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Transcriptome Profiling, Molecular Biological, and Physiological Studies Reveal a Major Role for Ethylene in Cotton Fiber Cell Elongation [W] [OA]

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Upland cotton (*Gossypium hirsutum*) produces the most widely used natural fibers, yet the regulatory mechanisms governing fiber cell elongation are not well understood. Through sequencing of a cotton fiber cDNA library and subsequent microarray analysis, we found that ethylene biosynthesis is one of the most significantly upregulated biochemical pathways during fiber elongation. The *1-Aminocyclopropane-1-Carboxylic Acid Oxidase1-3* (*ACO1-3*) genes responsible for ethylene production were expressed at significantly higher levels during this growth stage. The amount of ethylene released from cultured ovules correlated with *ACO* expression and the rate of fiber growth. Exogenously applied ethylene promoted robust fiber cell expansion, whereas its biosynthetic inhibitor L-(2-aminoethoxyvinyl)-glycine (AVG) specifically suppressed fiber growth. The brassinosteroid (BR) biosynthetic pathway was modestly upregulated during this growth stage, and treatment with BR or its biosynthetic inhibitor brassinazole (BRZ) also promoted or inhibited, respectively, fiber growth. However, the effect of ethylene treatment was much stronger than that of BR, and the inhibitory effect of BRZ on fiber cells could be overcome by ethylene, but the AVG effect was much less reversed by BR. These results indicate that ethylene plays a major role in promoting cotton fiber elongation. Furthermore, ethylene may promote cell elongation by increasing the expression of sucrose synthase, tubulin, and expansin genes.

INTRODUCTION

Cotton plants produce the most prevalent natural fiber used in the textile industry and are one of the mainstays of the global economy. Cotton fibers, commonly known as cotton lint, are single-celled trichomes differentiated from the ovule epidermis. Upland cotton (*Gossypium hirsutum*) generally grows up to 30 to 40 mm in length and to ~15 μm in thickness at full maturity and accounts for 90% of the production in the world (Basra and Malik, 1984; Tiwari and Wilkins, 1995), while a further 5 to 8% is produced from another tetraploid species, *Gossypium barbadense*.

The quality and productivity of cotton depends mainly on two biological processes: fiber initiation, which determines the number of fibers present on each ovule, and fiber elongation, which determines the final length and strength of each fiber. During the most active elongation period (5 to 20 d postanthesis [DPA]), vigorous cell expansion with peak growth rates of >2 mm/day are observed in upland cotton (John and Keller, 1996; Ji et al., 2002). As one of the most elongated plant cells, cotton fiber is considered a model system for studying cell elongation and cell wall biogenesis (Kim and Triplett, 2001).

Cell elongation and expansion contribute significantly to the growth and morphogenesis of higher plants since cells usually undergo substantial enlargement when they exit the meristems and differentiate. The extent of elongation depends on the cell type and is often regulated by environmental cues and endogenous hormones. Auxin (indole-3-acetic acid), gibberellin (GA), and brassinosteroids (BRs) have long been known to play pivotal roles in plant cell expansion or elongation (Phinney, 1984; Evans, 1985; Crozier et al., 2000; Wang and He, 2004). In vitro application of GA and BR promotes cotton fiber elongation, while treatment of cotton floral buds with brassinazole (BRZ; a brassinosteroid biosynthesis inhibitor) results in a complete absence of fiber differentiation. Sun et al. (2005) concluded that BR is required for both fiber initiation and elongation. However, the endogenous involvement of the above plant hormones during

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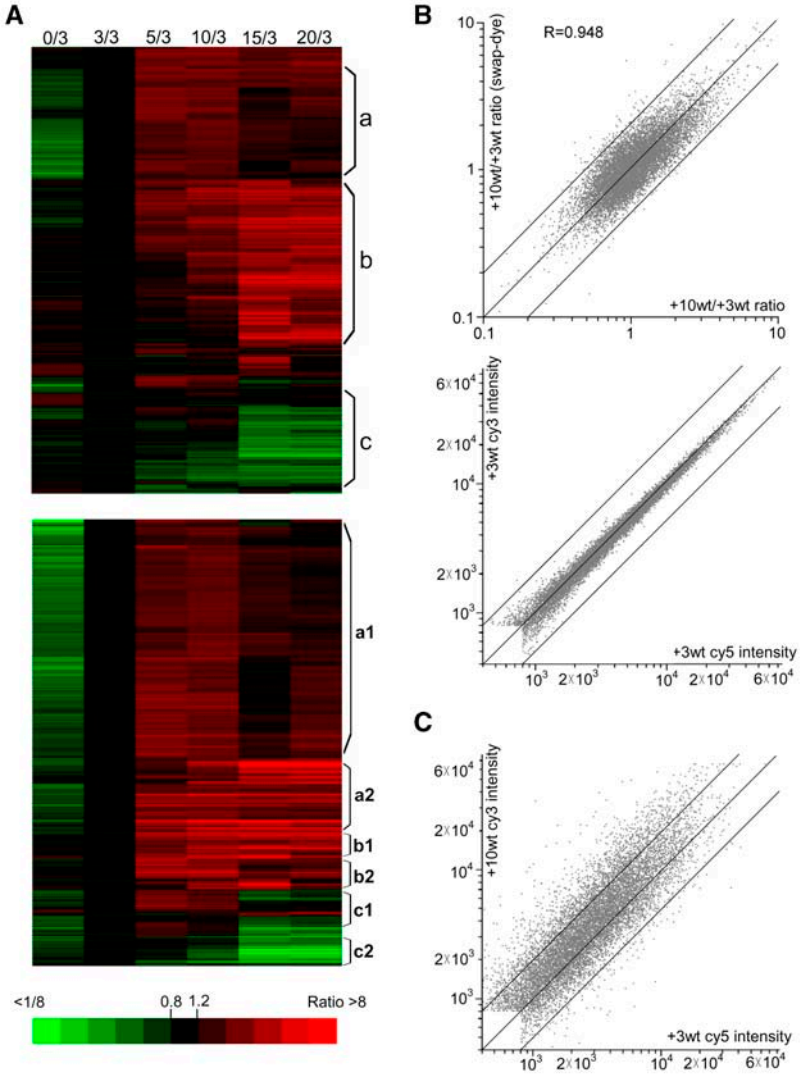

Figure 1. TreeView Representation of Fiber-Specific Cotton ESTs and Analysis of Data Quality.

(A) Top panel: hierarchical clustering of 2522 ESTs that showed FDR-corrected P values <0.001 in at least one of the growth stages. The signals are shown in a red-green color scale, where red represents higher expression and green represents lower expression. The numbers represent the DPA of ovule harvest of the hybridizing RNA. An RNA sample from 3-DPA ovules was used as the reference for each hybridization. a and b, genes induced before or after 3 DPA and maintained at relatively high levels throughout the experimental period; c, genes induced before 3 DPA and repressed drastically around 10 DPA. Bottom panel: hierarchical clustering of 778 ESTs that were developmentally upregulated in wild-type ovules but not in the mutant. a1, genes induced at 3 DPA with peak levels found at 5 to 10 DPA; a2, genes induced at 3 DPA and peaking around 10 to 20 DPA; b1, genes induced at 5 DPA and peaking around 10 to 20 DPA; b2, genes induced at 5 DPA with peak levels found at 5 to 10 DPA; c1, genes repressed at 15 DPA; c2, genes repressed at 5 or 10 DPA.
(B) Experimental variation and reproducibility assessment from randomly chosen microarray hybridizations. Top panel: comparisons of expression ratios obtained from swap-dye experiments to show the labeling efficiency of different dyes. Bottom panel: self-hybridization results obtained after probing the microarray with the same RNA sample prepared from 3-DPA wild-type ovules and labeled separately with either Cy3 or Cy5 dye.
(C) Scatterplot comparisons of 10/3-DPA hybridization data showing systematic upregulation of a large fraction of ESTs during the fast cell elongation period.

cotton fiber elongation is largely unknown. Several attempts have been made to alter the expression of genes involved in auxin and cytokinin biosynthesis in the fibers, but no favorable phenotypic changes were observed in the resultant transgenic plants (John, 1999). Ethylene is another phytohormone that has been extensively studied in fruit ripening, dormancy release, flower senescence and abscission, and stress responses (Bleecker and Kende, 2000; Crozier et al., 2000). Recent literature indicates that ethylene also acts as a positive regulator of root hair, apical hook, and hypocotyl development

Table 1. Correlation Coefficients Obtained from Microarray Hybridization Experiments with Total RNA Samples Prepared from Wild-Type or Mutant Cotton Ovules Harvested at Different Growth Stages

	Biological Coefficients			Technical Coefficients		
Microarray Samples[a]	(1,2)	(2,3)	(1,3)	(1,1′)	(2,2′)	(3,3′)
+3wt/0wt	0.924	0.928	0.938	0.968	0.966	0.957
+3wt/+3wt	0.982	0.989	0.933	0.959	0.965	0.956
+3wt/+3*fl*	0.925	0.918	0.931	0.960	0.940	0.925
+5wt/+3wt	0.933	0.926	0.917	0.962	0.946	0.937
+10wt/+3wt	0.912	0.932	0.924	0.963	0.945	0.948
+15wt/+3wt	0.966	0.964	0.951	0.976	0.964	0.977
+20wt/+3wt	0.965	0.945	0.966	0.963	0.945	0.954
+10wt/+10*fl*	0.963	0.951	0.952	0.965	0.956	0.937

[a] 0wt, +3wt, +5wt, +10wt, +15wt, and +20wt, samples derived from 0-, 3-, 5-, 10-, 15-, and 20-DPA wild-type upland cotton ovules; +3*fl* and +10*fl*, samples derived from 3- and 10-DPA *fuzzless-lintless* (*fl*) mutant upland cotton ovules.

