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GhDET2, a steroid 5α-reductase, plays an important role in cotton fiber cell initiation and elongation

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Summary

Cotton (*Gossypium hirsutum* L.) fibers, one of the most important natural raw materials for textile industry, are highly elongated trichomes from epidermal cells of cotton ovules. DET2, an Arabidopsis steroid 5d-reductase, is considered to catalyze a major rate-limiting in brassinosteroid (BR) biosynthesis. To understand the role of BRs in cotton fiber development, *GhDET2*, which putatively encodes a steroid 5α-reductase by sequence comparison, was cloned from developing fiber cells. *In vitro* assessment of GhDET2 protein activity confirmed that *GhDET2* encodes a functional steroid 5α-redutase. High levels of *GhDET2* transcript were detected during the fiber initiation stage and the fiber rapid elongation stage. Antisense-mediated suppression of *GhDET2* inhibited both fiber initiation and fiber elongation. Similarly, treating cultured ovules with finasteride, a steroid 5α-reductase inhibitor, reduced fiber elongation. Inhibition of fiber cell elongation by expression of antisense *GhDET2* or the finasteride treatment could be reversed by epibrassinolide, a biologically active BR. Furthermore, seed coat-specific expression of *GhDET2* increased fiber number and length. Therefore, *GhDET2* and BRs play a crucial role in the initiation and elongation of cotton fiber cells, suggesting that modulation of BR biosynthesis factors may improve fiber quality or yield.

Keywords: GhDET2, cotton fibers, brassinosteroids, fiber initiation, fiber elongation.

Introduction

Brassinosteroids (BRs) are a class of plant steroidal hormones that are involved in a wide variety of physiological and developmental processes, including cell division and elongation, vascular differentiation, reproductive development, senescence, and biotic and abiotic tolerance (Anuradha and Rao, 2001; Clouse, 2000, 2002; Clouse and Sasse, 1998; Dhaubhadel *et al.*, 2002; Grove *et al.*, 1979; Haubrick and Assmann, 2006; Mandava, 1988; Mazorra *et al.*, 2002; Nakashita *et al.*, 2003; Sasse, 2003).

To date, more than 50 BRs have been isolated and identified from numerous plants (Bajguz and Tretyn, 2003); of these, brassinolide (BL) is the most biologically active. The first reaction towards BL formation is the conversion of campesterol (CR) to campestanol (CN). CN is converted to castasterone (CS) through either early or late C6 oxidation, and then CS is converted to BL (Fujioka and Yokota, 2003). The rate-limiting step in BR biosynthesis is catalyzed by a steroid 5α-reductase, DET2, which hydrogenates a (24*R*)-24-methylcholest-4-en-3-one intermediate to convert CR into CN (Chory and Li, 1997; Fujioka *et al.*, 1997; Li *et al.*, 1997; Noguchi *et al.*, 1999). Reduced levels of endogenous BRs are linked to loss of DET2 activity (Fujioka *et al.*, 1997).

Cotton is the world's leading fiber crop. Cotton fiber is an elongated cell of the ovule epidermis that undergoes rapid and synchronous elongation. Cotton fiber development includes four discrete yet overlapping stages: initiation, elongation, secondary cell-wall accumulation, and maturation (Basra and Malik, 1984). Fiber initiation is visible on the epidermal surface of ovules on the day of anthesis, and is followed by cell elongation (Ryser, 2000). Fiber elongation proceeds at a fast rate, uninterrupted by cell division, for a relatively long period, (Ruan *et al.*, 1997, 2001; Russell and Wilson, 1994). Due to its highly elongated structure, cotton fiber provides an ideal system for studying cell elongation

(Kim and Triplett, 2001). Fiber number and fiber length, determined by fiber initiation and fiber elongation, respectively, are important traits in agronomy (John, 2000). However, the mechanisms controlling cotton fiber initiation and elongation are poorly understood.

Analyses of endogenous plant hormones in cotton boll or fiber, as well as exogenous applications of plant hormones or growth regulators to cotton flowers, squares or cultured ovules, suggest that gibberellins (GAs) and indole-3-acetic acid (IAA) may promote cotton fiber development (Basra and Sukumar, 2000; Beasley and Ting, 1973, 1974; Davidonis, 1993; Gialvalis and Seagull, 2001; Liu *et al.*, 1999; Seagull and Giavalis, 2004). Recent microarray analysis of the gene expression profiles of cultured cotton ovules from Xuzhou 142 and its fuzzless–lintless mutant (*fl*) indicates that ethylene may play a role in cotton fiber elongation (Shi *et al.*, 2006). Results of exogenous application of BRs or BR biosynthesis inhibitors to regulate the fiber growth (Clouse, 2003; Kasukabe *et al.*, 2001; Khripach *et al.*, 2000; Sun *et al.*, 2004, 2005), and the modest increase in BR biosynthetic gene expression during cotton fiber elongation (Shi *et al.*, 2006), suggest that BR may also be involved in cotton fiber development. However, the specific role of endogenous BRs and genes involved in BR biosynthesis in cotton fiber development remains unknown.

To improve our understanding of the role of BRs and BR biosynthesis in cotton fiber development, we cloned a steroid 5α-reductase cDNA, designated *GhDET2*, from developing fiber of cotton (*Gossypium hirsutum* L.). Using the *GhDET2* cDNA sequence, sense and antisense *GhDET2* transgenic cotton plants were created. Characterization of the transgenic plants has revealed that BRs and *GhDET2* play an important role in the initiation and elongation of cotton fiber cells.

Results

Molecular cloning of GhDET2 *and demonstration of its function* in vitro

To obtain a steroid 5α-reductase gene from upland cotton (*Gossypium hirsutum* L.), six cotton ESTs were identified according to their amino acid sequence similarity to AtDET2, an Arabidopsis steroid 5α-reductase (Li *et al.*, 1996), using the TBLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/). An 899 bp cDNA fragment, named *GhDET2* (GenBank accession number AY141136), was then cloned from cotton fibers (6 days post-anthesis) using RACE (Frohman *et al.*, 1988). *GhDET2* encodes a polypeptide of 258 amino acid residues with a predicted molecular mass of 30 kDa. The deduced amino acid sequence of GhDET2

