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ORIGINAL RESEARCH

Profiling Gene Expression During Gland Morphogenesis of a Glanded and a Glandless Upland Cotton

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Abstract The pigment gland is an important character of the *Gossypium* plant. With the aim of identifying genes involved in pigment gland morphogenesis in cotton, gene expression during pigment gland morphogenesis in Chuan 2802, which is glanded both in seed and plant, and a glandless line N5 was profiled using Affymetrix Cotton microarray. The results showed that there were 564 differentially expressed genes greater than twofold during gland morphogenesis. About 60.2% of these genes shares similarity with known genes on GenBank and about 39.8% with no functional description in the database. These described genes may play roles in defense response, response to oxidative stress, peroxidase activity, and the other metabolic pathways. The KEGG Orthology-Based Annotation System indicated that these above twofold expressed genes involved seven biochemical pathways on KEGG. These findings suggest that a complicated regulation is associated with pigment gland morphogenesis and the associated defense response including gossypol biosynthesis in cotton.

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Introduction

Cotton, *Gossypium* L., is a leading fiber and potential food crop. Besides cotton fiber, cotton seed is among the most abundant protein meal and it represents 6.9% of world protein meal production only next to soybeans and rapeseed (Ash and Dohlman 2006), and it has compared favorably with other traditional food sources as a source of protein in several human nutritional studies (Lusas and Jividen 1987). However, the cotton seed has not been utilized as a source of food and feed because of the presence of gossypol, a terpenoid aldehyde toxic to nonruminant animals and humans in pigment glands of cotton seeds (Weinbauer et al. 1983; Heywood 1988; Shandilya et al. 1982; Sang et al. 1980; Du et al. 2004; Stipanovic et al. 2006). The pigment glands, as the storage organs of terpenoid aldehydes, including gossypol, are on the surfaces of cotton organs or tissues (Punit et al. 1991).

The studies on the relationship between gossypol and its storage organ gland showed that some genetic regulation exists between glands and gossypol contents (Zhu and Chen 2005). Martin et al. (2003) have reported a proposed pathway for the biosynthesis of the compounds in gland, and (+)-δ-cadinene was found to play a key role in biosynthesis of gossypol. Research on the relationship between glands and their secondary inclusions at the molecular level would be one approach for genetic engineering to control the glands and gossypol content. In order to understand the molecular mechanism of gland morphogenesis in Chuan 2802, in this study, we performed the genome-wide analysis of cotton seed transcriptome

divergence between glanded Chuan 2802 and glandless N5 using Affymetrix Cotton GeneChip. A serial of genes with differential expression greater than twofold during gland morphogenesis were revealed; these genes may play roles in defense response, response to oxidative stress, peroxidase activity, and the other metabolic pathways. The results suggest that a complicated regulation is associated with pigment gland morphogenesis and the related defense response including gossypol biosynthesis in cotton.

Materials and Methods

Materials

Cotton seeds of Chuan 2802 (a germplasm glanded both in seed and plant) and N5 (glandless both in seed and plant) were obtained from the Cotton Institute of the Sichuan Academy of Agricultural Sciences (Chengdu, China) and the Cotton Institute of the Chinese Academy of Agricultural Sciences (Anyang, China), respectively.

Total RNA Extraction

Cotton seeds were disinfected in a solution containing 70% (*v*/*v*) ethanol and 15% H_2O_2 solution then dipped in sterilized water and began to bud in the plates containing sterilized filter paper and water. The pigment glands began to develop on the plate at 50 h or so and temperature of 25°C after budding. Total RNA was isolated by the total plant RNA extraction kit (Watson Biotech, Shanghai China) according to the manufacturer's instructions. The purity and concentration of each RNA sample were detected by agarose gel electrophoresis and ultraviolet spectrophotometer.

GeneChip Analysis

Total RNA was used to generate biotinylated cRNA targets for the cotton GeneChip analysis (Affymetrix), which comprised 23,977 probe sets representing 21,854 cotton transcripts. All these procedures were carried out as suggested by the manufacturers. After hybridization, the arrays were washed, stained using GeneChip fluidics station 450, and scanned on a GeneArray™ scanner 3000. After the arrays were scanned, the signals generated were determined and analyzed by the Affymetrix GeneChip® Operating Software. N5 was used as the baseline and Chuan 2802 was compared to this. One repeat experiment was performed on each of the biological samples. After normalization, scaling, and log transformation, the gene expression data among two samples were compared. The threshold ($P<0.01$) for definition as an upregulated gene was a combined change greater than twofold.