(Raz and Ecker, 1999; Cho and Cosgrove, 2002; Achard et al., 2003; Seifert et al., 2004; Grauwe et al., 2005). Mutants deficient in ethylene responses were shown to have significantly shorter root hairs, whereas exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) resulted in longer or ectopic root hairs (Tanimoto et al., 1995; Pitts et al., 1998).

A number of genes expressed in developing cotton fibers have been isolated. The expression of a plasma membrane proton-translocating ATPase, a vacuolar ATPase, a phosphoenolpyruvate

Table 2. Representative Fiber-Preferential Metabolic Pathways Identified by KOBAS

KEGG Pathways[a]	No. of Cotton Genes Located in Various Pathways	No. of Fiber-Upregulated Genes	P Value	FDR-Corrected P Value
Total	2914	162	—	—
Ethylene biosynthesis	5	3	0.0016	0.0295
γ-Hexachlorocyclohexane degradation	49	8	0.0049	0.0376
Cytoskeleton	75	10	0.0077	0.0376
Fatty acid biosynthesis and elongation	64	9	0.0080	0.0376
Glycosaminoglycan degradation	16	4	0.0099	0.0376
Stilbene, coumarine, and lignin biosynthesis	73	9	0.0183	0.0522
Ascorbate and aldarate metabolism	63	8	0.0217	0.0522
DDT degradation	12	3	0.0256	0.0522
Fluorene degradation	42	6	0.0267	0.0522
Androgen and estrogen metabolism	5	2	0.0275	0.0522
N-Glycan degradation	23	4	0.0356	0.0615
BR biosynthesis	6	2	0.0397	0.0629
Atrazine degradation	1	1	0.0556	0.0813
Pentose and glucuronate interconversions	28	4	0.0665	0.0819
Glycerolipid metabolism	65	7	0.0665	0.0819
ABC transporters, eukaryotic	8	2	0.0690	0.0819
Fructose and mannose metabolism	58	6	0.1002	0.1076
Riboflavin metabolism	10	2	0.1031	0.1076
Glycosphingolipid metabolism	33	4	0.1076	0.1076
Terpenoidbiosynthesis	11	2	0.1215	0.1140
Bile acid biosynthesis	23	3	0.1325	0.1140
Benzoate degradation via hydroxylation	12	2	0.1407	0.1140
Cell division	12	2	0.1407	0.1140
Auxin biosynthesis	3	0	—	—
GA biosynthesis	4	0	—	—
RNA polymerase	20	0	—	—
Replication, recombination, and repair factors	23	0	—	—

[a] All KEGG pathways were retrieved from KEGG Release 35.0 on July 1, 2005. Some plant-specific pathways were computed manually by extracting genes from the category named ''enzymes'' in KEGG due to insufficient pathway annotations. Pathways with a P value higher than that of ''cell division'' were not listed consecutively to save space.

carboxylase, a major intrinsic protein, and an α-tubulin gene was found to culminate during peak fiber expansion (Smart et al., 1998). Using the PCR-select cDNA subtraction method, 172 fiber-specific genes were identified, many of which encode components involved in cell expansion, lipid biosynthesis, and cell wall loosening. Among them, 29 cDNAs were found to increase >50-fold during the rapid fiber elongation period (Ji et al., 2003). Other related studies indicate that *G. hirsutum ACTIN1*, *Gossypium arboreum MYB2*, a sucrose synthase gene, and several tubulin genes are preferentially expressed in developing fibers and likely participate in various fiber elongation pathways (Ji et al., 2002; Li et al., 2002b, 2005; Ruan et al., 2003; Feng et al., 2004; Wang et al., 2004). A microarray study based on 12,227 ESTs from a diploid *G. arboreum* cotton species has identified >80 genes that are significantly upregulated during the time of secondary cell wall synthesis (at 24 DPA) (Arpat et al., 2004).

Although these molecular and genomic studies shed light on mechanisms responsible for fiber cell differentiation and expansion, a systematic survey of the genes crucial for this important process has yet to be performed. In order to identify genes regulating fiber cell elongation, we obtained 12,233 unique ESTs (uniESTs) from fast elongating fiber cells of a tetraploid species (*G. hirsutum*) that is widely used for industrial cotton lint production. Based on these ESTs, a custom-designed cDNA microarray was used for expression profiling of genes related to fiber elongation. After probing the cDNA microarrays with RNA samples from wild-type ovules harvested at different developmental stages and from a fiberless mutant, a large number of genes upregulated in elongating fiber cells was identified. Analysis of these genes revealed important roles of hormone biosynthesis during cotton fiber elongation, which was confirmed by further biochemical and physiological studies.

RESULTS

Obtaining 12,233 uniESTs by Sequencing 36,000 cDNAs from 5- to 10-DPA Cotton Ovules

A high-quality cDNA library was constructed using RNA samples harvested from 5- to 10-DPA tetraploid wild-type upland cotton ovules with their fiber attached (Lu, 2002). Random sequencing of 15 × 2400 bacterial colonies after in vivo excision of the λ-ZAP DNA resulted in 29,992 high-quality ESTs that were clustered into 12,233 uniESTs that included 4648 clusters and 7585 singletons. Clusters and singletons with alternatively spliced isoforms were reassembled manually to produce another set of 11,719 uniESTs that were used for PCR amplification and subsequent microarray construction.

Identification of 778 cDNAs Preferentially Expressed in Developing Cotton Fibers

Most of the uniESTs (11,692 out of 11,719) were successfully amplified by PCR and printed on amino silane slides to

Table 3. FDR-Corrected P Values for cDNAs Encoding Key Enzymes in Various Plant Hormone Biosynthesis Pathways

		FDR-Corrected P Value[a]							
Array ID	Enzyme	0wt/3wt	5wt/3wt	10wt/3wt	15wt/3wt	20wt/3wt	10wt/10*fl*	P < 0.005	P < 0.001
Ethylene biosynthesis									
CM106A07	ACS6	0.0410	0.0890	0.1200	0.0028	0.0083	0.0023	Y[b]	N
CM027E10	ACO1	0.1200	0.0000	0.0000	0.0000	0.0000	0.0000	Y	Y
CM050E03	ACO2	0.0000	0.0004	0.0013	0.0000	0.0100	0.0000	Y	Y
CM094A02	ACO3	0.0062	0.2000	0.0045	0.0009	0.0490	0.0005	Y	Y
CM034H03	ACO4	0.0320	0.0380	0.0140	0.3000	0.3500	0.0026	N	N
BR biosynthesis									
CM005B02	SMT1	0.0011	0.0000	0.0000	0.0000	0.0000	0.0000	Y	Y
CM021F11	DWF5	0.0092	0.1300	0.0170	0.6800	0.3800	0.0060	N	N
CM010A11	DWARF1	0.5400	0.0800	0.9993	0.2200	0.0390	0.2300	N	N
CM054H06	DEM1	0.0300	0.1200	0.0320	0.4600	0.1200	0.0410	N	N
CM010F11	DET2	0.0011	0.0033	0.0007	0.0050	0.0320	0.0000	Y	N
CM117F12	DDWF	0.0250	0.0004	0.0022	0.0078	0.0010	0.0760	N	N
GA biosynthesis									
CM092E03	GA20ox1	0.3100	0.0170	0.1200	0.0000	0.0000	0.0008	Y[b]	Y[b]
CM122F06	GA20ox2	0.0010	0.1900	0.0022	0.0120	0.4700	0.0000	Y[b]	N
CM041F09	GA3ox1	0.0200	0.0031	0.0056	0.0000	0.0000	0.0005	Y[b]	Y[b]
CM054D10	GA3ox2	0.2600	0.0130	0.0110	0.0000	0.0001	0.3400	N	N
Auxin biosynthesis									
CM099B04	NIT	0.1300	0.9600	0.0084	0.0024	0.0028	0.0190	N	N
CM040A08	FMO1	0.3900	0.5600	0.0360	0.0810	0.2900	0.1400	N	N
CM089E02	TDC	0.0670	0.0330	0.0066	0.0005	0.0074	0.1900	N	N

[a] FDR-corrected P values <1e-6 were shown as 0.

