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# Brassinosteroid Regulates Fiber Development on Cultured Cotton Ovules

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**Our 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. In order to elucidate the role of brassinosteroid in cotton fiber development further, we have performed a more detailed analysis of the effects of these chemicals on cultured cotton ovules. Our 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 — Brassinosteroid — Cell elongation — Cotton fiber — *Gossypium hirsutum* — Ovule culture.

Abbreviations: ACP, acyl carrier protein; AGP, arabinogalactan protein; BL, brassinolide; BR, brassinosteroid; Brz, brassinazole2001; DPA days post-antithesis; EXP, expansin; LM, light microscopy; NAA, naphthalene-1-acetic acid; RT–PCR, reverse transcription–PCR; SEM, scanning electron microscopy; XTH, xyloglucan transferase/hydrolase.

## Introduction

Cotton fibers are highly elongated trichomes that arise from the seed integument. Development of cotton fibers is positively affected by a number of phytohormones including auxin, gibberellins and brassinosteroids (BRs) (Delanghe 1986, Sun et al. 2004). BRs consist of a family of phytosterols found throughout the plant kingdom (Mandava 1988). Recent genetic data confirm that BR is required for normal plant growth and development (for a review see Schumacher and Chory 2000). In general, BR has effects similar to auxin. BR stimulates germination, shoot elongation, vascular development and pollen formation. BR inhibits root elongation and promotes secondary root initiation. BR accelerates cell elongation and affects cytoskeletal and cell wall structure. BR promotes the transverse orientation of microtubules in azuki bean (Mayumi and Shibaoka 1995), and the brassinolide (BL)-induced epicotyl growth in *Cicer arietinum* is accompanied by increased β-tubulin expression (Munoz et al. 1998). The expression of a β-tubulin gene (*TUB1*) is suppressed in the BR-deficient mutant *dim* in Arabidopsis (Takahashi et al. 1995). Among the first BR-responsive genes to be identified, *BR-unregulated gene 1* (*BRU1*) from soybean (Zurek and Clouse 1994) was identified as a xyloglucan endo-transglycosylase gene (now called xyloglucan transferase/hydrolase; XTH). These enzymes are involved in cell wall loosening (Fry et al. 1992) and provide a direct link between BR and its effects on cell wall development. Microarray analysis of BR-up-regulated genes showed that many cell wall-related genes, including expansin (EXP, At2g40610), fatty acid elongase (At1g01120), arabinogalactan protein (AGP, At5g10430), acyl carrier protein (ACP, At3g25110) and a number of XTHs (U43488, At1g65310 and At5g57550), are up-regulated by BL in Arabidopsis (Goda et al. 2002, Goda et al. 2004). Interestingly, the cotton counterparts of these genes were also reported to be preferentially expressed in cotton fibers (Ji et al. 2003). This correlation indicates that BR may regulate fiber elongation by up-regulating the expression of genes involved in cell expansion.

Cotton fiber development can be divided into four overlapping stages: fiber initiation, elongation, secondary wall deposition and maturation. Fiber elongation is controlled by the interaction of cell turgor and cell wall extensibility (Smart et al. 1998). Shimizu et al. (1997) reported that both endo-1,4-β-glucanse and expansin mRNA levels were high during the fiber elongation stage. Plasma membrane proton-translocating ATPase, vacuole-ATPase, proton-translocating pyrophosphatase (PPase), phosphoenolpyruvate carboxylase, major intrinsic protein and α-tubulin also accumulated to high levels during fiber elongation (Smart et al. 1998). Direct evidence for

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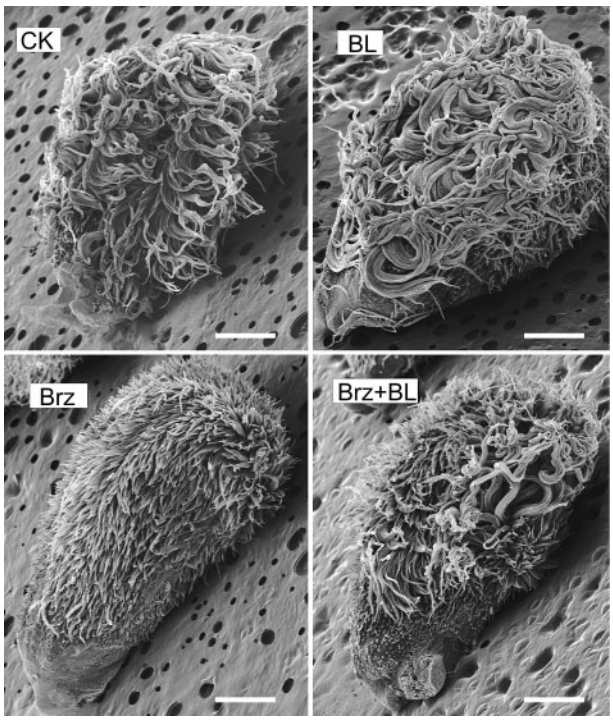