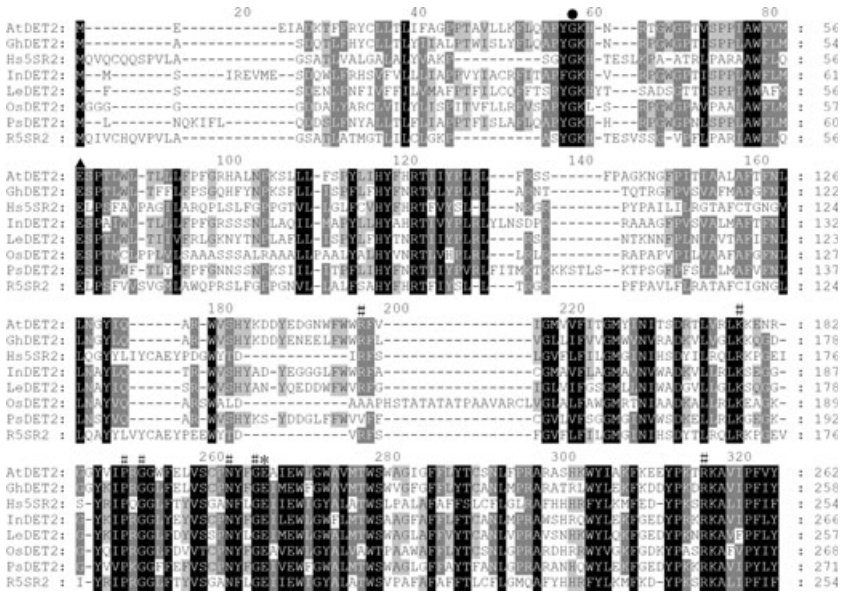

Figure 1. Amino acid sequence alignment of GhDET2 (AY141136) with previously identified steroid 5α-reductases.
The ClustalW method of the DNASTAR package DNASTAR Inc., Madison, WI, USA was used to align the amino acid sequence of GhDET2 (AY141136) with those of Arabidopsis DET2 (*Arabidopsis thaliana* AtDET2, U53860), tomato DET2 (*Lycopersicon esculentum*/*Solanum lycopersicum*, LeDET2, AJ786362), morning glory DET2 (*Ipomoea nil*, InDET2, AB106360) and pea DET2 (*Pisum sativum*, PsDET2, AY573897), a putative DET2 homologous protein from rice (*Oryza sativa*, OsDET2, NM190631), a putative DET2 homologous protein from soybean (*Glycine max*, GmDET2, AF203341), and mammalian steroid 5α-reductases from human (*Homo sapiens*, H5SR2, MIM607306) and rat (Rattus norvegicus R5SR2, NM022711). Black and grey shading indicate identical and similar residues, respectively. Dashes indicate gaps introduced to maximize alignment. The highly conserved Glu residue (Li *et al.*, 1996, 1997) is indicated by an asterisk. The Gly34 residue of GhDET2, which is important for sterol substrate binding (Rosati *et al.*, 2005; Russell and Wilson, 1994), is marked by a black dot. Seven co-factor binding sites originally identified in Hs5SR2 are indicated by #. Glu55, a conserved amino acid for InDET2 function (Glu62, Suzuki *et al.*, 2003), is indicated by a solid triangle.

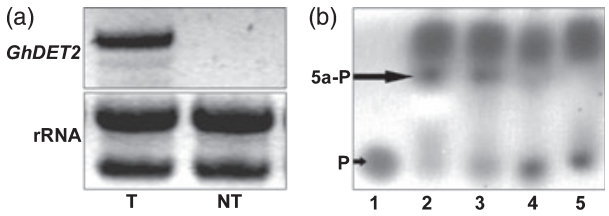

Figure 2. *In vitro* analysis of steroid 5α-reductase activity in *GhDET2*-expressing CHO cells.
(a) RT-PCR analysis of *GhDET2* transcripts. *GhDET2* was expressed in CHO cells transfected with *GhDET2* (T) but not in untransfected CHO cells (NT).
(b) TLC assay for 5α-reductase activity of CHO cells transfected with *GhDET2*. Lane 1, progesterone; lane 2, female rabbit liver homogenate, a positive control for steroid 5α-reductase activity (Russell and Wilson, 1994) +127 μM progesterone; lane 3, transfected cell lysate + 127 μM progesterone; lane 4, transfected cell lysate + 127 μM progesterone + 100 μM finasteride (an inhibitor of 5α-reductase); lane 5, untransfected cell lysate + 127 μM progesterone. P, progesterone; 5a-P, 5α-dihydroprogesterone, a product of the 5α-reductase-mediated conversion of progesterone. Note that 5α-dihydroprogesterone was produced in lanes 2 and 3.

is 66% identical and 81% similar to that of AtDET2. As shown in Figure 1, there are two invariant Glu residues (Glu55 and Glu200) in the deduced sequence that are absolutely required for steroid 5α-reductase activity (Russell and Wilson, 1994; Suzuki *et al.*, 2003). In addition, the sequence contains six (Arg148, Pro184, Gly186, Asn196, Gly199 and Arg250) of seven conserved residues (Arg145, Arg171, Pro181, Gly183, Asn193, Gly196 and Arg246) that are part of a co-factor-binding domain typical of mammalian steroid 5α-reductases (Russell and Wilson, 1994). These characteristics suggest that GhDET2 is probably a steroid 5α-reductase.

Southern blot hybridization result indicated that *GhDET2* is a single-copy gene in cotton (see Figure S1).

To determine whether *GhDET2* encodes a functional steroid 5α-reductase, the coding region of *GhDET2* was cloned into a mammalian expression vector, pcDNA3.1. The resulting plasmid was introduced into Chinese hamster ovary (CHO) cells, which have been used previously to measure steroid 5α-reductase activity (Rosati *et al.*, 2003). *GhDET2* transcript was detected in CHO cells transfected with *GhDET2* (Figure 2a). Extracts of CHO cells expressing *GhDET2* were used to measure the conversion of progesterone, a substrate of steroid 5α-reductases, to 5α-dihydroprogesterone, by thin-layer chromatography (Figure 2b). Finasteride, a specific inhibitor of steroid 5α-reductase, reduced the conversion of progesterone to 5α-dihydroprogesterone. These results demonstrate that *GhDET2* encodes a functional steroid 5α-reductase.

Expression of GhDET2 *in cotton plants*

Quantitative real-time RT-PCR was used to investigate the *GhDET2* expression pattern in cotton plants. *GhDET2* was readily detected in roots, hypocotyls, leaves and flowers at 0 days post-anthesis (DPA), and in ovules and fibers, but the expression level was very low in cotyledons (Figure 3a). In ovules, *GhDET2* expression increased from 0 DPA (the stage of fiber initiation) to 10 DPA, then decreased. By 22 DPA, *GhDET2* expression in ovules had declined to 12.1% of that of 10 DPA ovules. In fibers, the *GhDET2* transcript was very high at 5 DPA and remained elevated until 10 DPA (the stage of rapid elongation of fiber cells). The transcript level in fibers declined sharply to 9.9% of that in 5 DPA fibers by 20 DPA. Transcript levels in fibers were also analyzed by Northern blotting. Results were consistent with the RT-PCR pattern (Figure 3b).

In an *in vitro* assay for steroid 5α-reductase activity, cell extracts from 10 DPA fibers of wild-type plants were capable of converting progesterone (a typical substrate of steroid 5α-reductase) to 5α-dihydroprogesterone in a finasteride-sensitive manner (Figure 3c), indicating that steroid 5α-reductase is active in elongated fibers. *GhDET2* is highly expressed in 0–13 DPA ovules and 5–10 DPA fiber cells, implying that the *GhDET2* gene may play a role in both initiation and elongation of cotton fibers.