[b] Genes showed higher expression levels in 10-DPA mutant ovules compared with that of the wild-type tissue and were not considered as fiber-specific genes.

construct a cDNA microarray that was used to identify genes specifically or preferentially expressed in developing cotton fibers. First, we identified developmentally upregulated genes by hybridizing the microarray with RNA samples from wild-type ovules harvested at 0, 3, 5, 10, 15, and 20 DPA, using a 3-DPA sample as a reference for each hybridization. A total of 2522 genes passed multiple testing (with false discovery rate [FDR]–corrected P values <0.001 in at least one of the above growth stages) on the MAANOVA program and were considered developmentally upregulated (Figure 1A, top panel). To identify fiber-specific ESTs, we further probed the microarray with RNA samples of 3- and 10-DPA wild-type ovules against that of the *fl* mutant, which fails to initiate fiber cells, harvested at the same growth stage. Genes that showed simultaneous upregulation in the wild type and in the mutant (with FDR-corrected P values <0.001) were considered unrelated to fiber development and were excluded from the second clustering. The resulting final group contained 778 genes that showed increased expression during fiber elongation but were not upregulated in the ovules of the mutant (Figure 1A, bottom panel; see Supplemental Table 1 online).

The quality of the microarray data was assessed in several ways. Correlation coefficients (*r* values) calculated from different samples were used as measures of biological reproducibility, and *r* values obtained from swap-dye experiments of individual biological samples were used as measures of technical reproducibility (Table 1). Figure 1B (top panel) shows results obtained from one randomly chosen swap-dye experiment for visual assessment of the technical reproducibility. All but one data point obtained after self-hybridization of Cy3- and Cy5-labeled probes prepared using the same RNA sample from 3-DPA wild-type ovules were scattered inside the ±twofold lines (Figure 1B, bottom panel), indicating that our microarray experiments were precisely executed. Because an extensive expression pattern shift was recorded in mRNA populations of late developmental stages (shown in Figure 1C as an example), we applied a linear normalization strategy (van de Peppel et al., 2003) instead of the nonlinear global intensity-based LOWESS program (Yang et al., 2002). Evenly distributed signal intensities obtained for the 40 internal control genes after linear normalization (see Supplemental Table 2 online) indicated that it was a suitable method for cotton fiber transcriptome analysis.

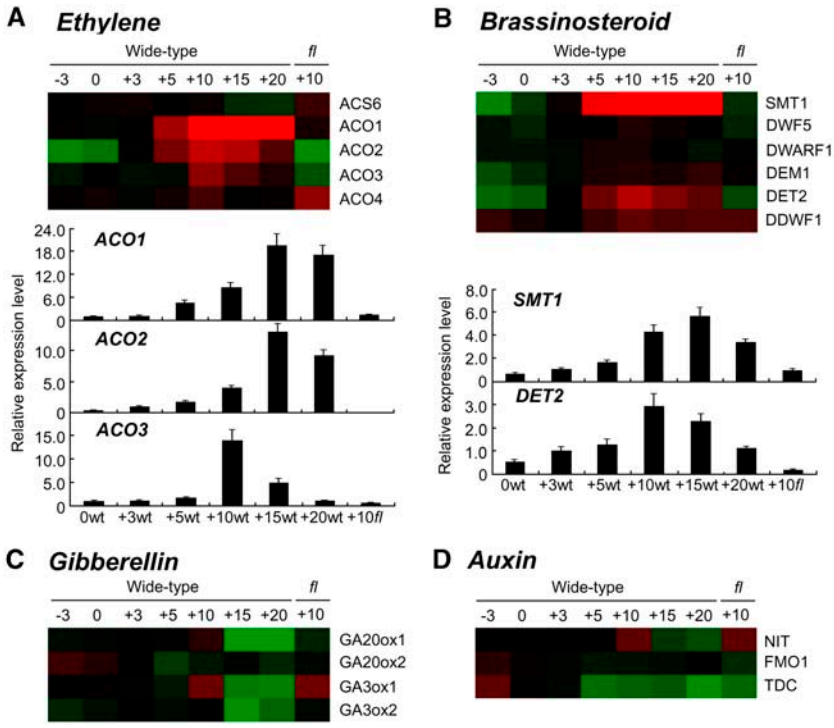

Figure 2. Detailed Expression Profiles of Genes in Major Plant Hormone Biosynthetic Pathways That Showed FDR-Corrected P Values <0.001.

(A) to **(D)** Comparison of expression ratios obtained from six microarray hybridizations for genes involved in ethylene, BR, GA, and auxin biosynthesis. Bottom panels of **(A)** and **(B)**: For data verification, QRT-PCR analysis was performed on *ACO1-3*, *SMT1*, and *DET2*, which were regarded as fiber-preferentially expressed genes after analysis of microarray hybridization data with FDR-corrected P values <0.001. Relative expression levels were determined after normalizing all data to that of 3-DPA wild-type ovules, which was set to 1.0. Error bars represent SD for three independent experiments. The time (DPA) of ovule collection is indicated. ACS6, ACC synthase 6; ACO, ACC oxidase; SMT1, 24-sterol C-methyltransferase; DEM1, steroid demethylase; DWF5, sterol δ7 reductase; DWARF1, C-24 sterol reductase; DET2, steroid 5-α-reductase; DDWF1, putative cytochrome P450 gene catalyzing typhasterol to castasterone; GA20ox, GA 20-oxidase; GA3ox, GA 3-hydroxylase; NIT, nitrilase; FMO1, flavin-containing monooxygenase; TDC, tryptophan decarboxylase.

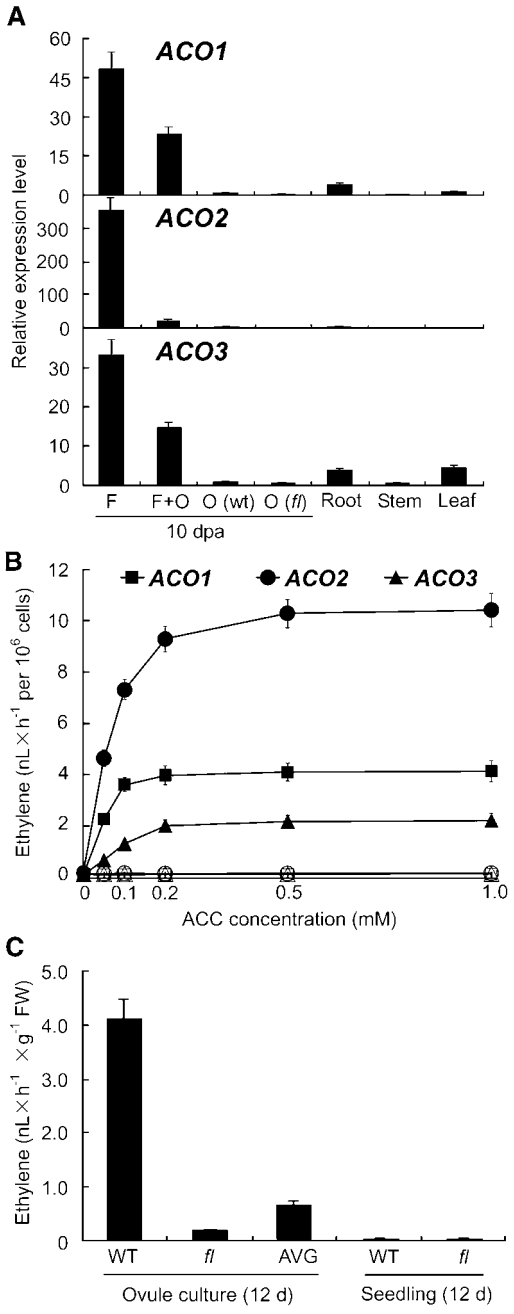

Figure 3. Tissue-Specific Expression, Enzymatic Activity of *ACO1-3* in Yeast, and Ethylene Production from Different Cotton Samples.

(A) *ACO1-3* transcripts are specifically expressed in developing cotton fibers. *ACO1-3* expression levels are quantified by QRT-PCR, and data of all samples are normalized to that of 10-DPA wild-type ovules with fiber cells removed, which is set to 1.0. Error bars represent SD for three independent experiments. F, fibers only; F+O, fibers attached on the ovules; O, ovules with fibers removed.