**Fig. 1** Scanning electron microscopic analysis of fiber development on 3-d cultured cotton ovules. Control ovules (CK) were grown on liquid medium containing auxin and GA. Ovules treated with 0.1 μM brassinolide (BL) had more extensive fiber development while ovules treated with 10 μM brassinazole2001 (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.

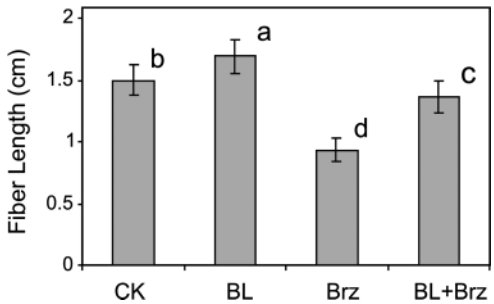


**Fig. 2** Average fiber length of cultured cotton ovules grown under control conditions (CK) or with added BL, Brz or both as described in Fig. 1. The values are means of measurements of 10 randomly selected ovules for each treatment in three independent experiments. Error bars represent the SD. Letters represent statistically significant differences (Students *t*-test).

the role of BR-stimulated gene expression in cotton fiber development comes from transgenic cotton plants that overexpress a cotton XTH known as KC22. These plants produce fibers that are significantly longer than those from control plants (Allen et al. 2000).

To better understand the role of BR in cotton fiber development, we have used the cotton ovule culture system developed by Beasley and Ting (1973). Ashcraft (1996) reported that the mean fiber length from cultured ovules treated with 0.1 μM BL increased by 11% relative to untreated control ovules. Expression of the BR-responsive *XTH* gene *KC22* was also increased in BL-treated cotton ovules. Sun et al. (2004) recently reported that treatment of cultured ovules with the BR biosynthesis inhibiter brassinazole2001 (Brz, Sekimata et al. 2001) strongly inhibited cotton fiber development. Simultaneous addition of Brz and BL to the cultured ovules partially restored fiber development, indicating that the inhibition of fibers by Brz is, at least for the most part, due to its inhibition of BR biosynthesis. Here we present a more detailed analysis of the effects of BR treatments on fiber development in cultured ovules. Our results confirm that Brz inhibits fiber elongation and this effect can be reversed by addition of BL. In addition, the effects of these treatments on the expression of selected genes confirm that the expression of BR-responsive genes correlates with fiber elongation in this system.

## Results

*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). Fertilized ovules were floated on the surface of a defined liquid medium to develop fibers (Beasley and Ting 1973). Ovule epidermal cells differentiate into fiber initials before anthesis, and initiation of fiber elongation requires exogenous auxin. While addition of BR is not required for fiber initiation, it does promote fiber elongation (Ashcraft 1996).

To test further the effects of BR on cotton fiber development, cultured ovules were grown on liquid media containing optimum concentrations of auxin [5 μM naphthalene-1-acetic acid (NAA)] and gibberellin (0.5 μM $GA_3$). 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 0.1 μM 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 (data not shown).