The twofold change genes were used for further analysis of related pathways using the KEGG Orthology-Based Annotation System (KOBAS; http://kobas.cbi.pku.edu.cn/; Mao et al. 2005; Wu et al. 2006). KOBAS assigns a given set of genes to pathways by first matching the genes to similar genes (as determined by a BLAST similarity search with cutoff *e* values $<1e^{-5}$, rank <5, and sequence identity >30%) in known pathways in the KEGG database.

RT-PCR Validation

To analyze and confirm the expression pattern of change genes, gene-specific primers were designed from four randomly selected ESTs. The first-strand cDNA was synthesized using 1 μg of total RNA with SuperScript™ II Reverse Transcriptase (Promega) in a 20-μl reaction volume. Semiquantitative RT-PCR was performed at 94°C for 5 min followed by 25 cycles of amplification (95°C for 30 s, 55°C for 30 s, and 72°C for 1 min). The 18S rRNA gene was used as a loading control. Primers are shown in Table 1.

Results

Analysis of Cotton GeneChip Expression

The pigment gland of cotton is responsible for storing the complex mixture of secondary metabolites such as gossypol involved defense response. The studies using Affymetrix GeneChip with glanded and glandless cotton will help understand the molecular mechanism of gland morphogenesis and the relationship between secondary metabolites of defense material and their storage organ (tissues).

Table 1 Primers for RT-PCR

Gene ID	Forward primer	Reverse primer
DW224803	GCTAAGATAGGTGCTGGT	GTCTGGCAATAATGGAAA
DN803199	CTTTGATTCCCATGTTGA	AAGTTCTTGTAATAGTAGCG
AF487461	CAATGGCAATGATACAAACC	GACCGAACCAGTTGACGA
DT463838	AAAAGGAGGAAGGAAGAG	GAGTACCTGGCAGAAATG
18S rRNA	TCGTAGTTGGACTTAGGGTGGG	CAAATGCTTTCGCAGTTGTTCG

The GeneChip result of the Chuan 2802 and N5 were analyzed by using the Affymetrix GeneChip® Operating Software. There are 3,264 differentially expressed genes comparing the Chuan 2802 to N5, including 1,509 upregulated genes and 1,755 downregulated genes, but only 219 upregulated and 345 downregulated, respectively, out of those that exhibited differential signal above twofold.

Functional Classification of the Genes with Greater than Twofold Expression Level

The twofold genes sequence of the differentially expressed genes above was downloaded from GenBank and Blastx and Blastn were performed against the GenBank database (Blast *e* value $<e^{-10}$). Among the 564 twofold genes, 340 (60.3%) genes showed a high similarity to database entries, indicating that they are either the same gene or belong to the same gene family. However, the remaining 238 have no homologies with any genes in the database, which might represent previously uncharacterized sequences; it may have new function of defense and others.

The GeneChip probes ID of these genes were then uploaded to NetAffx (the net analysis center of Affymetrix) for functional analysis. The 564 represent 39 functional categories, which include response to biotic stimulus, photosynthesis, biosynthetic process, response to oxidative stress, defense response, regulation of transcription, DNA-dependent, respiratory gaseous exchange, regulation of transcription, response to desiccation, polysaccharide catabolic process, carbohydrate metabolic process, chitin catabolic process, metabolic process, cell wall catabolic process, nucleus, mitochondrial envelope, monolayer-surrounded lipid storage body, integral to membrane, photosystem II, membrane, receptor activity, peroxidase activity, iron ion binding, oxidoreductase activity, metal ion binding, nutrient reservoir activity, hydroxymethylglutaryl-CoA reductase (NADPH) activity, nucleotide binding, ATP binding, transcription factor activity, sequence-specific DNA binding, DNA binding, zinc ion binding, oxygen binding, heme binding, chitinase activity, chitin binding, hydrolase activity, hydrolase activity, and acting on glycosyl bonds among others (Table 2).

Validation of Gene Expression

To validate the expression patterns observed by microarray, RT-PCR was utilized. The total RNA was isolated from Chuan 2802 and N5 seeds during gland morphogenesis, respectively. As seen in Fig. 1a, the relative expression levels of four genes were various in two materials. The scaling signal intensities of four genes were obtained from microarray data (Fig. 1b). The results showed that the expression patterns of RT-PCR results were similar to that of microarray data, confirming its reliability.