(B) Enzyme activity in yeast cells expressing each of the *ACO* genes. Air samples (100 μL) from each reaction containing 10^6 yeast cells, cofactors, and various amounts of the substrates were removed and injected into the column of a gas chromatograph for ethylene measurements. Means ± SD (bars) obtained for each substrate concentration point were calculated from measurements of three different cultures of the same transformant cell line with triplicate measurements of each cell culture. Closed symbols indicate results obtained from galactose-induced cells, and open symbols indicate that of noninduced cells.

(C) Only wild-type ovules produced substantial amounts of ethylene. Mutant or wild-type ovules (~30 collected from two flowers) were cultured for a total of 12 d, and ethylene production was analyzed in a gas chromatograph by injecting 100 μL of the head air from the culture flasks (with total available volume of ~50 mL) directly to the HP-PLOTQ column. Wild-type ovules were also cultured in the presence of 1.0 μM AVG for ethylene measurements. Means ± SD (bars) were calculated from measurements of three different ovule cultures (or closed glass jars for seedlings) with triplicate measurements for each sample. FW, fresh weight.

Several Biochemical Pathways Are Significantly Upregulated during Fiber Development

All cotton cDNAs on the microarray were analyzed using KOBAS (for KEGG Orthology Based Annotation System) (Mao et al., 2005) to identify the metabolic pathways in which they function. KOBAS mapped 2914 of the complete set of 11,692 ESTs to 168 KEGG pathways, including 162 fiber-specific genes to 102 pathways. Twelve of these pathways were significantly upregulated ($P < 0.05$) during the fiber elongation period if judged by P value based on hypergeometric distribution. Five of these pathways had P values <0.05 after FDR correction (Table 2). Three of five genes in ethylene biosynthesis were induced significantly so that this pathway ranked number one with an FDR-corrected P value of 0.0295. The cytoskeleton, fatty acid biosynthesis, and elongation pathways showed P values <0.05 as well. BR biosynthesis had a P value of 0.0629 after FDR correction and ranked number 12 among all pathways identified (Table 2).

Characterization of the Expression Patterns of Phytohormone Biosynthetic Genes

Since KOBAS analysis revealed the potential importance for both ethylene and BR during cotton fiber development, we obtained complete cDNA sequences from plasmids containing genes encoding enzymes responsible for catalyzing various steps in ethylene, BR, GA, and auxin biosynthesis. Of the 18 genes analyzed, 11 putative full-length cDNAs were recovered based on GenBank annotations of genes from other plant species. Most of the homologs were found to share >70% sequence identity. Although cDNAs corresponding to *GA20ox1*, *GA3ox1*, and *GA3ox2* shared only 31 to 32% overall sequence identity with homologous genes in other plant species, all of them showed conserved functional domains specific for their particular biochemical reactions (see Supplemental Table 3 online).

Analysis of the microarray data using FDR-corrected P values <0.001 as the significance criterion indicated that the expression of *ACO1-3*, *SMT1*, and *DET2* was upregulated in at least one of the time points during fiber elongation and was reduced in the *fl* mutant (Table 3). By contrast, *ACS6*, *GA20ox1*,

GA20ox2, and *GA3ox1* genes showed higher levels of expression in mutant ovules than in the wild-type tissue so that they were not considered as fiber-preferential genes (Table 3). Expression of the other putative hormone biosynthetic genes did not show significant changes during fiber elongation (Figure 2, Table 3).

The expression levels of *ACO1-3*, *SMT1*, and *DET2* were further quantified by quantitative real-time RT-PCR (QRT-PCR) (Figures 2A and 2B, bottom panels). Transcript levels of *ACO1* and *2* reached peak values around 15 DPA, which were ~18 or 13 times that found at 3 DPA. *ACO3* transcript levels increased to a similar extent but peaked around 10 DPA (Figure 2A, bottom panel). The relative levels of *SMT1* and *DET2* mRNA increased by sixfold and threefold at 15 and 10 DPA, respectively, compared with that at 3 DPA (Figure 2B, bottom panel). The genes potentially involved in the GA or auxin biosynthetic pathways were not significantly upregulated during fiber development (Figures 2C and 2D).

To confirm the fiber specificity of the *ACO* genes, fiber cells were harvested from ovules and analyzed separately. QRT-PCR results showed that the expression levels of *ACO1*, *2*, and *3* were 48.2 ± 6.5, 357.1 ± 42.5, and 33.1 ± 4.2 folds higher, respectively, in separated fiber cells than in ovules with fibers removed (Figure 3A). Interestingly, 10-DPA wild-type ovules with fiber cells removed and 10-DPA *fl* mutant ovules contained almost identical levels of *ACO1*, *2*, and *3* transcripts (Figure 3A). *ACO1* and *3* were expressed highly in elongating fiber cells, with very small amounts found in roots, stems, and leaves, whereas *ACO2* was only detected in fibers (Figure 3A).

All Three Cotton *ACO* Genes Encode Functional Enzymes

To determine whether the cotton *ACO1-3* genes encoded functional ACC oxidases, they were introduced into yeast cells behind an inducible promoter and assayed for ethylene production from ACC (Hamilton et al., 1991). Upon galactose induction, substantial amounts of ethylene were produced in yeast cells transformed with expression vectors carrying each of the *ACO* genes (Figure 3B). Nontransformed yeast

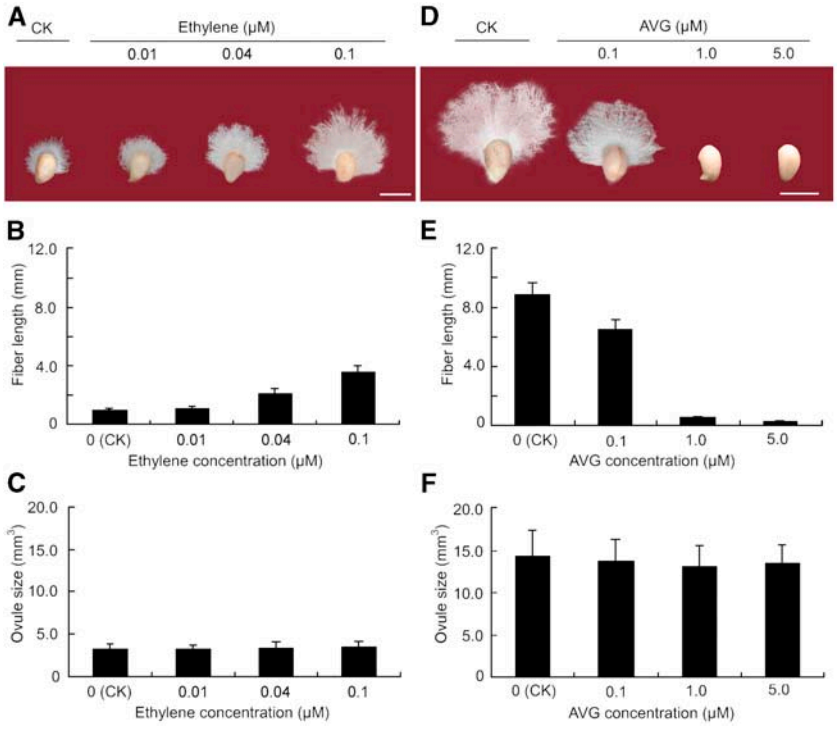

Figure 4. Exogenous Ethylene Promotes and AVG Inhibits Fiber Cell Elongation.

(A) Phenotypes of 7-d-old wild-type ovules (collected at 1 DPA) cultured with or without (CK) ethylene supplementation. Bar = 2.5 mm.
(B) Final fiber lengths measured at the end of the 6-d culture period.
(C) Ovule sizes measured at the end of the 6-d culture period.
(D) Phenotypes of 13-d-old wild-type ovules (collected at 1 DPA) cultured with or without (CK) AVG. Bar = 5 mm.
(E) Final fiber lengths measured at the end of the 12-d culture period.
(F) Ovule sizes measured at the end of the 12-d culture period.
Ovules were cultured for a longer period in **(D)** to **(F)** to maximize the differences in fiber length between the AVG-treated and the non-AVG-treated ovules. Each data point in **(B)** and **(E)** is the average of three independent ovule culture experiments, with a total of 90 fiber cells measured on three individual ovules every time. Each data point in **(C)** and **(F)** is the average of 30 ovules obtained from three independent culture experiments. Error bars indicate SD ($n = 30$).

produced no detectable amount of ethylene (data not shown), and transformed but not induced yeast cells produced negligible amounts of ethylene (Figure 3B). Yeast expressing *ACO2* showed the highest enzyme activity, with *ACO1* and *3* having somewhat lower activities (Figure 3B). Protein gel blotting analysis indicated that different levels of protein expression might be responsible for the different activities detected from the three ACOs (data not shown).