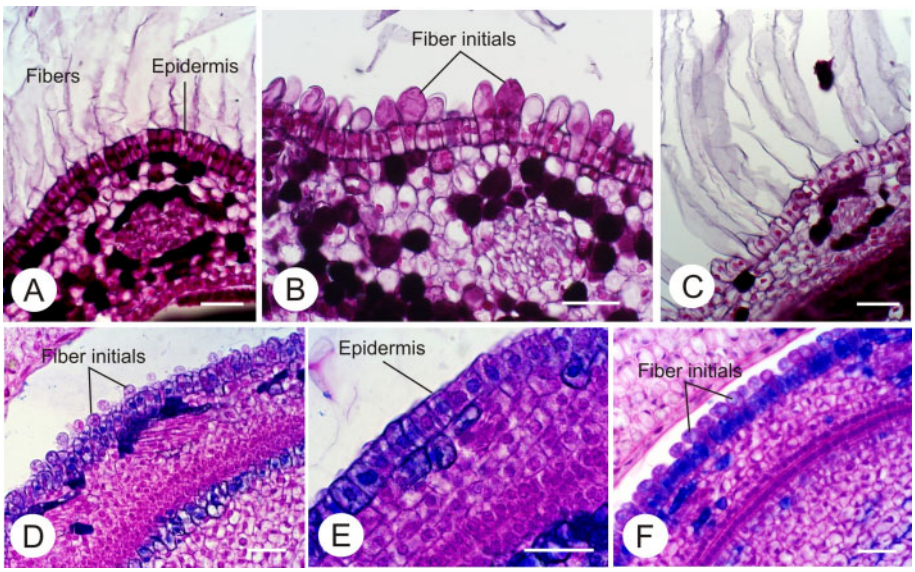


**Fig. 3** Structural analysis of cotton fiber elongation in Brz-treated cultured cotton ovules. Ovules cultured in the presence of 0.1 μM BL for 3 d show extensive fiber growth (A), while ovules treated with 10 μM Brz show numerous fiber initials that fail to elongate as evidenced by their protrusion from the epidermal surfaces (B). Treatment of ovules with both 10 μM Brz and 0.1 μM BL restored fiber development (C). Scale bars = 50 μm. 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).

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.

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. 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 mock-treated 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. Though preliminary, these results indicate that

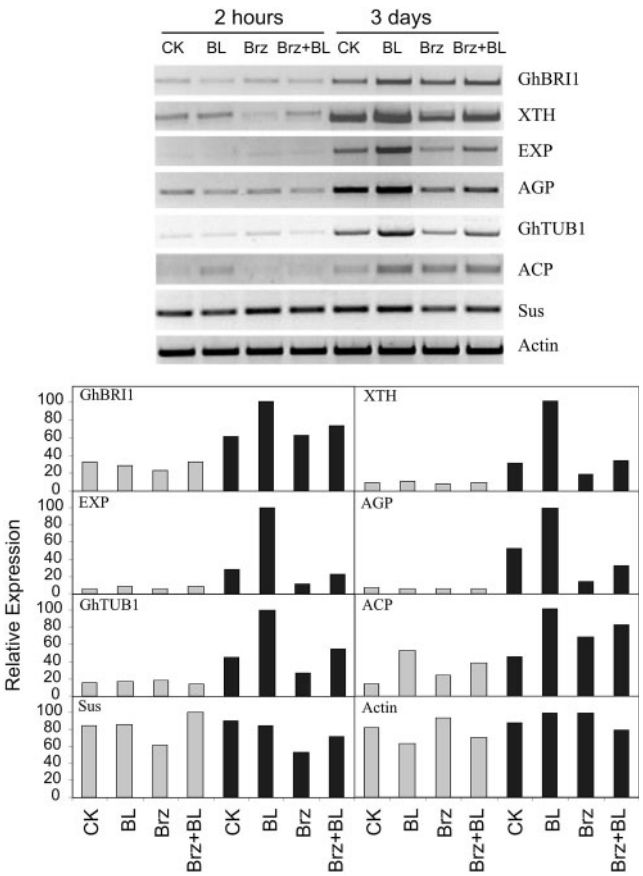


**Fig. 4** Reverse transcription–PCR analysis of mRNA levels in cotton ovules cultured for 2 h or 3 d with and without BL and/or Brz treatment. The images of the RT–PCR gel were scanned under an Eagle Eye II System, and the relative band density was quantified using Kodak 1D Image Analysis Software. The actin mRNA level was used to normalize for RNA input. The relative mass value for each primer pair is shown at 2 h (shaded bars) and 3 d (black bars) as a percentage of the maximum value.

BR is likely to play a critical role in the early differentiation of fiber cells, as well as their elongation.

*Effects of BR on gene expression*

In order to correlate the effects of the BL and Brz treatments on cotton fiber development with BR-responsive gene expression, levels of mRNAs derived from *GhBRI1* (Sun et al. 2004) along with several fiber-expressed and BR-responsive genes in cultured cotton ovules were assayed by reverse transcription–PCR (RT–PCR) analyses (Fig. 4). Expression of transcripts for the putative BR receptor *GhBRI1* was detectable in both 2-h and 3-d cultured ovules. Expression of *GhBRI1* transcripts modestly increased, relative to control samples, in 3-d cultured ovules treated with BL and was somewhat lower in samples treated with Brz.