Constitutive suppression or over-expression of GhDET2 *leads to abnormal growth of transgenic cotton plants*

35S::antisense and 35S::sense *GhDET2* genes were constructed using the GhDET2 cDNA. Cassettes containing antisense or sense *GhDET2* along with the CaMV 35S promoter::GUS and nos promoter::*NPTII* genes were delivered into the cotton genome by *Agrobacterium*-mediated transformation. Ten sense and ten antisense GUS-positive transgenic cotton plants were obtained. Six antisense plants were extremely stunted and did not survive after being transferred to a greenhouse. Using total RNA from leaves, transcript levels of *GhDET2* were determined by quantitative real-time RT-PCR. Levels of endogenous *GhDET2* transcript were reduced in all antisense plants, and were almost completely suppressed in the AD4 plant (Figure 4d). In contrast, *GhDET2* expression levels were greatly increased in the sense transgenic plants (Figure 5c).

Morphologically, the antisense *GhDET2* plants were much shorter than control plants transformed with an empty vector (Figure 4a). The antisense *GhDET2* plants exhibited reduced leaf size as well as shortened internodes and branches (Figure 4b,c). I_2-KI staining indicated that pollen grains from antisense plants were sterile (Figure 4f). Also, spinules could easily be observed on the surface of pollen grains from control plants (Figure 4e,g), but could not be identified on the surface of pollen from antisense cotton plants (Figure 4f,h). Cotton bolls produced from the antisense *GhDET2* plants aborted within 3–5 DPA, even if the plants were pollinated with wild-type pollen. Sense *GhDET2* transgenic plants

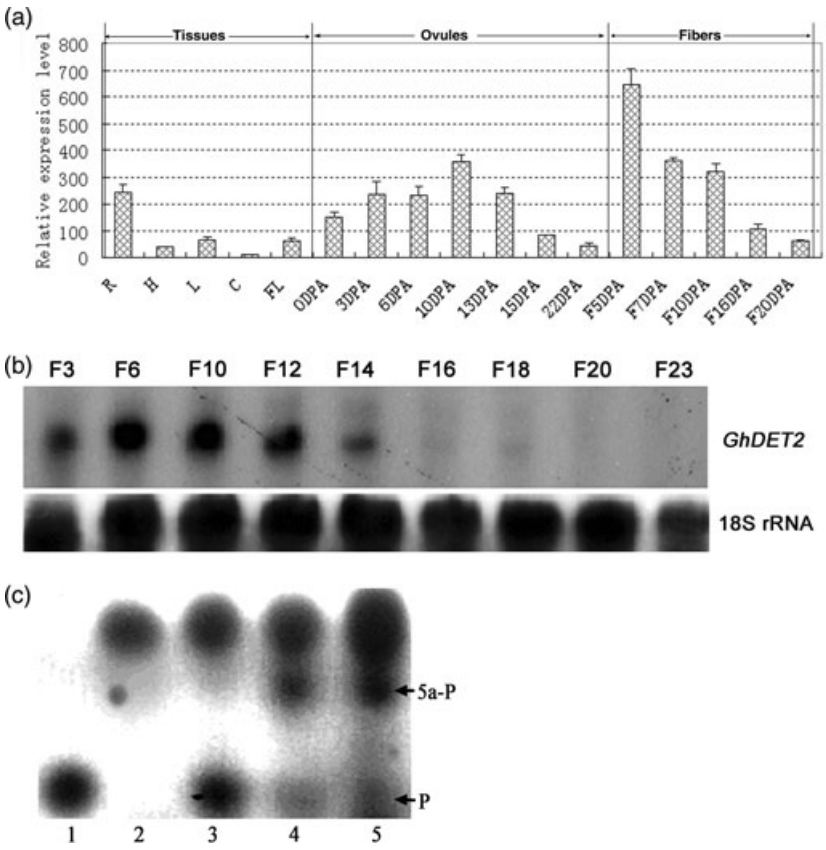

Figure 3. Expression of *GhDET2* in cotton plants.
(a) *GhDET2* transcript levels in various organs determined by quantitative real-time RT-PCR. Amplification of *GhDET2* cDNA derived from total RNA of roots (R), hypocotyls (H), leaves (L), cotyledons (C), 0 DPA flowers (FL), ovules at 0–22 DPA (0DPA–22DPA), and fibers at 5–20 DPA (F5DPA–F20DPA). Error bars represent SD for three independent experiments.
(b) Northern blot of *GhDET2* transcript in 3–23 DPA fiber cells (F3–F23). The full-length cDNA of *GhDET2* radiolabeled with α-^{32}P was used as probe.
(c) TLC assay for steroid 5α-reductase activity in 10 DPA fiber cells. Lane 1, progesterone only; lane 2, 10 DPA cotton fiber cell homogenate without progesterone (a steroid 5α-reductase substrate); lane 3, 10 DPA cotton fiber cell homogenate + 127 μM progesterone + 100 μM finasteride (a steroid 5α-reductase inhibitor); lane 4, 10 DPA cotton fiber cell homogenate + 127 μM progesterone; lane 5, female rabbit liver homogenate + 127 μM progesterone as positive control; P, progesterone; 5a-P, 5α-dihydroprogesterone, a product of the steroid 5α-reductase-mediated conversion of progesterone.

displayed almost normal growth (Figure 5a,b). However, the branches of constitutively over-expressing plants were shorter (Figure 5d,e), and the size of the main stem leaf was smaller than that of controls (Figure 5f). Similar to antisense plants, pollen from sense *GhDET2* transgenic cotton was sterile, and boll drop occurred within a few days after anthesis.

Fiber initiation and elongation are drastically reduced in antisense GhDET2 *plants but can be rescued by 24-epibrassinolide*

Scanning electron microscopy was used to examine ovules where fibers are initiated. On the day of anthesis, the density of fiber initials on an antisense *GhDET2* plant ovule was greatly reduced relative to that of a control plant ovule (Figure 6a–d). On average, there were approximately 1600 fiber cells per ovule in antisense *GhDET2* plants, much less than the approximately 5600 per ovule in the control ovules (Figure 7a). Many fiber cells from the ovules of the antisense *GhDET2* plant appeared smaller than those from ovules of control plants.

Fiber cell elongation was also inhibited in plants expressing antisense *GhDET2*. Fiber cells on the 1 DPA control plant ovules were obviously elongated, whereas those on the antisense *GhDET2* plant ovules were severely stunted (Figure 6e,f). An ovule culture method (Beasley and Ting, 1973; Kim and Triplett, 2001) was used to examine the effect of BR on fiber elongation. Fiber cell elongation was significantly reduced in the antisense *GhDET2* plant ovules (Figures 7b and 8b). However, addition of 100 nM 24-epibrassinolide (EBL, a biologically active BR) to the culture medium restored fiber cell elongation in antisense *GhDET2* plant ovules to near wild-type levels (Figures 7b and 8c).

A steroid 5α-reductase inhibitor, finasteride, blocks fiber elongation

To further examine the role of *GhDET2* and BRs on fiber elongation, finasteride, an inhibitor of steroid 5α-reductase (Rosati *et al.*, 2005), was used to suppress endogenous GhDET2 activity in cotton ovules. Ovules from wild-type plants at 2 DPA, a time at which many fiber cells have already initiated, were cultured for 5 days on medium containing different concentrations of finasteride. Higher finasteride concentrations produced an even greater inhibition of fiber elongation (Figure 8f). Little fiber cell elongation was observed in ovules cultured for 5 (Figure 8g) or 15 days (Figure 8i) with 159 μM finasteride. Finasteride-mediated inhibition of fiber elongation was partially overcome by 100 nM EBL (Figure 8j).