Ovules with Elongating Fibers Release Significantly Higher Amounts of Gaseous Ethylene

We next measured the amount of gaseous ethylene produced from ovules with or without fibers or from seedlings to determine whether increased expression of *ACO* genes leads to increased hormone biosynthesis. The final ethylene concentration in the headspace of flasks containing wild-type cotton ovules (0.02 g average weight) cultured for 12 d was 4.11 ± 0.36 nL $\times$ $h^{-1} \times g^{-1}$ fresh weight. We recorded only 0.19 ± 0.02 and 0.67 ± 0.06 nL $\times$ $h^{-1} \times g^{-1}$ fresh weight of ethylene from cultures of the *fl* mutant and the wild type, respectively, that received L-(2-aminoethoxyvinyl)-glycine (AVG) treatment (Figure 3C). Both wild-type and *fl* mutant seedlings grown in a closed jar did not release detectable levels of ethylene in our system (Figure 3C). These results clearly indicated that a substantial amount of ethylene was synthesized only in elongating fiber cells.

Ethylene Promotes Cotton Fiber Cell Elongation

Increased expression of *ACO* genes and production of ethylene in elongating fiber cells suggest that ethylene may actually promote fiber cell elongation. We thus tested the effect of ethylene on fiber cell growth by treating in vitro–cultured ovules with ethylene. When increasing concentrations of ethylene were applied in the culture flasks for 6 d, fiber lengths increased in a dose-dependent manner (Figures 4A and 4B). Treatment with 0.1 μM ethylene increased the fiber length by threefold (3.60 ± 0.40 mm versus 1.2 ± 0.1 mm without ethylene). Accordingly, application of the ethylene biosynthetic inhibitor AVG reduced the fiber length significantly. When 1 or 5 μM of AVG was added in the culture media, no fiber elongation was visible after 12 d of culture, whereas fibers grew to ~8.5 mm in the absence of AVG (Figures 4D and 4E). Addition of either ethylene or AVG did not affect the final size of the cultured ovules (Figures 4C and 4F), suggesting that the treatment did not have toxic effects and that ethylene plays a specific and important role in promoting fiber cell elongation.

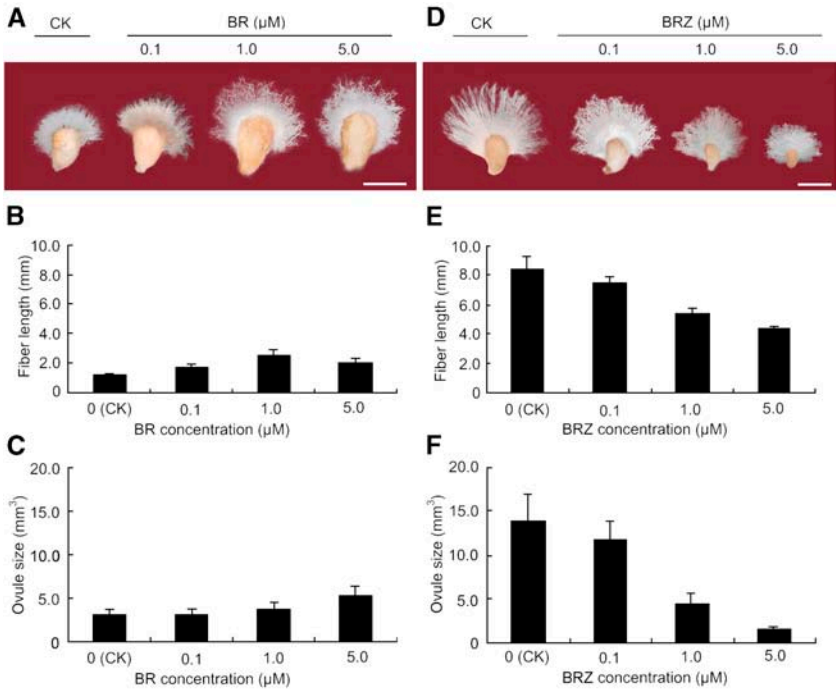

Figure 5. Effects of Exogenously Applied BR and BRZ on Fiber Cell Elongation.

(A) Phenotypes of 7-d-old ovules (collected at 1 DPA) cultured with and without (CK) BR supplementation. Bar = 2.5 mm.
(B) Final fiber lengths measured at the end of the 6-d culture period.
(C) Ovule sizes measured at the end of the 6-d culture period.
(D) Phenotypes of 13-d-old ovules (collected at 1 DPA) cultured with or without (CK) BRZ. Bar = 5 mm.
(E) Final fiber lengths measured at the end of the 12-d culture period.
(F) Ovule sizes measured at the end of the 12-d culture period.
See legend of Figure 4 for details on sample preparation and measurements. Error bars indicate SD ($n = 30$).

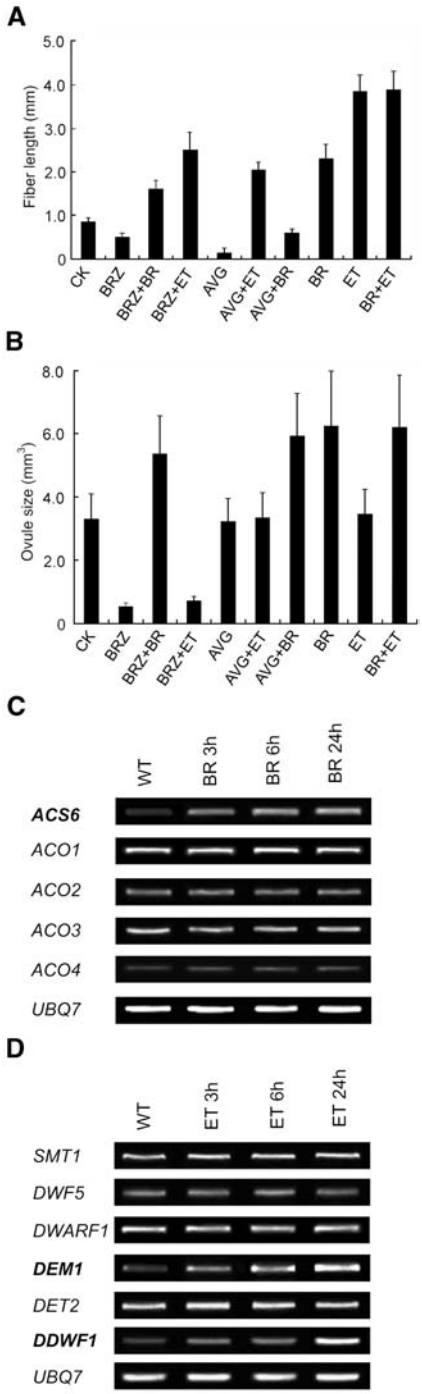

Figure 6. Ethylene and BR Interact during Fiber Elongation, Ovule Cell Expansion, and Gene Expression.

(A) Final fiber lengths measured at the end of a 6-d culture period with ethylene, BR, or their biosynthetic inhibitors added individually or in combination. Wild-type ovules collected at 1 DPA were used throughout the study. Ethylene (0.1 μM), BR, and the two inhibitors (1 μM) were added in the culture as defined in the figure. CK, no chemicals added.
(B) Ovule sizes measured at the end of a 6-d culture period with ethylene, BR, or their biosynthetic inhibitors added individually or in combination. See legend of Figure 4 for details on sample preparation and measurements. Error bars indicate SD ($n = 30$).
(C) The effect of ethylene on BR biosynthetic gene expression.
(D) The effect of BR on ethylene biosynthetic gene expression. Total RNA samples prepared from 1-DPA ovules after culturing for the specified time (h) in the presence of 5 μM ethylene (ET) or 5 μM BR were used for RT-PCR analysis. Data are representative of three amplifications from independent RNA preparations. *UBQ7* was used as a normalization control. Significantly upregulated genes upon respective hormone treatment are shown in bold in **(C)** and **(D)**.

The Interactions between Ethylene and BR during Cotton Fiber Elongation

Increased expression of BR biosynthetic genes, such as *DET2* and *SMT1*, during the fiber elongation period (Figure 2B) suggests that BRs play a role in fiber cell development. BR treatment induced a modest increase in the length of the fiber cells (Figures 5A and 5B), whereas BRZ reduced the fiber length (Figures 5D and 5E). BR and BRZ increased and reduced, respectively, the size of the ovules drastically (Figures 5C and 5F), consistent with BR being an essential growth-promoting plant hormone. The inhibitory effects of AVG or BRZ on fiber elongation were nullified when ethylene or BR was added to the same culture flasks simultaneously (Figure 6A), suggesting that the observed effects of the biosynthetic inhibitors are due to its inhibition of hormone biosynthesis.