Primers used for the specific amplification of transcripts for fiber-expressed proteins include *XTH* (Allen et al. 2000),

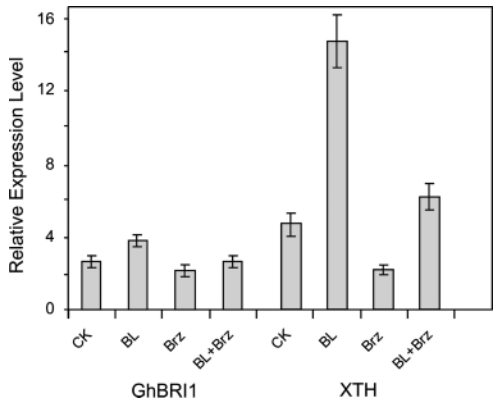


**Fig. 5** Real-time PCR using TaqMan assays for quantitative analysis of *GhBRI1* and *XTH* expression levels in cotton ovules cultured for 3 d with and without BL and/or Brz treatment. Data represent means ± SD.

*EXP* (Ji et al. 2003), *AGP* (Ji et al. 2003), β-tubulin 1 (*GhTUB1*; Li et al. 2002), *ACP* (Song and Allen 1997) and sucrose synthase (Sus; Ruan et al. 2003). Primers for actin were used for normalization of RNA levels in the assays. As can be seen in Fig. 4, the levels of actin mRNA were relatively constant in all of the RNA samples analyzed.

Expression of *XTH*, *EXP*, *AGP*, *GhTUB1* and *ACP* mRNAs was very low in 2-h cultured ovules, but levels of these mRNAs were much higher in 3-d cultured ovules. Expression of *XTH* and *ACP* transcripts increased somewhat in response to BL treatment in 2-h cultured ovules and decreased in response to Brz treatment. However, expression of all five transcripts was substantially increased in response to BL treatment in 3-d cultured ovules relative to control ovules and they were strongly suppressed in ovules treated with Brz. Levels of these mRNAs in ovules treated with both Brz and BL were comparable with those in control ovules. On the other hand, *Sus* transcripts were expressed at constitutively high levels in all of the samples tested, and were somewhat reduced in ovules treated with Brz.

To determine the magnitude of changes in gene expression in response to BL and Brz treatments more accurately, quantitative real-time PCR was used to assay the expression levels of *XTH* and *GhBRI1* (Fig. 5). Although the magnitude of the response was small, expression of *GhBRI1* was increased by about 40% in BL-treated 3-d cultured ovules relative to control ovules. The level of *GhBRI1* mRNA was reduced by 25% in ovules treated with 10 μM Brz, while its expression in ovules treated with both Brz and BL was similar to that in control ovules.

As can be seen in Fig. 5, steady-state levels of *XTH* mRNAs were three times higher in 3-d cultured ovules treated with 0.1 μM BL than in control ovules. *XTH* expression was reduced by about 2-fold in cultured ovules treated with 10 μM Brz relative to controls. Expression of XTH mRNA in 3-d

cultured ovules treated with both Brz and BL was similar to that in control ovules, indicating that the Brz-dependent suppression of *XTH* expression was reversed by BL. These results clearly indicate that *XTH* gene expression is regulated by BR and provide correlative evidence for the role of BR in fiber development.

## 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 us 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 our analysis of ovules from emasculated flowers 1 d after anthesis, which showed numerous fiber initials.

To correlate the developmental effects of BR alteration with gene expression, the impact of BL and Brz treatments on the expression of a number of genes was examined in cultured ovules. Recent genetic analysis in Arabidopsis has led to the identification of several critical proteins that are involved in the BR signal transduction cascade (Bishop and Koncz 2002). *GhBRI1*, a functional ortholog of the Arabidopsis BR receptor gene (Li and Chory 1997), is expressed at relatively high levels in cotton ovules and fibers during the elongation phase of fiber development then decreases during secondary wall formation (Sun et al. 2004). *GhBRI1* was expressed in both 2-h and 3-d cultured ovules and was only slightly affected by treatment with BL and Brz. These results confirm that this critical component of the BR signal transduction pathway is expressed in developing ovules during culture.