Discussion

Along with the decrease in available arable lands and the need for biofuels from plants, the world is now threatened by insufficient food. Making cotton crop both a fiber and a food crop could help ease the problem. In this regard, researchers have focused on breeding cotton varieties with glandless seed and glanded plant through both conventional breeding and genetic engineering in recent years. Understanding the molecular mechanism of gland morphogenesis and the relationship between gossypol and its storage organ, glands, at the molecular level in cotton will be beneficial to create the new type variety of glandless seed and glanded plant.

In this study, two GeneChip were used to explore the gene expression profiles during gland morphogenesis. The differential genes during gland morphogenesis of the two materials most probably were what we anticipated, which were presumably involved in cotton gland morphogenesis. However, there is currently no report on gene expression profile comparisons between the glanded and glandless materials.

As mentioned above, 564 above twofold difference expressed genes were obtained. In Table 2, the main GO categories were biological process and molecular function; the description function includes response to biotic stimulus, defense response transcription factor activating, peroxidase, PR protein and so on.

Ethylene response factors (ERFs) play important roles in regulating plant biotic and abiotic stress tolerance. The research confirmed the ethylene response factors 2 (GbERF2) gene that plays an important role in response to ethylene stress and fungal attack in cotton (Zuo et al. 2007). Whether the biosynthesis of gossypol is directly or indirectly induced by ethylene is an interesting research topic and needs further exploration.

A large number of class I chitinase were found in this stage; it may be a member of the biosynthesis of gossypol phytoalexins (Dowd et al. 2004).

UV-B represses plant genes related to photosynthesis and drought resistance but induces expression of genes responsible for pathogen defense, auxin response, and mRNA translation (Liu et al. 2002). So we think that the UV-B gene may be involved in the biosynthesis of gossypol.

The result indicated that the pathogenesis-related 10 protein (PR 10) is involved in the response of cotton to *F. oxysporum* f. sp. *vasinfectum* infection (Dowd et al. 2004). PR10, PR2, and PR3 all have been found to be induced by