When ethylene and BR were both added to the culture media, fiber cells did not grow any longer than those treated with ethylene alone (Figure 6A). The fiber growth-promoting effect of each hormone was not compromised in the presence of its respective biosynthetic inhibitors (Figure 6A). Ethylene was more potent than BR in regulating fiber cell elongation, as 0.1 μM of ethylene stimulated more fiber elongation than 1 μM of BR. Also, the inhibitory effect of BRZ on fiber cells could be negated to a large extent by adding ethylene, whereas the AVG effect was much less reversed by BR supplementation (Figure 6A). BR was more potent in regulating ovule cell expansion, as shown in Figure 6B.

The effect of BR on ethylene biosynthetic genes and ethylene on BR genes was studied using RT-PCR analysis. BR induced the expression of the ACC synthase gene *ACS6*, as reported in mung bean (*Vigna radiata*) previously (Yi et al., 1999), but did not affect the expression of *ACO*s over the 24-h experimental period (Figure 6C). Ethylene treatment induced the expression of *DEM1* and *DDWF1* but not *SMT1* and *DET2* in the BR biosynthetic pathway (Figure 6D). These results suggest that ethylene and BR may promote the biosynthesis of each other.

Ethylene May Promote Fiber Elongation by Activating Genes Important for Cell Wall Synthesis, Wall Loosening, or Cytoskeleton Arrangement

Further RT-PCR analysis showed that a sucrose synthase gene (*SuSy*), a tubulin gene (*TUB1*), and two expansin genes (*EXP1* and *2*) were expressed at significantly higher levels in wild-type ovules than in the *fl* mutant (Figure 7). Treatment of cultured

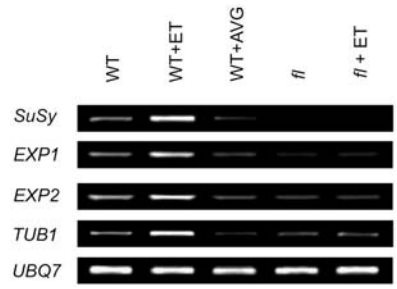

Figure 7. Expression Profiling of Five Downstream Genes Important for Fiber Elongation in Wild-Type and *fl* Mutant Ovules after Ethylene or AVG Treatment.

Total RNA samples prepared from 1-DPA ovules after a 3-d culture with or without adding 5 μM ethylene (ET) or 1 μM AVG were used for RT-PCR analysis. Data are representative of three amplifications from independent RNA preparations.

ovules with 5 μM ethylene resulted in a significant accumulation of the transcript levels for these four genes in wild-type ovules but not in the mutant. Treatment with AVG suppressed the expression of these genes to comparable levels as in the mutant (Figure 7).

DISCUSSION

In this work, we found a prominent role for the plant hormone ethylene in promoting cotton fiber cell elongation. This conclusion is based on genomic, genetic, molecular biological, and physiological studies. We fabricated a cotton cDNA microarray and screened it systematically with RNA samples prepared throughout the period of active fiber elongation. We also used a genetic mutant that produced no fiber cells to confirm the fiber specificity of the ESTs (Figure 1). Genes obtained through these analyses were used to characterize upregulated metabolic pathways during cotton fiber development. Among the 15 fiber-upregulated metabolic pathways, ethylene biosynthesis ranked the highest (Table 2). Further analysis showed that the expression of *ACO1-3* increased significantly during the fiber elongation phase, with peak levels at ~10 to 15 DPA, and decreased as fiber cells entered the maturation phase after 20 DPA (Figure 1; see Supplemental Table 1 online). All three genes were not induced in the fiberless mutant harvested either at 3 or 10 DPA (see Supplemental Table 1 online).

In concert with the upregulated expression of *ACO* genes during fiber elongation, wild-type cotton ovules with elongating fiber cells were found to release large amounts of ethylene. By contrast, mutant ovules, wild-type ovules treated with AVG, or seedlings of either genotype produced very low amounts of the hormone (Figure 3C). These results indicate that increased expression of the *ACO* genes leads to increased ethylene biosynthesis in elongating fiber cells. Exogenously applied ethylene was found to strongly promote fiber elongation, whereas its biosynthetic inhibitor AVG suppressed fiber elongation specifically (Figure 4). Although a review on cotton fiber (Kim and Triplett, 2001) states that cytokinins, abscisic acid, and ethylene inhibit fiber development, this statement was based on experiments that did not in fact include ethylene in the ovule culture studies (Beasley, 1971; Beasley and Ting, 1973, 1974).

Based on the Yang cycle of the ethylene biosynthetic pathway (Yang and Hoffman, 1984), the first committed step of ethylene biosynthesis is the conversion of *S*-adenosyl-L-methionine to ACC by ACC synthase (ACS) (*S*-adenosyl-L-methionine methylthioadenosine-lyase; EC 4.4.1.14). ACC is further oxidized by ACO to form ethylene, CO_2, and cyanide (Yang and Hoffman, 1984; Wang et al., 2002). While the ACS step is often regarded as rate limiting in ethylene biosynthesis, the regulation of *ACO* expression also has functional significance (Prescott and John, 1996). At least two *ACO* genes in *Arabidopsis thaliana* are ethylene inducible (Alonso et al., 2003; Zhong and Burns, 2003). One of these genes, *ACO2*, is induced primarily in the apical region of seedlings and correlates with differential cell expansion and apical hook formation (Silk and Erickson, 1978; Raz and Ecker, 1999).

G. arboreum diploid cotton microarray data have been published, but data for *G. hirsutum* tetraploid cotton have not (Arpat et al., 2004). Detailed sequence comparisons showed that 7339 ESTs on our *G. hirsutum* microarray were homologous to the *G. arboreum* data set if the cutoff e-value was 10^{-2}. The number decreased to 6367 or 5934 when the cutoff e-values were increased to 10^{-10} or 10^{-20}, respectively, indicating that significant sequence divergence exists between the two species. Apart from the obvious differences in the genomes used, the previous work (Arpat et al., 2004) only analyzed genes involved in secondary cell wall biosynthesis. That study compared gene expression at 24 to 10 DPA (active secondary cell wall synthesis versus fast cell elongation). This work, however, concentrated on elucidating mechanisms of fiber cell elongation by probing the microarrays with RNA samples covering the active elongation phase. Nonetheless, many fiber upregulated genes previously reported by Smart et al. (1998), Li et al. (2002a), and Ji et al. (2003) were found in this work (see Supplemental Table 1 online).

BR is a growth-promoting hormone required for normal cell elongation or expansion (Wang and He, 2004). The increased expression of BR biosynthetic genes, such as *DET2* and *SMT1*, suggests that BR biosynthesis may be increased in elongating fiber cells as well. However, the role of BR in fiber cell elongation appears to be less prominent than that of ethylene, for it was only modestly effective in our experiments (Figures 5 and 6). Furthermore, BR and BRZ affected both fiber length and ovule size, whereas ethylene and AVG affected only fiber length (Figures 4 to 6). Although the actions of BR and ethylene on fiber elongation are not interdependent, it appears that they do not act completely independently either. We found that BR induces the expression of *ACS6* and that ethylene induces the BR biosynthetic genes *DEM1* and *DDWF1* (Figures 6C and 6D), suggesting that each hormone positively modulates the synthesis of the other. Such positive interactions between the two hormones potentially contribute to the extreme elongation of fiber cells. A similar interaction between BR and ethylene has been reported. Ethylene was shown to increase the expression of the BR biosynthetic gene *CPD* (Grauwe et al., 2005), and BR was shown to increase the expression of several ACS genes in mung bean (Yi et al., 1999).

Compared with BR, ethylene plays a major role in fiber elongation, as it was able to override the inhibitory effect of BRZ, whereas BR could not reverse the fiber cell growth arrest caused by AVG (Figure 6A). Also, ethylene induced the expression of *SuSy*, *TUB1*, and *EXPs* (Figure 7), which are involved in cotton fiber elongation or submergence-induced petiole elongation in *Rumex palustris* (Ji et al., 2002, 2003; Ruan et al., 2003; Vreeburg et al., 2005). These results indicate that ethylene may promote fiber cell elongation by activating genes that mediate cell wall synthesis, wall loosening, or cytoskeleton arrangement. Interactions between the ethylene and BR pathways in cotton fibers may provide a model system for studies of how hormones regulate cell elongation.

METHODS

Plant Materials

Upland cotton (*Gossypium hirsutum* cv Xuzhou 142) and the *fl* mutant, originally discovered in the same cotton field in China (Zhang and Pan, 1992), were grown in soil mixture in a fully automated green house. Ovules at different growth stages were excised from bolls on the cotton plant. For in vitro ovule cultures, 1-DPA fresh ovules picked from the plants were used. For RNA extraction, 5- to 10-DPA ovules were frozen and stored in liquid nitrogen immediately after harvest.