Ji et al. (2003) reported that mRNAs for cell wall-loosening enzymes such as XTH and EXP, and fiber-specific proteins such as ACP and AGP are preferentially expressed in elongating fibers. EXPs mediate cell wall extension in plants by breaking the hydrogen bonds between cellulose and hemicellulose, allowing these polymers to slip relative to each other (Mason and Cosgrove 1995). Cosgrove (2000) asserted that EXPs are the most important wall-loosening factors in turgor-driven cell wall extension, while XTHs and other glucanases act secondarily in cell wall-loosening and reconstruction. AGPs are proteoglycans that play a potential role as cell adhesion molecules that are important for cell expansion (Schultz et al. 2000, Shi et al. 2003) while ACPs function in the biosynthesis of cellular lipids, including phospholipids necessary for the expansion of plasma membrane and tonoplast (Song and Allen 1997). Arabidopsis orthologs of these four genes are also up-regulated by BR (Goda et al. 2002, Goda et al. 2004). Therefore, we predicted that BR may regulate fiber development, at least in part, by controlling the expression of genes involved in cell expansion. In addition, a cotton β-tubulin gene, *GhTUB1*, was preferentially expressed in fiber (Li et al. 2002). Microtubules play a number of important roles in a wide variety of processes in plant cells, including cell elongation. Cortical microtubules are thought to be involved in determining the orientation of cell wall expansion and cellulose synthesis (Whittaker and Triplett 1999). Sucrose synthase is the primary enzyme for sucrose catabolism in fibers and may channel UDP-glucose directly to the cellulose synthase complex during fiber maturation (Haigler et al. 2001, Ruan et al. 2001, Koch 2004). Cotton plants that express an antisense *Sus* transgene are reported to have a fiberless phenotype (Ruan et al. 2003).

Expression of *XTH*, *EXP*, *AGP*, *GhTUB1* and *ACP* mRNAs was low in 2-h cultured ovules, but the levels of these mRNAs was considerably higher in 3-d cultured ovules (Fig. 4). This increase in expression correlates with increased rates of fiber elongation and increases in the amount of fiber tissue on the ovule surface during this time. Expression of each of these transcripts was substantially increased in BL-treated ovules relative to control ovules and they were strongly sup-

**Table 1** Primers used for reverse transcriptase–PCR analysis of gene expression

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *GhBRI1* | 5′-AAGAGGGCTGGCGTTTCTTC-3′ | 5′-TGCAAAAGAATGTTCTCTGA-3′ |
| *XTH* | 5′-CATGGGTATGGGTTTAAGGAAT-3′ | 5′-TCAGATGATGGACATGCACTC-3′ |
| *EXP* | 5′-AGTCGAACCATAACCGTGACAGCC-3′ | 5′-CCCAATTTCTGGACATAGGTAGCC-3′ |
| *AGP* | 5′-ATGAGGCAACAATATGTCTTCAC-3′ | 5′-TCACAAAGAAAGTGCAATAACTAG-3′ |
| *GhTUB1* | 5′-ATGAGAGAAATCCTTCACATCC-3′ | 5′-TTAAGCCTCTGCCTCGTATTC-3′ |
| *ACP* | 5′-ATGGCTTCTATTGCTGGTTCAT-3′ | 5′-TAGGCACTTTTCTCACTGCA-3′ |
| *SUS* | 5′-GCCTGCATTGTATGAAGCCTT-3′ | 5′-TTACTCCTCTGCCAATGGAAC-3′ |
| *Actin* | 5′-CTACGGTAACATTGTGCTCAG-3′ | 5′-GGACCCATCATCTTAATGGTG-3′ |

pressed in ovules treated with Brz. These results support our hypothesis that BR promotes fiber elongation by up-regulating the expression of genes involved in cell expansion and cell wall reconstruction. On the other hand, *Sus* transcripts were expressed constitutively in all of the samples tested, which suggests that Sus may play a broader role in ovule differentiation and seed formation that is not dependent on BR.

The results presented here establish a clear connection between BR and cotton fiber development. We anticipate that ongoing experiments, including overexpression and suppression of BR signaling components in cotton, will elucidate this relationship further. We expected that a more complete understanding of the role of BR in cotton fibers will provide opportunities to alter cotton fiber development through transgenic and/or molecular breeding techniques.

## Materials and Methods

*Plant growth conditions*

Cotton plants (*Gossypium hirsutum* cv. Coker 312) were grown in potting soil in a greenhouse at 28°C. Flowers were tagged on the day of anthesis.

Exogenous application of Brz to flowers prior to anthesis was performed according to the methods of Seagull and Giavalis (2004). Daily application of 250 μl of 2.5 μM Brz (RIKEN, Saitama, Japan) to the 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.

*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 $GA_3$ in 50 ml flasks (Beasley and Ting 1973, Ashcraft 1996). The ovules were incubated at 34°C in the dark without agitation. Preliminary experiments indicated that addition of 0.1 μM 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 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 post-fixation 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.