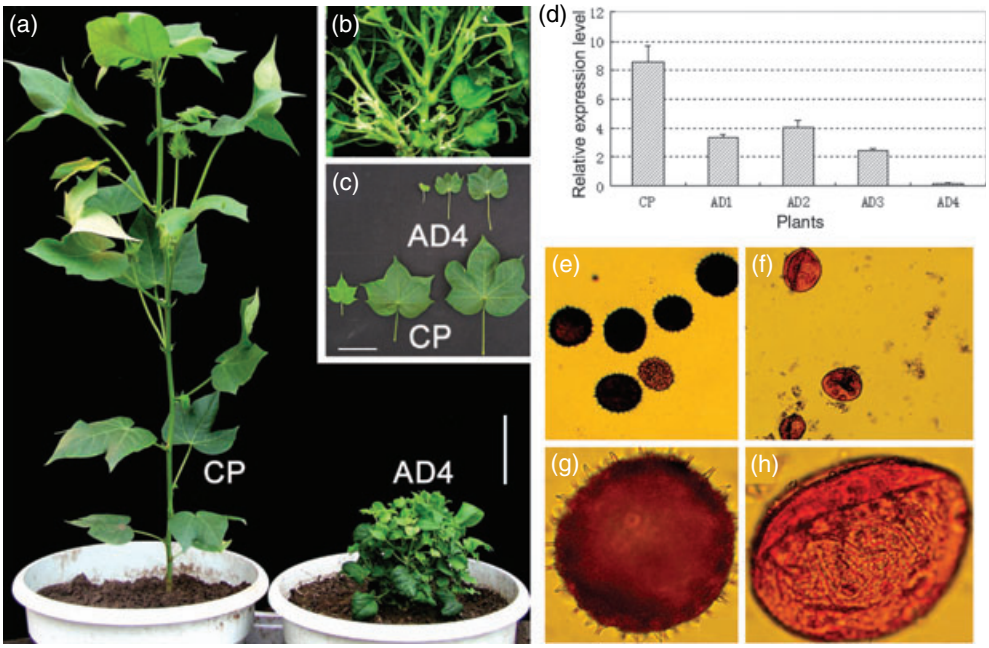

Figure 4. Comparison of *GhDET2* transcript levels and phenotypes of antisense transgenic cotton and control plants.
(a) Phenotypes of control and antisense transgenic cotton plants. CP, control plant; AD4, antisense *GhDET2* transgenic plant number 4. Scale bar = 10 cm.
(b) Branches and internode of a transgenic cotton plants.
(c) Leaves derived from transgenic cotton and control plants. CP, control plant; AD4, antisense *GhDET2* transgenic plant number 4.Scale bar = 5 cm.
(d) Real-time RT-PCR analysis of *GhDET2* transcript in antisense transgenic cotton and control plants. Total RNA was extracted from the leaves of cotton plants. To measure the expression level of endogenous *GhDET2* and eliminate interference from antisense *GhDET2* in transgenic cotton plants, the cDNAs of *GhDET2* were synthesized using GD2 primer (see Experimental procedures). Error bars represent SD for six independent experiments. CP, control plant; AD1–4, antisense *GhDET2* transgenic plants numbers 1–4.
(e)–(h) Pollen grains from a control plant were viable, as indicated by dark staining with I_2-KI, 6.5g iodine and 17.5g KI dissolved in 100 ml water (e), and produced many surface spinules (g). Pollen grains from the AD4 plant were not viable, as indicated by light-color staining with I_2-KI (f), and they produced few surface spinules (h).

Ectopic expression of GhDET2 *under the control of a seed coat-specific promoter, pFBP7, increases fiber number and fiber length*

Over-expression of sense *GhDET2* leads to sterility and boll abortion in transgenic cotton plants. Because cotton fiber is an elongated epidermal cell of the seed coat, a petunia-derived seed coat-specific gene (*FBP7*) promoter, pFBP7 (Colombo *et al.*, 1997) was used to study the effects of *GhDET2* on fiber initiation and elongation in the absence of developmental defects. To verify the tissue specificity of pFBP7 in cotton, this promoter was cloned upstream of a GUS reporter gene, and the fused gene cassette was delivered into cotton by *Agrobacterium*-mediated transformation. Under the control of the pFBP7 promoter, GUS staining was observed in cotton seed coats and fiber cells only, and acted from 1 day before anthesis to at least 10 DPA (see Figure S2).

Based on the expression pattern of GUS in pFBP7::GUS transgenic cotton plants, the pFBP7 promoter can drive expression of the target gene tissue-specifically in cotton seed coat. To up-regulate *GhDET2* in cotton seed coat cells, pBI121-GN-pFBP7-GD was constructed by replacing the 35S promoter with pFBP7 in the pBI121-GN-GD vector. We obtained 14 pFBP7::*GhDET2* transgenic cotton plants by *Agrobacterium*-mediated transformation. As expected, all T_0 transgenic plants harboring the pFBP7::*GhDET2* cassette had normal morphology and growth. Segregation analysis was performed by GUS staining of the selfed progenies (T_1). The homozygosity of T_2 plants was determined by staining T_1 selfed seeds. The homozygous T_2 generation lines were used for further study. To determine the level of *GhDET2* expression in these transgenic cotton plants, total RNA was extracted from 10 DPA fibers and subjected to quantitative real-time RT-PCR analysis. Three transgenic lines showed an obvious increase in *GhDET2* transcript levels (Figure 9a). Fiber number and fiber length were increased in the three resulting transgenic T_2 lines; for example, the fiber number and the fiber length of the F2-7-10 transgenic line were 6240 ± 610 and 32.98 ± 0.85 mm, respectively, representing 22.6% and 10.7% increases relative to control plants (5090 ± 380 fibers and 29.78 ± 1.10 mm fiber length; Figure 9b,c). However, no obvious improvement of fiber number and length was observed in transgenic lines in which no obvious change of *GhDET2* transcript was detected (data not shown). The effects of up-regulating of *GhDET2* specifically in cotton fibers further confirm that this gene is involved in fiber cell initiation and elongation.