RNA Isolation, cDNA Library Construction, and Sequencing

Total RNA samples were prepared from 1 g cotton fibers harvested from 5- to 10-DPA ovules using a modified hot borate method (Lu, 2002). cDNA was synthesized and cloned into the *Eco*RI-*Xho*I sites of the ZAP Express vector using a cDNA synthesis kit, and the ligation mixture was packaged using a ZAP-cDNA Gigapack Gold III cloning kit (Stratagene). Bacterial colonies containing fiber cDNAs, with average insert size of 1.6 to 1.7 kb, were obtained after in vivo excision.

EST Processing and Annotation

A total of 36,000 clones were subjected to single-pass sequencing reactions from the 5′ end. Vectors and sequences shorter than 300 bp or containing >1.5% of imprecise nucleotides were removed. The remaining ESTs were compared with the GenBank database using BLASTX (http://www.ncbi.nlm.nih.gov/BLAST/), with 10^{-2} as the cutoff e-value, and named after the homologous sequences in GenBank. ESTs with e-values higher than 10^{-2} were designated as unknown. EST assembly was performed to obtain uniESTs using Stackpack2.1 software (Christoffels et al., 2001). Clusters and singletons with alternatively spliced isoforms were reassembled manually.

Cotton Fiber cDNA Microarray Construction

Individual bacterial clones containing 11,719 cotton uniESTs were selected and distributed into 96-well plates. Amplification of cDNA by PCR with primers specific to the plasmid vector sequences flanking the insert cDNA (M13 forward primer, 5′-CTGCAAGGCGATTAAGTTGGGTAAC-3′; M13 reverse primer, 5′-GTGAGCGGATAACAATTTCACACAGGAAACAGC-3′) were performed in 96-well PCR plates in a Perkin-Elmer 9600 thermocycler in 50-μL reactions containing 1× PCR buffer (TaKaRa), 2.0 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate (dNTP), 10 pmol each of the primers, 5 units Taq polymerase, and 10 ng plasmid template. PCR was performed as follows: at 95°C for 3 min, then 35 cycles at 95°C for 1 min, 55°C for 20 s, and 72°C for 90 s, and a final extension at 72°C for 5 min. Aliquots (1 μL) of the PCR reactions were analyzed in a 1% agarose gel to verify the success of the PCR. The remaining cDNA was precipitated with addition of 100 μL anhydrous ethanol and resuspended in 15 μL 50% DMSO for arraying.

Eight sequences derived from intergenic regions of the yeast genome showing no significant homology with any existing cotton sequences in GenBank were used as external controls. These sequences were PCR amplified and cloned into plasmid pSP64Poly(A) (Promega) to produce poly(A)-RNA after in vitro transcription. PCR products representing control sequences were spotted 12 times onto the microarray in different subgrids.

Preparation of Fluorescent Dye–Labeled DNA and Hybridizations

cDNA labeled with a fluorescent dye (Cy5 and Cy3-dCTP) was produced by Eberwine's linear RNA amplification method and subsequent enzymatic reaction. In detail, double-stranded cDNAs (containing the T7 RNA polymerase promoter sequence) were synthesized from 10 μg total RNA using the cDNA synthesis system according to the manufacturer's protocol (TaKaRa). A T7-oligo(dT) primer (5′-AAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGCGCTTTTTTTTTTTTTTTT-3′) was used instead of the poly-T primer provided in the kit.

After completion of double-stranded cDNA synthesis, cDNA products were purified using a PCR purification kit (Qiagen) and eluted with 60 μL elution buffer. One-half of the eluted double-stranded cDNA products were vacuum evaporated to 8 μL and subjected to 20-μL in vitro transcription reactions at 37°C for 3 h using the T7 RiboMAX Express large scale RNA production system (Promega). The amplified RNA was purified using the RNeasy mini kit (Qiagen). Klenow enzyme labeling strategy was adopted after reverse transcription. Briefly, 2 μg amplified RNA was mixed with 2 μg random hexamers, denatured at 70°C for 5 min, and cooled on ice. Then, 4 μL of first-strand buffer, 2 μL of 0.1 M DTT, 1 μL 10 mM dNTP, and 1.5 μL SuperScript II (Invitrogen) were added. The mixtures were incubated at 25°C for 10 min, then at 42°C for 60 min. The cDNA products were purified using a PCR purification kit (Qiagen) and vacuum evaporated to 10 μL. The cDNA was mixed with 2 μg random nanomer, heated to 95°C for 3 min, and snap cooled on ice. Then, 10 μL buffer, dNTP, and Cy5-dCTP or Cy3-dCTP (Amersham Pharmacia Biotech) were added to final concentrations of 120 μM dATP, 120 μM dGTP, 120 μM dTTP, 60 μM dCTP, and 40 μM Cy-dye. Klenow enzyme (1 μL; TaKaRa) was then added, and the reaction was performed at 37°C for 60 min. Labeled cDNA was purified with a PCR purification kit (Qiagen) and resuspended in elution buffer. Labeled controls and test samples were quantitatively adjusted based on the efficiency of Cy-dye incorporation and mixed with 30 μL hybridization solution (50% formamide, 1× hybridization buffer; Amersham Biosciences). DNA in hybridization solution was denatured at 95°C for 3 min prior to loading onto a microarray. Arrays were hybridized at 42°C overnight and washed with two consecutive solutions (0.2% SDS, 2× SSC at 42°C for 5 min, and 0.2× SSC for 5 min) at room temperature.

Image Acquisition, Data Processing, and Clustering

All microarrays were scanned with a ScanArray Express scanner using ScanArray 2.0 software (Packard Bioscience). We quantified signal intensities of individual spots from the 16-bit TIFF images using GenePix Pro 4.0 (Axon Instruments). The microarray slides were hybridized with RNA prepared from three biological replicate samples for each growth stage or tissue type. As a measure of technical replication, one swap-dye experiment was performed on each of the biological samples so that a total of six data points were available for every EST on the microarrays. The linear normalization method was used for data analysis, based on the expression levels of 40 cotton housekeeping genes in combination with the yeast external controls. Normalized data was log transformed and

loaded into MAANOVA under R environment for multiple testing, by fitting a mixed effects ANOVA model (Wu et al., 2003). Microarray spots with FDR-corrected P values <0.001 in the *F*-test were regarded as differentially expressed genes. Hierarchical clustering with the average linkage method was employed only on those genes that showed fiber-specific expression in one or more stages defined as 0/3 (0 DPA versus 3 DPA RNA samples, etc.), 5/3, 10/3, 15/3, and 20/3 as well as in 3wt/3*fl* and 10wt/10*fl*. We visualized the cluster data by the Treeview program (Eisen et al., 1998).

Identifying Fiber-Preferential Pathways Using KOBAS

We used the software KOBAS (Mao et al., 2005) to identify biochemical pathways involved in cotton fiber development and to calculate the statistical significance of each pathway. KOBAS assigns a given set of genes to pathways by first matching the genes to similar genes (as determined by BLAST similarity search with cutoff e-values $<1e^{-5}$, rank <10, and sequence identity >30%) in known pathways in the KEGG database. We also manually reviewed all identified pathways for quality control. KOBAS ranks pathways by the statistical significance of whether a pathway contains higher ratio of fiber-preferential genes among all genes mapped to it when compared with that of all fiber-preferential genes in the whole data set. The P value we obtained by running the KOBAS software is designed to test whether data from a particular pathway fits the null hypothesis or the alternative hypothesis defined as

$$H_0: p_0 = p_1$$

$$H_1: p_0 \neq p_1,$$

where $p_0 = m/M$, $p_1 = n/N$, m is the number of fiber-preferential genes mapped to the pathway under investigation, M is the number of genes on the microarray that were mapped to that particular pathway, n is the total number of fiber-preferential genes mapped to all pathways, and N is the total number of genes on the microarray mapped to all pathways. The P value of a particular pathway corresponds to a test statistic following a hypergeometric distribution:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}.$$

Since a large number of pathways are involved, we implemented FDR correction to control the overall Type I error rate of multiple hypotheses testing using GeneTS (2.8.0) in the R (2.2.0) statistics software package (Storey, 2002; Wichert et al., 2004). Pathways with FDR-corrected P values <0.05 were considered statistically significant.