Table 2 Functional classification of 564 genes

GO Cotegory	GeneChip ID	Descriptions	GenBank ID
Biological process			
Response to biotic stimulus	GbaAffx.201.1.S1_at	PR10-5-like protein	AY560553.1
Response to biotic stimulus	Ghi.8071.1.S1_at	PR protein class 10	AF305067.1
Response to biotic stimulus	GbaAffx.201.1.S1_s_at	PR10-5-like protein	AY560553.1
Response to biotic stimulus	Ghi.6523.1.S1_s_at	PR protein class 10	AF305064.1
Photosynthesis	Ghi.8119.1.S1_at	Ultraviolet-B-repressible protein	AY551823.1
Biosynthetic process	GraAffx.17247.2.S1_s_at	*Gossypium barbadense* 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR3)	DQ350145
Biosynthetic process	Gra.1436.4.A1_s_at	*Gossypium barbadense* 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR3)	DQ350145
Response to oxidative stress	Ghi.8104.1.S1_at	Bacterial-induced peroxidase (pod4)	AF155124.1
Response to oxidative stress	Ghi.3169.1.S1_at	Bacterial-induced peroxidase (pod2)	AY074794.1
Defense response	GbaAffx.201.1.S1_at	PR10-5-like protein	AY560553.1
Defense response	Ghi.8071.1.S1_at	PR protein class 10	AF305067.1
Defense response	GbaAffx.201.1.S1_s_at	PR10-5-like protein	AY560553.1
Defense response	Ghi.6523.1.S1_s_at	PR protein class 10	AF305064.1
Regulation of transcription, DNA-dependent	GbaAffx.196.1.A1_at	Ethylene response factor 2	AY572462.1
Regulation of transcription, DNA-dependent	GbaAffx.196.1.A1_s_at	Ethylene response factor 2	AY572462.1
Respiratory gaseous exchange	Ghi.3408.1.A1_at	*Gossypium hirsutum* alternative oxidase (AOX1)	DQ250028
Regulation of transcription	Ghi.10061.1.S1_s_at	*Gossypium arboreum* transcription factor WRKY1	AY507929.2
Response to desiccation	Ghi.6722.1.S1_s_at	Group 4 late embryogenesis-abundant protein	M88322.1
Polysaccharide catabolic process	Ghi.6521.1.S1_at	*Gossypium hirsutum* class I chitinase	CD486070
Carbohydrate metabolic process	Ghi.6521.1.S1_at	*Gossypium hirsutum* class I chitinase	CD486070
Chitin catabolic process	Ghi.6521.1.S1_at	*Gossypium hirsutum* class I chitinase	CD486070
Metabolic process	Ghi.6521.1.S1_at	*Gossypium hirsutum* class I chitinase	CD486070
Metabolic process	Gra.1544.1.A1_s_at	*Gossypium hirsutum* gibberellic acid receptor	DQ829776.1
Cell wall catabolic process	Ghi.6521.1.S1_at	*Gossypium hirsutum* class I chitinase	CD486070
Cellular component			
Nucleus	Ghi.10061.1.S1_s_at	*Gossypium arboreum* transcription factor WRKY1	AY507929.2
Nucleus	GbaAffx.196.1.A1_at	Ethylene response factor 2	AY572462.1
Nucleus	GbaAffx.196.1.A1_s_at	Ethylene response factor 2	AY572462.1
Mitochondrial envelope	Ghi.3408.1.A1_at	*Gossypium hirsutum* alternative oxidase (AOX1)	DQ250028
Monolayer-surrounded lipid storage body	Ghi.8035.1.S1_at	16.4 kDa oleosin (MatP7)	L00934.1
Integral to membrane	Ghi.8035.1.S1_at	16.4 kDa oleosin (MatP7)	L00934.1
Photosystem II	Ghi.8119.1.S1_at	Ultraviolet-B-repressible protein	AY551823.1
Membrane	Ghi.8119.1.S1_at	Ultraviolet-B-repressible protein	AY551823.1
Membrane	Ghi.8035.1.S1_at	16.4 kDa oleosin (MatP7)	L00934.1
Molecular function			
Receptor activity	Gra.1544.1.A1_s_at	*Gossypium hirsutum* gibberellic acid receptor	DQ829776.1
Peroxidase activity	Ghi.8104.1.S1_at	Bacterial-induced peroxidase (pod4)	AF155124.1
Iron ion binding	Ghi.8104.1.S1_at	Bacterial-induced peroxidase (pod4)	AF155124.1
Iron ion binding	Ghi.8085.1.S1_at	Nonsymbiotic hemoglobin protein	AY899302.1
Iron ion binding	Ghi.3169.1.S1_at	Bacterial-induced peroxidase (pod2)	AY074794.1
Oxidoreductase activity	Ghi.8104.1.S1_at	Bacterial-induced peroxidase (pod4)	AF155124.1
Oxidoreductase activity	Ghi.3169.1.S1_at	Bacterial-induced peroxidase (pod2)	AY074794.1
Nutrient reservoir activity	GhiAffx.48249.1.S1_s_at	Proline-rich protein	AY560547.1
Nutrient reservoir activity	GbaAffx.198.1.S1_s_at	Proline-rich protein	AY560547.1
Hydroxymethylglutaryl-CoA reductase (NADPH) activity	GraAffx.17247.2.S1_s_at	*Gossypium barbadense* 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR3)	DQ350145

Table 2 (continued)

GO Cotegory	GeneChip ID	Descriptions	GenBank ID
Hydroxymethylglutaryl-CoA reductase (NADPH) activity	Gra.1436.4.A1_s_at	*Gossypium barbadense* 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR3)	DQ350145
Nucleotide binding	Ghi.1206.1.S1_at	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase 2	AF329935.1
ATP binding	Ghi.1206.1.S1_at	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase 2	AF329935.1
Transcription factor activity	Ghi.10061.1.S1_s_at	*Gossypium arboreum* transcription factor WRKY1	AY507929.2
Transcription factor activity	GbaAffx.196.1.A1_at	Ethylene response factor 2	AY572462.1
Transcription factor activity	GbaAffx.196.1.A1_s_at	Ethylene response factor 2	AY572462.1
Sequence-specific DNA binding	Ghi.10061.1.S1_s_at	*Gossypium arboreum* transcription factor WRKY1	AY507929.2
DNA binding	GbaAffx.186.1.S1_s_at	Fiber protein Fb37	AY429441.1
Zinc ion binding	GbaAffx.186.1.S1_s_at	Fiber protein Fb37	AY429441.1
Oxygen binding	Ghi.8085.1.S1_at	Nonsymbiotic hemoglobin protein	AY899302.1
Heme binding	Ghi.8085.1.S1_at	Nonsymbiotic hemoglobin protein	AY899302.1
Metal ion binding	Ghi.8085.1.S1_at	Nonsymbiotic hemoglobin protein	AY899302.1
Metal ion binding	Ghi.3169.1.S1_at	Bacterial-induced peroxidase (pod2)	AY074794.1
Metal ion binding	Ghi.8104.1.S1_at	Bacterial-induced peroxidase (pod4)	AF155124.1
Chitinase activity	Ghi.6521.1.S1_at	*Gossypium hirsutum* class I chitinase	CD486070
Chitin binding	Ghi.6521.1.S1_at	*Gossypium hirsutum* class I chitinase	CD486070
Hydrolase activity	Ghi.6521.1.S1_at	*Gossypium hirsutum* class I chitinase	CD486070
Hydrolase activity	Gra.1544.1.A1_s_at	*Gossypium hirsutum* gibberellic acid receptor	DQ829776.1
Hydrolase activity, acting on glycosyl bonds	Ghi.6521.1.S1_at	*Gossypium hirsutum* class I chitinase	CD486070

