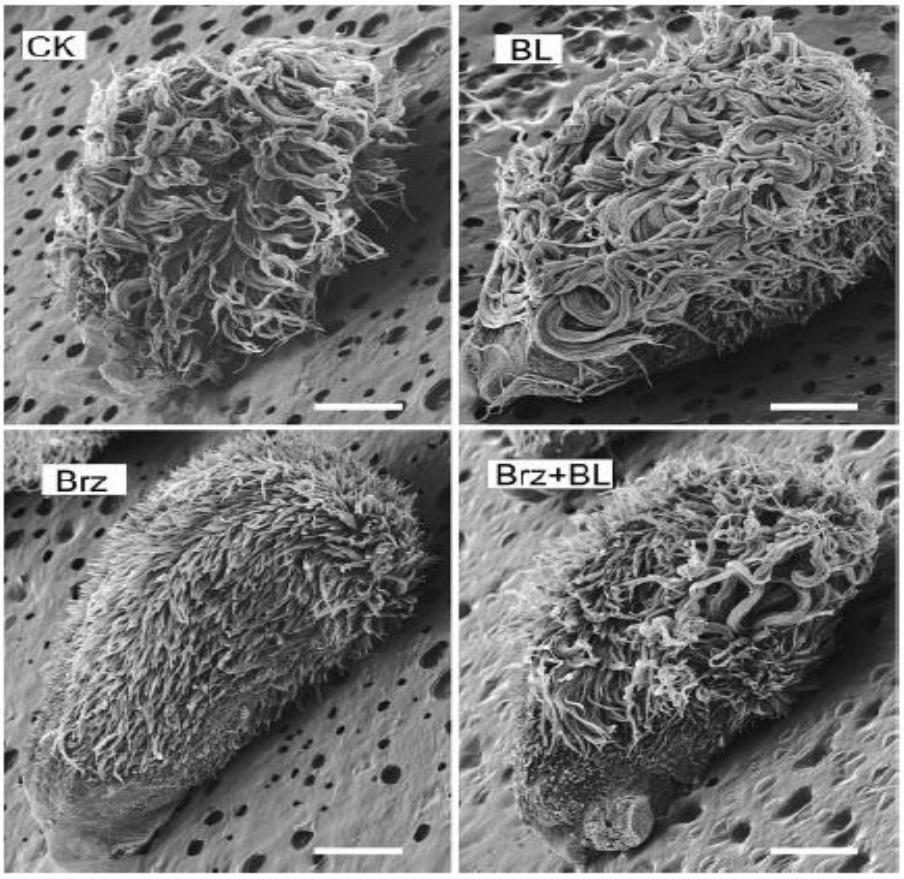

Fig. (1) : Scanning electron microscopic analysis of fiber development n 3-d cultured cotton ovules. Control ovules (CK) were grown on liquid medium containing auxin and GA. Ovules treated with 0.1 µM brassinolide (BL) had more extensive fiber development while ovules treated with 10 µM brassinazole 2001 (Brz) had reduced fiber length. Fibers on ovules treated with both Brz and BL (Brz + BL) had fibers similar to control ovules. Scale bars = 0.5 mm.

Average fiber length of cultured cotton ovules grown under control conditions (CK) or with added BL, Brz or both is shown in Fig.(2). The values are means of measurements of 10 randomly selected ovules for each treatment in three independent experiments. Error bars represent the SD. Letters represent statistically significant differences (Students t-test). Brassinosteroid affects cotton fibers. For quantitative analysis, ovules were randomly sampled from the cultures after 14 d and soaked in 95°C water for 5 min to relax the fibers. The fibers were then spread and measured. Mean fiber lengths from the three different experimental treatments are shown in Fig.(2). Each value represents the mean of measurements from 10 ovules for

each treatment from three independent experiments. Under these conditions, the mean fiber length for ovules cultured without exogenous BL was 15.0 mm while the mean fiber length from ovules cultured with 0.1 µM BL was 16.9 mm. Therefore, exposure of cultured ovules to BL increased fiber length by 12.7%. Treatment of cultured ovules with 10 µM Brz reduced the mean fiber length to 9.3 mm, a 38% decrease in fiber length relative to control samples, while ovules treated with both Brz and BL had a mean fiber length of 13.7 mm , an increase of 47 % relative to Brz - treated ovules.