RT-PCR and QRT-PCR Analysis

First-strand cDNA was synthesized from 5 μg total RNA using the Superscript first-strand synthesis system for RT-PCR (Invitrogen). Gene-specific RT-PCR primers were synthesized commercially (Bioaisa) as listed in Supplemental Table 3 online. Parallel reactions using cotton *UBQ7* primers were performed to normalize the amount of template cDNA added in each reaction. QRT-PCR was performed using the SYBR Green PCR kit (Applied Biosystems) in a DNA Engine Opticon continuous fluorescence detection system (MJ Research) as previously described (Qin et al., 2005). The QRT-PCR cycles were as follows: initiation with a 10-min denaturation at 95°C, followed by 42 cycles of amplification with 10 s of denaturation at 94°C, 20 s of annealing according to the melting temperatures provided in Supplemental Table 3 online, 20 to 30 s of extension at 72°C, and reading the plate for fluorescence data collection at 78 to 80°C. After a final extension at 72°C for 5 to 10 min, a melting curve was performed from 65 to 95°C (1 s hold per 0.2°C increase) to check the specificity of the amplified product.

For QRT-PCR analyses of developmental stage-specific expression of *ACO1-3*, *SMT1*, and *DET2*, total RNA samples prepared from 0- to 20-DPA wild-type ovules (with fibers attached) or from 10-DPA *fl* mutant ovules were used. For tissue-specific expression pattern studies of *ACO1-3*, total RNA samples prepared from 10-DPA wild-type cotton fibers not attached to the ovules, from wild-type ovules with fibers scraped off, from the *fl* mutant ovules harvested at the same growth stage, and from roots, leaves, and stems were used.

A literature search was conducted to identify putative downstream genes important for fiber or any other types of extreme cell elongation. RT-PCR was performed using gene-specific primers (shown in Supplemental Table 3 online), and RNA samples were prepared from wild-type and mutant ovules cultured in the presence of ethylene or AVG for 3 d. All RT-PCR and QRT-PCRs were performed in triplicate using independent RNA samples.

In Vitro Ovule Culture and Treatment with Plant Hormones or Hormone Inhibitors

Cotton ovules were collected half a day before or after flower opening (defined as 0 or 1 DPA, respectively), soaked in 70% ethanol for 1 min, rinsed in distilled and deionized water, and soaked again in 0.1% HgCl solution containing 0.05% Tween-80 for 20 min to sterilize. Ovules (~0.02 g) were placed in liquid media formulated by Beasley and Ting (1973) in 50-mL flasks under aseptic conditions. Media compositions given in Table 1 of their original publication were followed except that 120 mM D-glucose was used instead of 100 mM D-glucose plus 20 mM D-fructose (Beasley and Ting, 1974). The ovules generally floated on the surface of 20 mL of the liquid media containing various plant hormones or inhibitors at the specified concentrations and were cultured at 30°C in darkness without agitation. Gaseous ethylene was added in 2-liter glass containers to specified concentrations, disregarding the volume of the 50-mL culture flasks sitting inside. The large container was sealed gastight, while the culture flasks were left open for gas exchange. No GA or naphthylacetic acid was added in the culture media. Cultured ovules were rinsed with distilled and deionized water, heated to 100°C for 10 min, and transferred to 45% acetic acid after cooling to room temperature. The lengths of fibers from ovules treated with different chemicals were measured manually under a bright-field microscope after combing the cells to upright positions. Gaseous ethylene (99.9%) was purchased from Qianxi Chemicals. BR (brassinolide) and AVG were purchased from Sigma-Aldrich, and BRZ was kindly provided by T. Asami (Asami et al., 2000).

Yeast Constructs and *ACO1-3* Enzyme Activity Measurements

ACO1-3 open reading frames were amplified with gene-specific primers: *ACO1*, 5′-CACCACAAAATGGAGCTCACTTTCCCTGTAATC-3′ (forward [F]) and 5′-AACAGTTGCAATAGGACCCAAGTT-3′ (reverse [R]); *ACO2*, 5′-CACCACAAAATGGAGGTAGCTTTCCCTGTTATT-3′ (F) and 5′-AACAGTTGCAATAGGACCCAAGCT-3′ (R); *ACO3*, 5′-CACCACAAAATGGCTACTTTCCCAGTGATCAAC-3′ (F) and 5′-AGCTGTTGCAATGGGAGCAGTAGC-3′ (R). The PCR products were purified using the QIAquick gel extraction kit (Qiagen) and were cloned into pENTR/D/TOPO vectors (Invitrogen) to form the *pENTR-ACO1-3* vectors. These vectors were verified by sequence analyses using M13 primers. Yeast expression cassettes were generated using the *pENTR-ACO1-3* and pYTV vectors in Gateway LR Clonase enzyme mix (Invitrogen) as previously described (Gong et al., 2004). Untransformed yeast cells (strain PEP4) or cells transformed with the empty pYTV or the *pYTV-ACO1-3* vectors were grown in synthetic complete broth, with or without uracil, respectively, at 30°C with continuous agitation (250 rpm). Glucose (2% final concentration) was included in the media as the energy and carbon source for the

first 24 h (at which time most of the glucose had been consumed). Raffinose was then used to replace glucose for the next 16 h. Once the OD_{600} of the culture reached 0.8, 2% galactose was added to activate the *GAL1* promoter for 4 h. Samples ($\sim 10^6$ cells) were taken from the yeast culture at the end of the induction period, and the capacity of these cells to convert ACC to ethylene was determined in a reaction containing 50 mM ascorbate and 200 μM $FeSO_4$ as cofactors with varying amounts of ACC as the substrate. Samples were incubated with agitation (250 rpm) for 1 h at 30°C in 15 × 1.5-cm test tubes sealed with polyethyl cellulose film. Ethylene produced by different samples was determined as described in the following section after subtracting any amount of the hormone produced by the same noninduced yeast cell lines.

Total proteins extracted from 0.2 mL yeast cells (OD_{600} = 1.0) expressing *ACO1*, *2*, and *3* genes were fractionated on 12% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. Recombinant ACO1, ACO2, and ACO3 proteins were detected with monoclonal antipolyhistidine antibody (RandD Systems) and visualized after incubating with goat anti-mouse IgG conjugated with horseradish peroxidase (Promega) as previously described (Qin et al., 2005).

Ethylene Measurements

A gas chromatograph (GC-14C; Shimadzu) equipped with a flame-ionization detector with a 30-m HP-PLOT column (Agilent Technologies) was used to measure the amount of ethylene produced. Approximately 20 freshly collected 1-DPA wild-type or *fl* mutant ovules were cultured in 20 mL liquid media in 50-mL glass flasks in darkness for 12 d at 30°C. Plant hormones or hormone inhibitors were first dissolved in small amounts of ethanol before adding to the liquid media to final concentrations, as specified in the figure legends. To measure ethylene produced by the vegetative tissue, both wild-type and mutant seeds were sown in 1-liter airtight glass jars on solid media and were kept in a fully automated growth room for a total of 12 d. Seedlings were generally 15 cm in height and ~3 g in fresh weight. Air samples (100 μL) from each flask were removed and injected into the column of the gas chromatograph for ethylene measurements. All observations were recorded from at least three replicating flasks, and the whole experiment was repeated with comparable results. Standards of 0.1, 1, 10, and 50 ppm ethylene were used to verify the retention time and to quantify the amount of ethylene produced. Any amount of ethylene detected in the first day of culture was subtracted from the data to circumvent the problem of injury-related ethylene production. The sensitivity of our gas chromatograph was 0.01 ppm.

Accession Numbers

All 11,692 uniESTs were deposited in GenBank with accession numbers DR452281 to DR463972. All microarray data from this work were deposited to the National Center for Biotechnology Information GEO database in the MIAME format (GPL2610, GSE2901, GSM63341 to GSM63346, GSM63365 to GSM63379, and GSM63391 to GSM63420). Other accession numbers are as follows: *UBQ7*, DQ116441; *ACS6*, DQ122174; *ACO1*, DQ116442; *ACO2*, DQ116443; *ACO3*, DQ116444; *ACO4*, DQ122175; *SMT1*, DQ116445; *DET2*, DQ116446; *DDWF1*, DQ122176; *DEM1*, DQ122177; *DWARF1*, DQ122178; *DWF5*, DQ122179; *GA20ox1*, DQ122181; *GA20ox2*, DQ122188; *GA3ox1*, DQ122182; *GA3ox2*, DQ122183; *NIT*, DQ122184; *FMO1*, DQ122185; *TDC*, DQ122187; *Susy*, U73588; *TUB1*, AF521240; *EXP1*, AY189969; *EXP2*, DQ204496.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Statistics of 778 Both Developmentally and Genetically Upregulated Cotton ESTs.

Supplemental Table 2. Analysis of Expression Ratios of 40 Internal Control Genes during Fiber Development in Wild-Type and the Mutant Cotton Ovules.

Supplemental Table 3. cDNAs Encoding Key Enzymes in Various Plant Hormone Biosynthetic Pathways.

Supplemental Table 4. Primers Used for RT- and QRT-PCR Analysis.

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