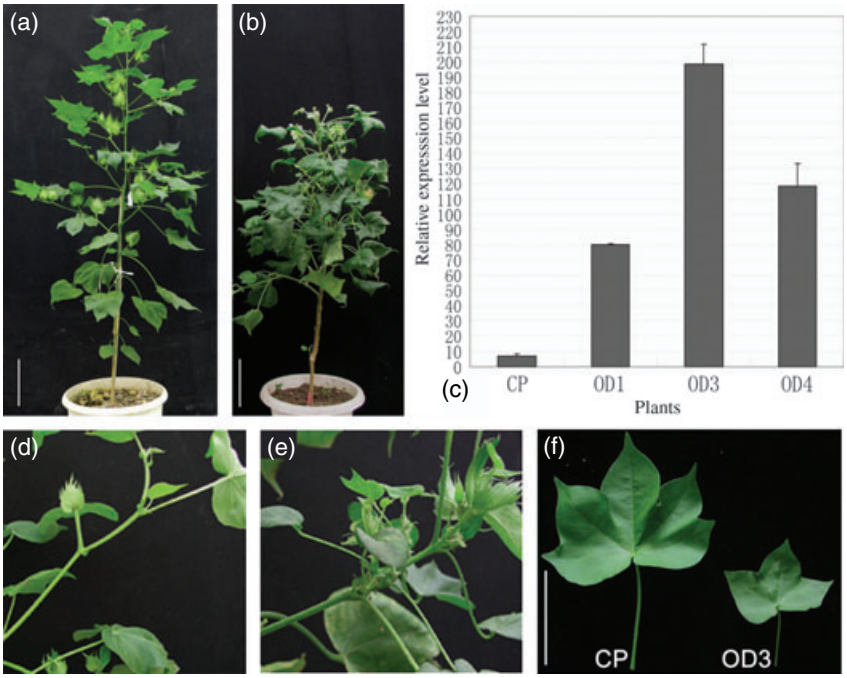

Figure 5. Comparison of *GhDET2* transcript levels and phenotypes in 35S::*GhDET2* transgenic cotton and control plants.
(a) Phenotype of a control plant. Scale bar = 20 cm.
(b) Phenotype of a transgenic cotton plant over-expressing *GhDET2*. Scale bar = 20 cm.
(c) Real-time RT-PCR analysis of the *GhDET2* transcript in transgenic cotton over-expressing *GhDET2* and control plants. CP, control plant; OD1–OD4, independent transgenic lines harboring 35S::sense *GhDET2*. Error bars represent SD for six independent experiments.
(d,e) Branches of a control plant (d) and a transgenic cotton plant (e).
(f) Leaves from a control plant (CP) and sense *GhDET2* transgenic line 3 (OD3). Scale bar = 10 cm.

Discussion

Recent studies have demonstrated that the reaction catalyzed by steroid 5α-reductases is the rate-limiting step in BR biosynthesis (Chory and Li, 1997; Li *et al.*, 1997). Consequently, loss of DET2 function often results in reduced endogenous BR levels (Fujioka *et al.*, 1997). To investigate the role of BRs in cotton fiber development, a steroid 5α-reductase gene, *GhDET2*, was cloned from cotton fibers and characterized. *GhDET2* shares high amino acid sequence similarity with previously cloned DET2 genes encoding steroid 5α-reductases. Functional assessment of *GhDET2* in CHO cells, a heterologous expression system, provides strong evidence that *GhDET2* encodes a steroid 5α-reductase. Furthermore, the alterations in plant growth and development observed in antisense *GhDET2* transgenic plants (e.g. smaller leaf size, reduced apical dominance with clusters of branches, shortened internodes and sterility) resemble the phenotype of Arabidopsis *det2* mutants. Therefore, we conclude that *GhDET2* encodes a functional steroid 5α-reductase.

Exogenous applications of BRs or BR inhibitors have been used to study the role of BRs in fiber development. Low concentrations of brassinolide (BL) can promote fiber elongation (Sun *et al.*, 2005), whereas treatment of cotton buds or cultured ovules with brassinazole (BRZ), a BR biosynthesis inhibitor (Asami *et al.*, 2000; Sekimata *et al.*, 2001), inhibited both differentiation and elongation of fibers on ovules (Shi *et al.*, 2006; Sun *et al.*, 2004). However, BRZ is structurally similar to uniconazole, a GA biosynthesis inhibitor. Furthermore, exogenous application of an active BR to BRZ-treated ovules only partially rescued fiber development (Sun *et al.*, 2004, 2005). Therefore, it is possible that the effect of BRZ is mediated, at least in part, by inhibition of GA biosynthesis (Sun *et al.*, 2005). Manipulating expression of BR biosynthetic genes in transgenic cotton plants provides a powerful tool for evaluating the role of BRs in fiber initiation and elongation. However, severe sterility and boll abortion were observed in transgenic antisense *GhDET2* cotton plants as well as transgenic plants over-expressing *GhDET2*. Similar phenotypes have been reported previously (He *et al.*, 2003; Schaeffer *et al.*, 2001) in the Arabidopsis *smt2* mutant or transgenic plants in which campesterol, a substrate of DET2 (Clouse and Sasse, 1998), was up- or down-regulated. Abnormalities resulting from constitutive over-expression or suppression of *GhDET2* may interrupt development of seeds and fiber cells, thereby making it infeasible to obtain further information on GhDET2 function in fiber development. To solve this problem, a seed coat-specific promoter, pFBP7, was used to up-regulate *GhDET2* specifically in cotton seed coat and fiber.

GhDET2 and BRs play an important role in the fiber cell initiation process. It has been well documented that fiber cells initiate on or before the day of anthesis (Ryser, 2000). Fiber initiation that takes place on 0 DPA is not a consequence of pollination because fertilization occurs at 2 DPA (Kim and Triplett, 2001). Using a quantitative real-time RT-PCR technique, we have shown that *GhDET2* expression is relatively high in 0 DPA ovules. More importantly, scanning electron microscopy of 0 DPA ovules revealed that the density of fiber cells on antisense *GhDET2* plant ovules was drastically reduced relative to