These results confirm the observation that exogenous BR promotes fiber growth, and inhibition of the endogenous biosynthesis of BR by treatment with Brz strongly inhibits fiber development.

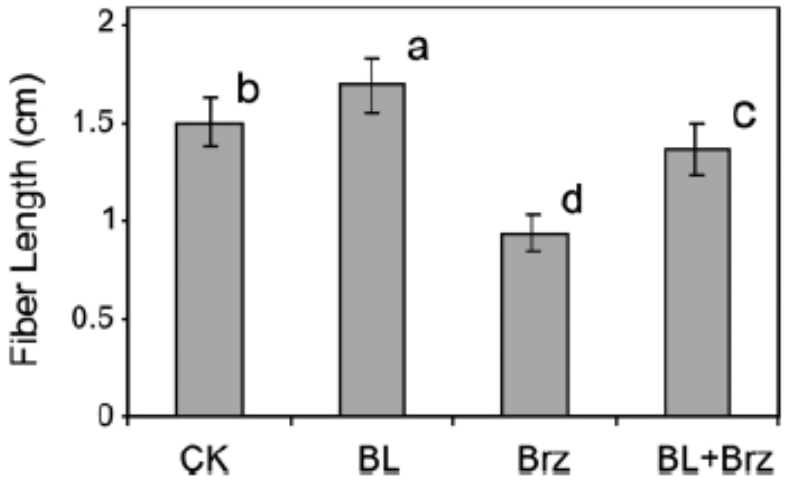

Fig. (2): Average fiber length of cultured cotton ovules under control conditions (CK) or with added BL, Brz or both. Te values are mean measurements of 10 randomly selected ovules for each treatment in 3 independent experiments. Error bars represent the SD. Letters represent statistically significant differences (Students t-test).

Structural analysis of the epidermis of 3-d cultured cotton ovules using tissue sections and LM was carried out to examine further the effects of BL and Brz treatment on fiber development (Fig. 3). BL-treated ovules and control ovules were structurally indistinguishable at this level; the epidermis of these samples is composed primarily of small cuboidal cells along with numerous highly elongated fiber cells (Fig. 3A). The elongated portions of the fiber cells are dominated by an enlarged central vacuole and contain no visible cytoplasmic features (Delanghe 1986). The effects of Brz treatment, however, could be clearly seen. Observations of areas of these ovules where fiber elongation was strongly inhibited revealed numerous fiber cells that had initiated expansion to produce rounded protrusions from the epidermal surface; however, further elongation was inhibited (Fig. 3B). These tissues resemble cotton ovules on the day of anthesis (Fig. 3D), indicating that fiber

elongation was blocked in these areas immediately upon exposure to Brz. Tissue sections from ovules treated with Brz and BL were structurally similar to untreated ovules (Fig. 3C). The effects of Brz treatment on fiber elongation can be easily studied using the cultured ovule system. However, since fibers are already initiated in ovules that are collected for culture on the day of anthesis, this system is less useful for the analysis of fiber initiation. Therefore, to begin to address the role of BR in fiber initiation, Brz was applied directly on a daily basis to developing cotton flowers from floral bud initiation until anthesis. Preliminary treatments showed that flowers are exceedingly sensitive to Brz treatment and virtually all treated floral buds abscised within a few days of the first treatment.