vascular wilt disease (*Verticillium dahliae*) in cotton stems (Hill et al. 1999; McFadden et al. 2001). In our study, PR 10-5-like protein and PR protein class 10 genes was found during gland morphogenesis and may play a key role in defense.

In plants, genes encoding HMGR have been shown to be differentially regulated at the transcriptional level by wound-derived and pathogen-derived signals (Burnett et al. 1993; Choi et al. 1994). Recent studies in diploid (Chen et al. 1995) and tetraploid (Joost et al. 1995) cotton revealed the simultaneous increase in transcription of genes encoding both HMGR and (+)-δ-cadinene synthase when cultured cells or intact plants are challenged with fungal elicitors. These results clearly suggested a coordinate regulation of the biochemical pathways for sesquiterpenoid phytoalexin synthesis (Loguercio et al. 1999). In this study, HMGR3 were found in this stage; it may play a key role in biosynthesis of isoprenoids and may be involved in a particular pathway in gossypol biosynthesis.

The (+)-δ-cadinene synthase (CAD1) gene is a key gene in the synthesis of gossypol of cotton; however, it was not found in this study. Yet, a transcription factor name WRKY1 has attracted some interest. The CAD1 gene

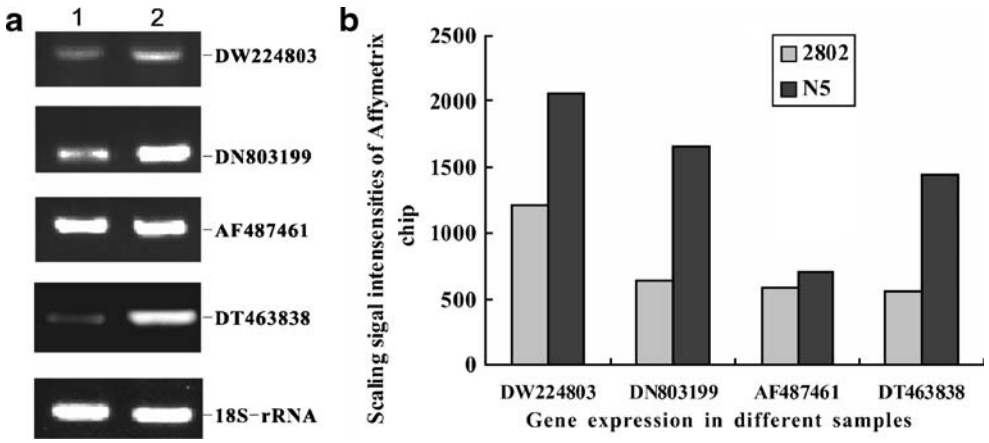

Fig. 1 The expression pattern of four genes. **a** Semiquantitative RT-PCR analysis of four genes. A cotton 18S rRNA gene was used to normalize the amount of template added in PCR reaction. *Lanes 1 and 2* represent the stage of gland morphogenesis in Chuan 2802 and N5, respectively. **b** The expression pattern of four genes in different samples detected by the Affymetrix chip

promoter contains a W-box palindrome with two reversely oriented TGAC repeats, which are the proposed binding sites of WRKY transcription factors. GaWRKY1 participates in regulation of sesquiterpene biosynthesis in cotton and CAD1-A is a target gene of this transcription factor (Xu et al. 2004). Similar to genes encoding enzymes of the cotton sesquiterpene pathway, GaWRKY1 was downregulated in a glandless cotton cultivar that contained much less gossypol (Xu et al. 2004). The same result was discovered in our study, the ratio of downregulated genes is 4.