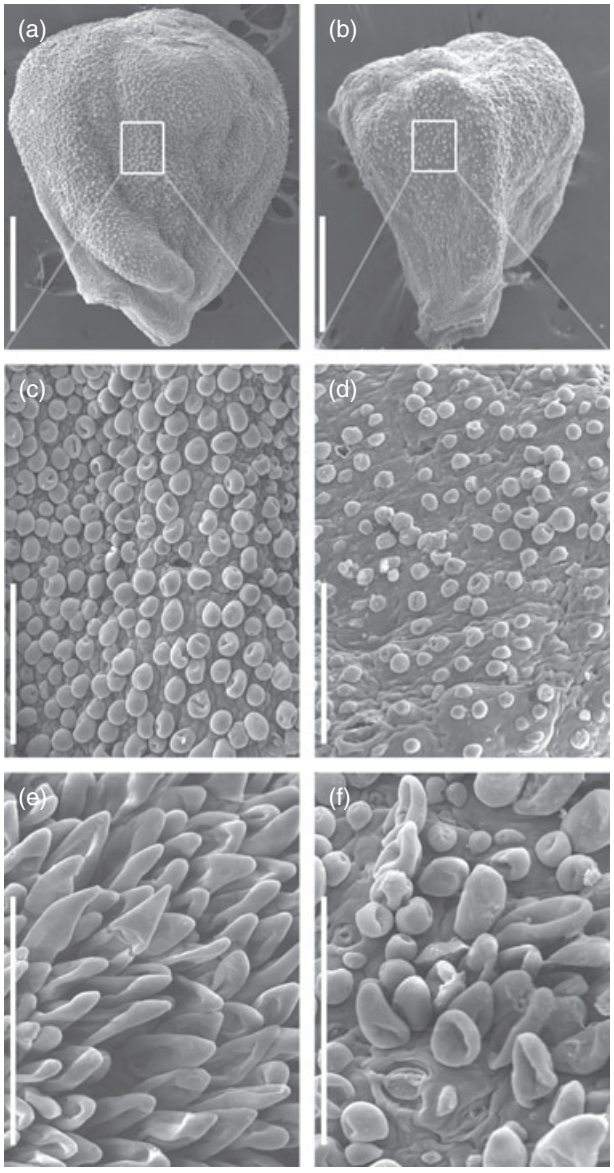

Figure 6. Scanning electron microscopy of ovules from control and antisense *GhDET2* plants.
(a) A typical 0 DPA ovule from a cotton plant. Scale bar = 500 μm
(b) A typical 0 DPA ovule from the AD4 transgenic plant. Scale bar = 500 μm.
(c) An enlarged area of the ovule shown in (a). Scale bar = 100 μm
(d) An enlarged area of the ovule shown in (b). Scale bar = 100 μm.
(e) Fiber cells from a 1 DPA ovule of a control cotton plant. Scale bar = 100 μm.
(f) Fiber cells from a 1 DPA ovule (same stage as shown in (e)) from the AD4 transgenic plant. Scale bar = 100 μm.

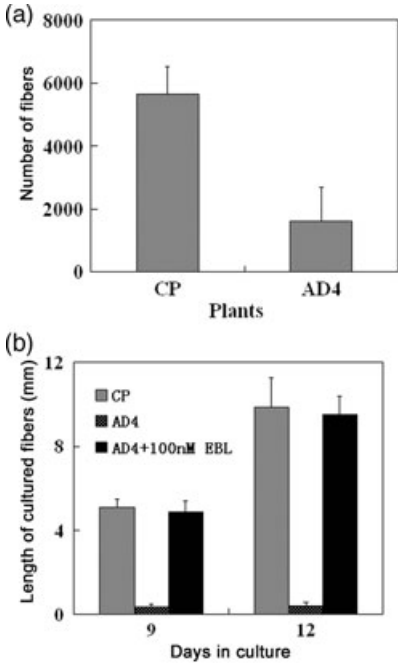

Figure 7. Effects of antisense *GhDET2* expression on fiber cell initiation (a) and elongation (b).
Fiber cells were counted at 2 DPA. Fiber length was determined according to the method described by Sun *et al.* (2005). Cultured ovules were treated with or without 100 nM 24-epibrassinolide (EBL) as indicated. CP, control plants transformed with empty vector; AD4, antisense *GhDET2* transgenic plant number 4. Error bars represent SD for five independent assays.

control ovules. Expression of 35S antisense *GhDET2* reduced 2 DPA fiber cell number many-fold compared to control plants under the same experimental conditions. Moreover, in pFBP7::*GhDET2* transgenic plants, which over-expressed *GhDET2* transcript specifically in seed coat and fibers, the number of fibers was enhanced. Regulation of fiber cells as a consequence of modulating *GhDET2* expression leads us to conclude that *GhDET2* and BRs are critically involved in the process of initiating fiber cells from cotton ovule surfaces.

GhDET2 and BRs also play an important role in fiber cell elongation. The log phase of fiber elongation takes place during the first 10 DPA (DeLanghe, 1986), and high expression of *GhDET2* in 5–10 DPA fibers correlates well with rapid elongation of fibers. In 35S-driven antisense *GhDET2* transgenic plants, fiber elongation is dramatically inhibited, and this effect can be overcome by exogenously applied active BR. Fibers are longer in transgenic plants over-expressing *GhDET2* specifically in the seed coats and fibers, consistent with previous reports that exogenous BL application increased fiber length (Kasukabe *et al.*, 2001; Sun *et al.*, 2004, 2005). Finally, finasteride, an inhibitor of steroid 5α-reductase (Rosati *et al.*, 2003, 2005), inhibited the elongation of initiated fiber cells under *in vitro* culture conditions, whereas application of EBL could reverse the effects of finasteride and restore normal fiber cell elongation.

Studies based on exogenous applications of BRs have also shown that BRs are involved in many developmental and physiological processes such as stem elongation, adventitious root development, and increase tolerance to drought, saline, chilling and heat shock stresses (Brosa,

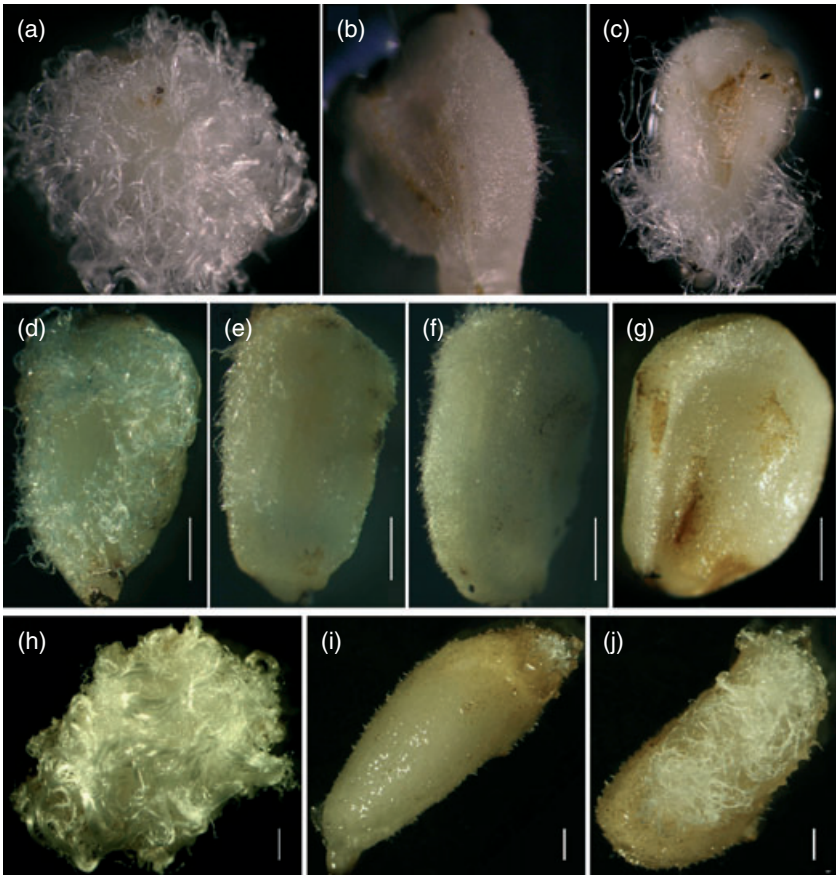

Figure 8. Effects of 24-epibrassinolide and finasteride on fiber elongation.
(a) A control plant ovule cultured for 12 days.
(b) A 35S::antisense-GhDET2 transgenic plant (AD4) ovule.
(c) A 35S::antisense-GhDET2 transgenic plant (AD4) ovule cultured for 12 days with 24-epibrassinolide (EBL).
(d)–(g) Fiber elongation on ovules from wild-type plants can be suppressed by the steroid 5α-reductase inhibitor, finasteride. Ovules from wild-
type plants were cultured for 5 days on medium with or without finasteride. (d) Without finasteride; (e) with 32 μm finasteride; (f) with 64 μm finasteride; (g) with 159 μm finasteride. Scale bar in (d)–(g) = 0.5 mm.
(h)–(j) Ovules from control plants were cultured for 15 days on medium with or without finasteride. (h) Without finasteride; (i) with 159 μm finasteride; (j) with 159 μm finasteride + 100 nm 24-epibrassinolide. Scale bar in (h)–(j) = 0.5 mm.