*Total RNA extraction*

Cotton ovule tissues were frozen in liquid nitrogen and and ground with a pestle to a fine powder in a cold mortar. Total RNA was extracted using the hot borate method (Wan and Wilkins 1994).

*Reverse transcription–PCR analysis*

Cotton RNA samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France). All cotton RNA samples were diluted to 200 ng $\mu l^{-1}$. A 1 μg aliquot of total RNA was used for a 50 μl RT–PCR. Reverse transcription of mRNA in all the samples was carried out using oligo(dT) primer and Multiscript reverse transcriptase (Applied Biosystems, Foster City, CA, USA). Gene-specific RT–PCR primer pairs are shown in Table 1. The number of thermal cycles used for each reaction was: *GhBRI1*, 27 cycles; *XTH*, 25 cycles; *EXP*, 23 cycles; *AGP*, 28 cycles; *GhTUB1*, 30 cycles; *ACP*, 28 cycles; *Sus*, 25 cycles. Parallel reactions using primers from a cotton actin gene (D88414) were used for RNA normalization (24 cycles). Images of RT–PCR gels were obtained using an Eagle Eye II System (Stratagene, La Jolla, CA, USA). The relative density of bands for each reaction was quantified using Kodak 1D Image Analysis Software (Eastman Kodak Company, New York, USA). The relative mass value of each band was normalized by dividing by the actin mass value for each RNA sample. For comparisons of bands for each primer pair across different RNA samples, the highest value was set to 100% and the other values are given as a percentage of that value.

*Real-time PCR analysis*

Quantitative real-time PCR was used to assay gene expression levels for two genes. The expression levels of the *GhBRI1* and *XTH* genes in different cotton ovule samples were detected by using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA) was used to design primers and probes for real-time PCR analysis. The *GhBRI1*-specific probe and forward and reverse primers were as follows: *GhBRI1*-FAM probe (FAM was used as first fluorescent reporter dye), 6FAM-AAGATCAAGCACCGGAA-CCTCGTTCC-TAMRA; *GhBRI1* forward primer, 5′-AGAATTCAC-AGCGGAAATGGAA-3′; *GhBRI1* reverse primer, 5′-CCCACCCTA-CAGTAACCCAAGAG-3′. The *XTH*-specific probe and primers were as follows: *XTH*-FAM probe, 6FAM-TCATCCCCGGCGACTCCGC-TAMRA; *XTH* forward primer, 5′-GACGTGTCAGCATGAAAAT-CAA-3′; *XTH* reverse primer, 5′-TCATATAAAAGGCGGTGACTG-TTC-3′; 18S rRNA VIC probe (VIC was used as second fluorescent reporter dye), 18S rRNA forward primer and 18S rRNA reverse primer (TaqMan Ribosomal RNA Control Reagents Kit, Applied Biosystems, Foster City, CA, USA) were used for RNA normalization. Reactions that lacked reverse transcriptase were included to check the possible amplification of genomic DNA contamination for each RNA sample.

TaqMan Reverse Transcription Reagent Kit (Applied Biosystems, Foster City, CA, USA) was used for RT–PCR. All cotton RNA samples were diluted to 100 ng $\mu l^{-1}$. Reverse transcription of all the RNA samples was carried out using random hexamers. The amplification reaction included the *GhBRI1* FAM-labeled probe (first probe), *GhBRI1* forward primer and reverse primer; or the *XTH* FAM-labeled probe (first probe), *XTH* forward primer and reverse primer; 18S rRNA VIC-labeled probe (second probe), 18S rRNA forward primer and reverse primer, AmpErase UNG (uracil-N-glycosylase), AmpliTaq Gold DNA polymerase and other components (TaqMan PCR Core Reagents Kit, Applied Biosystems, Foster City, CA, USA) were in the same tube for multiplex PCR. Each sample was assayed three times.

The relative expression levels of all the samples were calculated and analyzed (User Bulletin #2, ABI PRISM 7700 Sequence Detection System, Livak and Schmittgen 2001). The threshold cycles (Ct value) of the target genes and 18S RNA in different samples were obtained after quantitative real-time PCR. In brief, the first calculation is to subtract the normalizer 18S RNA Ct value from the Ct of the gene of interest (target gene) to produce the dCt value of the sample. The dCt value of the calibrator (the sample with the highest dCt value) was subtracted from every other sample to produce the ddCt value. $2^{-ddCt}$ was taken for every sample as the relative expression level.

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