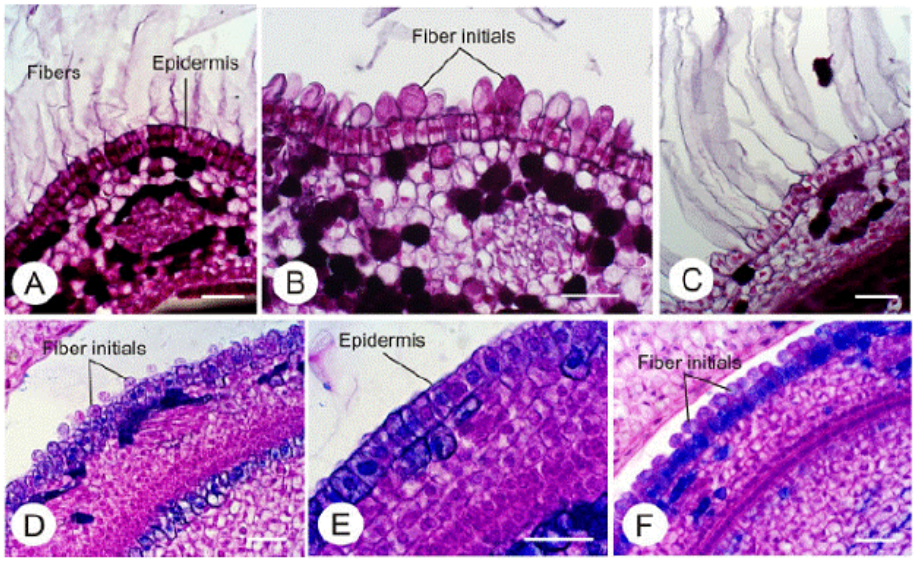

Fig. (3) : Structural analysis of cotton fiber elongation in Brz-treated cultured cotton ovules. Presence of 0.1 µM BL for 3 d show extensive fiber growth (A), while ovules treated with 10 µM Brz show numerous fiber initials that fail to elongate as evidenced by their protrusion from the epidermal surfaces (B). Treatment of ovules with both 10 µM Brz and 0.1 µM BL restored fiber development (C). Treatment of floral buds with Brz resulted in a loss of fiber initiation. Numerous cotton initials are seen protruding from the epidermis of ovules from untreated cotton ovaries on the day of anthesis (D) while the epidermis of ovules from cotton buds treated with 2.5 µM Brz show complete inhibition of fiber initiation (E). Ovules from emasculated flowers show numerous fiber initials, indicating that the loss of fiber initiation in Brz-treated flowers in not related to pollination (F). Scale bars = 50 µM .

Buds treated with a mock solution lacking Brz were retained, indicating that abscission was due to the Brz and not to the treatment procedure. However, at relatively low Brz concentrations (2.5 µM), approximately 10% of the treated buds were retained. While the epidermis of ovules from mocktreated flowers on the day of anthesis showed the presence of numerous fiber initials, the ovules from Brz-treated flowers showed no fiber differentiation (compare Fig. 3D and E). The epidermis of these ovules contained only cuboidal cells with no apparent cell wall protrusions. One possible explanation for the lack of fiber initials in these ovules is that Brz inhibits pollination so that the ovules are not fertilized. However, unfertilized ovules from emasculated flowers do show distinct fiber initials (Fig. 3F), indicating that fiber differentiation is not dependent on fertilization.Ovules cultured in the presence of 0.1 µM BL for 3 d show extensive fiber growth (A), while ovules treated with 10 µM Brz show numerous fiber initials that fail to elongate as evidenced by their protrusion from the epidermal surfaces (B). Treatment of ovules with both 10 µM Brz and 0.1 µM BL restored fiber development (C). Treatment of floral buds with Brz resulted in a loss of fiber initiation. Numerous cotton initials are seen protruding from the epidermis of ovules from untreated cotton ovaries on the day of anthesis (D) while the epidermis of ovules from cotton buds treated with 2.5 µM Brz show compete inhibition of fiber initiation (E). Ovules from emasculated flowers show numerous fiber initials, indicating that the loss of fiber initiation in Brz-treated flowers in not related to pollination (F). Brassinosteroid affects cotton fibers . BR is likely to play a critical role in the early differentiation of fiber cells, as well as their elongation.