1999). In our study, six out of ten transgenic cotton plants expressing antisense *GhDET2* died after transfer from tissue the culture vessel to greenhouse, indicating that *GhDET2* is required for normal plant growth and development. One possible explanation for the low survival rate of antisense *GhDET2* cotton plants is reduced tolerance to various environmental stresses, such as drought stress and temperature change, thus these plants do not survive well under greenhouse conditions. Further characterization of transgenic plants expressing antisense *GhDET2* may provide some insight into the role that DET2 and BR play in responses to various biotic stresses.

Additionally, modulation of fiber number and fiber length by controlling BR or other phytohormone biosynthetic genes specifically in the seed coat or fibers may improve fiber quality and yield in cotton.

Experimental procedures

Plant materials and growth conditions

Upland cotton (*Gossypium hirsutum* L.) cv. Jimian 14 was kindly provided by Dr M. Shiying (Hebei Agricultural University, China). Transgenic cotton plants were grown in the greenhouse under natural and additional artificial light (14 h light photoperiod at 150 μmol $m^{-2}s^{-1}$) at 28–34°C during the day and 24–27°C at night.

cDNA cloning and sequence analysis

To clone a gene encoding DET2 from upland cotton, the deduced amino acid sequence of an *Arabidopsis thaliana* steroid 5α-reductase gene (DET2, GenBank accession number U53860; Li *et al.*, 1996) was used for comparison with cotton EST sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov). Candidate ESTs identified were subjected to contig analysis using the SeqMan program (DNASTAR Inc., WI, USA). BLASTX analysis (http://www.ncbi.nlm.nih.gov/blast) was performed to determine the putative start codon (ATG). A specific primer GD1 (5′-GAAAATGGCCTCCGATCAGAC-3′), designed based on the putative start codon sequence, was used to amplify the cDNA sequence from 6 DPA fiber cells by the 3′ RACE method (TaKaRa, http://www.takara.com/). Alignment analysis of multiple amino acid sequences was performed with the Megalign program (DNASTAR).

RNA isolation, Northern blot hybridization and RT-PCR analyses

Total RNA was isolated from leaves, roots, hypocotyls, cotyledons, 0 DPA flowers, 0–22 DPA ovules and 5–20 DPA fibers according to the method described by Wan and Wilkins (1994), and converted to cDNA according to the manufacturer's instructions of cDNA synthesis kit (TaKaRa). For Northern analysis, 30 μg total RNA was fractionated in 1.5% w/v agarose gels with 2.2 M formaldehyde, and transferred to Zetaprobe nylon membranes (Bio-Rad; http://www.bio-rad.com/) (Sambrook and Russell, 2001). The full-length

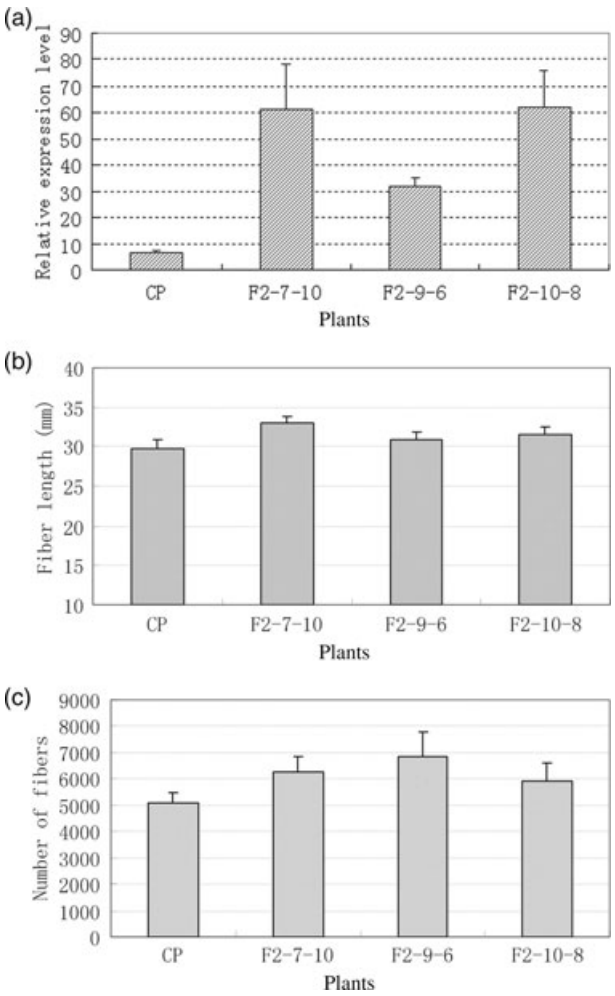

Figure 9. Up-regulation of *GhDET2* in fibers increases fiber length and fiber number in transgenic pFBP7::*GhDET2* lines

(a) Relative *GhDET2* expression measured by real-time RT-PCR in 10 DPA fibers from control plants and pFBP7-driven sense gene lines. Error bars represent SD for six independent experiments

(b) Fiber length in control plants and transgenic lines. Error bars represent SD for three independent experiments.

(c) Number of fibers in control plants and transgenic lines. Error bars represent SD for three independent experiments.

CP, control plant (empty vector transgenic cotton plants); F2-7-10, F2-9-6 and F2-10-8, transgenic lines (T_2) expressing pFBP7::*GhDET2*.

cDNA of *GhDET2* radiolabeled with α-^{32}P using Ready-To-Go DNA labelling beads (-dCTP) (Amersham, http://www.gehealthcare.com/lifesciences) was used as a probe. After hybridization, the membrane was washed twice with 2 × SSC and 0.1% SDS at 65°C for 10 min and once with 0.5 × SSC and 0.1% SDS at 65°C for 10 min (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate). Kodak x-ray films (Eastman-kodak, http://wwwcn.kodak.com) were exposed to the nylon membranes for 2 days with the aid of intensifier screens.

For quantitative real-time RT-PCR, approximately 5 μg of total RNA was used for first-strand cDNA synthesis (M-MLVRTase cDNA synthesis kit, TaKaRa), and 1 μl first-strand cDNA was used as a template for subsequent PCR amplifications using a quantitative real-time PCR kit (Bio-Rad). The PCR reactions were performed according to the manufacturer's instructions. Cotton *HISTONE3* (GenBank accession number AF024716) was used as a loading control to normalize samples. The primers used for *HISTONE3* PCR were GhHIS1 (5′-GAAGCCTCATCGATACCGTC-3′) and GhHIS2 (5′-CTACCACTACCATCATGGC-3′) as reported previously by Zhu *et al.* (2003). To determine the expression level of endogenous *GhDET2* and eliminate interference from the over-expressed antisense gene in transgenic cotton plants, *GhDET2* cDNAs for RT-PCR were synthesized using the GD2 primer (5′-CACCGAATCAATCATATGTGGC-3′).