Gibberellins (GAs) function not only to promote the growth of plant organs, but also to induce phase transitions during plant development. Recently, GA receptors of rice (GID1) and *Arabidopsis* have been discovered (Ueguchi-Tanaka et al. 2005; Nakajima et al. 2006). The receptors are able to interact, in a GA-dependent way, with the DELLA proteins (Ueguchi-Tanaka et al. 2005; Nakajima et al. 2006). DELLA proteins are inhibitors of GA responses, which are broken down in the presence of GAs, hence promoting germination and elongation growth (Silverstone et al. 2001). In this study, the GA receptor was downregulated and the ratio is 2.1. To our knowledge, no reports are available for the regulation system of GA receptors regulation in cotton.

We found two bacterial-induced peroxidases, which were named as pod 2 and pod 4 in this study. These two peroxidases belong to class III peroxidases (pod1–pod6, pod10) in the defense of cotton to bacterial blight (Delannoy et al. 2003). Pods 2, 3, 4, and 6 were reported to be activated in response to pathogen attacks and may serve in defense of *Xanthomonas* infections in cotton (Delannoy et al. 2003). The existence of class III peroxidase may be involved in the complex pathway of gossypol biosynthesis. However, the exact function of class III peroxidases during gland morphogenesis was unclear and further studies were needed.

The studies showed that the expression of the nonsymbiotic hemoglobin protein (nsHb) gene was responsive to fungus challenge (Dowd et al. 2004; Qu et al. 2005), implying that the cotton nonsymbiotic hemoglobin gene may play an important role in the defense responses against invasion of the fungus pathogens. Through our study, we found that nsHb was downregulated and the ratio is 2.6.

Phenylpropanoid biosynthesis is a key pathway in the synthesis of gossypol of cotton. In many researchers' studies, induction of phenylpropanoid synthesis under stress conditions is the result of increased transcription of genes encoding the corresponding biosynthetic enzymes (Kneusel et al. 1989; Dixon and Paiva 1995). Borevitz et al. (2000) suggested that the upregulation of pigmentation reflected a dominant mutation that resulted in massive activation of phenylpropanoid biosynthetic genes and enhanced accumulation of lignin, hydroxycinnamic acid esters, and flavonoids, including various anthocyanins that were responsible for the purple color. These complicated biosynthesis pathways that proved the interesting relationship between gossypol and the gland deserve further studies.

From Table 3, the related genes may distribute in various pathways and showed an extensive characteristic. The results may also indicate the complexity of the regulation in the network of pigment gland morphogenesis in cotton. Two peroxidase genes were involved in phenylpropanoid biosynthesis, methane metabolism, and phenylalanine metabolism pathway, which may imply a key role in regulation. The existence of PR proteins and peroxidases may give proof that gossypol and related terpenoid aldehydes in the cotton gland has the function to resist the attack of insect and pathogen.

RT-PCR analysis indicated that the four genes showed similar expressed trend with the GeneChip results during gland morphogenesis in glanded Chuan 2802 and glandless N5. These genes were confirmed by RT-PCR indeed.

In the studies by microarray analysis, we have identified a large number of genes preferentially expressed during gland morphogenesis. This information will facilitate a rapid and targeted reverse genetics approach for identifying key mediators of gland form and function. The differentially expressed genes may play key roles, both known and unknown, in gland morphogenesis and gossypol biosynthesis and related networks. The results suggest that the regulation of pigment gland morphogenesis and the

Table 3 Representative metabolic pathways identified by KOBAS

Pathway	Gene number	Pathway ID	GenBank ID	Definition
Phenylpropanoid biosynthesis	2	ko00940	AY074794.1;;AF155124.1	Peroxidase
Methane metabolism	2	ko00680	AY074794.1;;AF155124.1	Peroxidase
Biosynthesis of steroids	2	ko00100	DQ350145;; DQ350145	3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR3)
Phenylalanine metabolism	2	ko00360	AY074794.1;;AF155124.1	Peroxidase
Amino sugars metabolism	1	ko00530	CD486070	Class I chitinase
Other enzymes	1	ko00000	DQ250028	Alternative oxidase (AOX1)
Transcription factors	2	ko00000	AY572462.1;; AY572462.1	Ethylene response factor 2

associated defense response, including gossypol biosynthesis, is complex. The results of this study can serve as a guide for further gene function research and a basis for the related gene networks.

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