DISCUSSION

The rapid elongation of cotton fibers is among the most remarkable cell elongation events in the plant kingdom. While previous results with the cultured ovule system have clearly shown that auxin is required for fiber development (Beasley and Ting 1973), the effects of exogenous application of BL were more subtle (Ashcraft 1996). However, the strong inhibition of fiber development in cultured ovules treated with Brz (Sun *et al*. 2004) led to suspect that BR is also required for fiber formation. Unlike auxin, which must be added to cultured ovules to promote fiber elongation, BL has a relatively small effect on fiber development , while the inhibition of BR biosynthesis with Brz can, in some cases, almost completely abrogate fiber elongation. These results indicate that BR biosynthesis by the ovule is required for fiber development. Brz, which inhibits the cytochrome P450 monooxygenase encoded by the DET2 gene, is structurally related to the gibberellin biosynthesis inhibitor uniconazole (Rademacher 1989). While Brz is reported to be highly specific for the BR biosynthetic pathway (Sekimata *et al*. 2001), it remains possible that the suppression of fiber development by Brz could be an indirect effect, perhaps by inhibition of cytochrome P450 enzymes involved in other pathways. However, the observation that the addition of BL can rescue fiber elongation in Brz-treated ovules indicates that at least the

primary effect of Brz on fiber development is due to the loss of BR synthesis. Analysis of cultured cotton ovules treated with Brz confirms that fiber elongation was inhibited following fiber cell initiation, and co-treatment with Brz and BL resulted in normal fiber elongation. Based on these observations, it would appear that cell expansion is the primary target of the BR signaling pathway in developing fibers. However, analysis of ovules from floral buds treated *in situ* with Brz indicates that BR may have a broader effect on fiber development. While the epidermis of ovules from control plants 1 d after anthesis had numerous fiber initials, the epidermis of ovules from Brz treated plants consisted solely of cuboidal cells with no indication of initiated fiber cells (see Fig. 3E). Therefore, it is apparent that BR is required not only for fiber cell elongation following anthesis but also for the formation of fiber initials prior to anthesis. Although fiber elongation is initiated at pollination, it is clear that fiber initials form before anthesis (Delanghe 1986). This was confirmed by analysis of ovules from emasculated flowers 1 d after anthesis, which showed numerous fiber initials.

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تأثير البراسينوستيرويد علي نمو شعيرات القطن

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ترجع المعرفة الحالية لدور الهرمونات النباتية في نمو شعيرات القطن بشكل أساسي إلي نظام بحثي دقيق ، فيه تؤخذ بويضات نبات القطن يوم تفتح الأزهار ، وتنمي علي بيئات سائلة، حيث وجد أن إضافة الأوكسين والجبريللين تحت هذه الظروف يدفع تكشف واستطالة الشعيرات .وحديثاً ، وجد أيضاً أن إضافة تركيزات منخفضة من البراسينوليد (من البراسينوستروید) يدفع استطالة الشعيرات ، في حين يثبط البراسينوزول ٢٠٠١ (مثبط للتمثيل الحيوي للبراسينوستيرويد) نمو الشعيرات .

وتؤكد نتائج هذه الدراسة أن إضافة البراسينوليد تنشط استطالة الشعيرات ، في حين تثبطها المعاملة بالبراسينوزول ٢٠٠١ . فضلاً عن ذلك تعيق تماما معاملة البراعم الزهرية بالبراسينوزول ٢٠٠١ تكشف الشعيرات ، وهذا يبرهن علي أهمية البراسينوستيرويد في تكشف واستطالة شعيرات القطن ، كما يزيد تعبير العوامل الوراثية المسئولة عن استطالة الشعيرات في البويضات المعاملة بالبراسينوليد ، في حين تثبطها المعاملة بالبراسينوزول ٢٠٠١ ، مما يرجح الارتباط بين تعبير العامل الوراثي المنظم للبارسينوستيرويد واستطالة الشعيرات .

توضح نتائج هذه الدراسة العلاقة بين البارسينوستيرويد ونمو الشعيرات، كما تفتح الباب للتحليل الوراثي لنمو شعيرات القطن من خلال التحوير المباشر لمسار انتقال تعبير العامل الوراثي للبارسينوستيرويد .