For RT-PCR analysis of *GhDET2* transcript levels in transfected CHO cells, 5 μg total RNA was used for first-strand cDNA synthesis, and 1 μl first-strand cDNA was used as a template for subsequent PCR amplifications. To amplify a specific fragment from *GhDET2*, two primers, GD1 and GD2, were used.

Functional analysis of GhDET2 *expressed in CHO cells*

A *GhDET2* cDNA fragment in pUCm-T (including the ORF) was digested with *Not*I and *Xba*I and ligated into a mammalian expression vector, pcDNA3.1, that had been digested with the same enzymes. The resultant expression plasmid was transfected into CHO (Chinese hamster ovary) cells using a calcium phosphate procedure (Normington and Russell, 1992). The transfected cells were selected, amplified and harvested according to previous method (Normington and Russell, 1992). Then the cells were frozen in liquid nitrogen for storage at −80°C.Total RNA derived from transfected cells and non-transfected cells was used as a loading control.

Assay of steroid 5α-reductase activity was carried out using progesterone (Sigma; http://www.sigmaaldrich.com/) as a substrate. 5α-dihydroprogesterone (5α-P), the product of steroid 5α-reductase-mediated metabolism of progesterone, was separated from progesterone by thin-layer chromatography and visualized by I_2 vapor. Female rabbit liver homogenate was used as a positive control of steroid 5α-reductase activity. Preparation of cotton fiber cell homogenates and assessment of steroid 5α-reductase activity were performed according to the methods described by Rosati *et al.* (2003).

Construction of plant expression cassettes

To construct a convenient plant expression vector, plasmid pBI121 was modified. The modified vector, named pBI121-GN, contained CaMV 35S promoter::GUS::nos and nos promoter::*NPTII*::nos fusion genes. To construct the antisense *GhDET2* cassette, the *GhDET2* cDNA fragment was excised using *Bam*HI and *Eco*RI, and then inserted into pBI121-GN vector digested with the same enzymes. The resultant vector, named pBI121-GN-AGD, contained an antisense *GhDET2* sequence under the control of the CaMV 35S promoter. A pBI121-GN-GD vector containing a sense *GhDET2* sequence under the control of the CaMV 35S promoter was similarly constructed. To characterize the pFBP7 promoter, pBI121-pFBP7::GUS was constructed by replacing the 35S promoter with pFBP7 in pBI121. To up-regulate *GhDET2* in cotton seed coat cells, pBI121-GN-pFBP7-GD was constructed by replacing the 35S promoter with pFBP7 in the pBI121-GN-GD vector. These Ti plasmids were delivered into *Agrobacterium tumefaciens* strains (LBA4404), and the resulting *Agrobacterium* strain was used to produce transgenic cotton plants.

Genetic transformation of cotton

Cotton seeds (cv. Jimian 14) were surface-sterilized with 75% ethanol for 1 min followed by 0.1% $HgCl_2$ for 10 min. After

washing with sterile distilled water at least five times, the seeds were germinated on MS medium (Murashige and Skoog, 1962) at 28°C in the dark for 4-5 days. *A. tumefaciens* strain LBA4404 was used for transformation. The bacteria were grown in YEB (containing 100 mg/l kanamycin and 125 mg/l steptomycin) at 28°C for 20 h. The bacterial cells were harvested by centrifugation, and resuspended to an OD_{600} of 0.8–1.0 in MS medium supplemented with 30 g/l glucose and 100 μM acetosyringone. The hypocotyls were cut into 1 cm segments, and infected with *Agrobacterium* suspension for 20–30 min, blotted on sterile filter paper, and then transferred to co-cultivation medium (MS salt, B5 vitamins, 1.0 mg/l IBA, 0.5 mg/l KT, 30 g/l glucose, 100 μM acetosyringone, 2.0 g/l Gelrite, pH 5.4). They were maintained at 25°C in the dark for 2 days, and then transferred onto selection medium (MS salts, B5 vitamins, 1.0 mg/l IBA, 0.5 mg/l KT, 75 mg/l kanamycin, 400 mg/l cefotaxime, 30 g/l glucose, 2.0 g/l Gelrite, pH 5.8). Approximately 4 months later, embryogenic calli of the kanamycin-resistant calli formed on the selection medium. After GUS staining (Li *et al.*, 1992), the kanamycin-resistant and GUS-positive embryogenic calli were cultured in liquid MSB medium with 0.1 mg/l KT and 1.9 g/l KNO_3. These cultures were plated on elongation medium (0.5× MS salts, 15 g/l glucose, 15 g/l sucrose, 2.5 g/l Gelrite, pH 6.2). The elongated somatic embryos were transferred to SH medium (Shenk and Hildebrandt, 1972) containing 0.5% w/v activated carbon. Transgenic plantlets produced were identified by histochemical GUS staining of leaf tissues, and the GUS-positive plants were then transplanted and grown in the greenhouse.

Segregation analysis and homozygosity determination

Primary transgenic plants of pFBP7::sense *GhDET2* were confirmed by GUS staining. The segregation pattern of the selfed progeny (T_1) and the homozygosity of T_2 plants were also determined by GUS staining.

In vitro *ovule culture and 24-epibrassinolide and finasteride treatment*

Cotton ovule culture was carried out as described by Beasley and Ting (1974) with minor modifications. Briefly, cotton bolls were harvested at 2 DPA and surface-sterilized in 75% w/v ethanol for 1 min, rinsed in distilled deionized water, then soaked in 0.1% w/v $HgCl_2$ solution containing 0.05% w/v Tween-80 for 10 min for sterilization, followed by rinsing with sterile water at least five times. Ovules were separated from ovaries under sterile conditions, and then immediately placed on BT media (Beasley and Ting, 1974) supplemented with 5 μM NAA and 0.5 μM GA. The culture plates were incubated at 31°C in darkness without agitation.

For finasteride treatment, various concentrations of finasteride (*N*-(1,1-dimethylethyl)-3-oxo-4-azaandrost-1-ene-17-carboxamide) (Merck Sharp & Dohme Ltd) were added to BT media. For the rescue assay, antisense *GhDET2* or finasteride-treated ovules were treated with 100 nM 24-epibrassinolide (Yunda S & T Company) prior to measurement of fiber cell length., The culture medium contained 3.2 mM DMSO throughout.

Fiber cell determination and scanning electronic microscopy

For each transgenic event, the numbers of fiber initials per ovule were counted at 2 DPA as described previously (Gialvalis and Seagull, 2001; Seagull and Giavalis, 2004). To monitor the kinetics of fiber elongation, the cultured fiber lengths at 9 and 12 DPA were measured with a ruler after soaking fibers in 95°C water for 5 min (Sun *et al.*, 2005). For scanning electronic microscopy, samples were prepared as previously described (Sun *et al.*, 2005). The developing cotton ovules were examined and photographed with a Hitachi S-3000 N scanning electron microscope.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Southern blot of *GhDET2*.

Figure S2. GUS histochemistry of ovules and fibers from transgenic plants harboring the pFBP7::GUS cassette.

This material is available as part of the online article from http://www.blackwell-synergy.com

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