GbPDF1 Is Involved in Cotton Fiber Initiation via the Core cis-Element HDZIP2ATATHB2

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Cotton (Gossypium spp.) fiber cells are seed trichomes derived from the epidermal layer of the cotton seed coat. The molecular components responsible for regulating fiber cell differentiation have not been fully elucidated. A cotton PROTODERMAL FACTOR1 gene (GbPDF1) was found to be expressed preferentially during fiber initiation and early elongation, with highest accumulation in fiber cells 5 d post anthesis. PDF1 silencing caused retardation of fiber initiation and produced shorter fibers and lower lint percentage compared with the wild type, indicating that the gene is required for cotton fiber development. Further analysis showed that a higher accumulation of hydrogen peroxide occurred in the RNA interference transgenic cotton lines. Meanwhile, the expression of several genes related to ethylene and pectin synthesis or sugar transport during cotton fiber growth was found to be significantly reduced in the PDF1-suppressed cotton. Three proteins interacting with GbPDF1 in yeast and in planta might involve cellular signaling or metabolism. GbPDF1 promoter::GUS constructs in transgenic cotton were predominantly expressed in the epidermis of ovules and developing fibers. Progressive deletions of the GbPDF1 promoter showed that a 236-bp promoter fragment was sufficient for basal GbPDF1 transcription in cotton. Mutation of putative regulatory sequences showed that HDZIP2ATATHB2, an element within the fragment, was essential for PGbPDF1-1 expression. The binding activity between this cis-element and nuclear extracts from fiber-bearing cotton ovules at 5 d post anthesis was specific. We conclude that GbPDF1 plays a critical role together with interaction partners in hydrogen peroxide homeostasis and steady biosynthesis of ethylene and pectin during fiber development via the core cis-element HDZIP2ATATHB2.

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Cotton PROTODERMAL FACTOR1 (GhPDF1) is considered to be a potential “patterning” gene working in fiber cell primordia in conjunction with other proteins. The expression of GhPDF1 is strongly induced in immature ovules, prior to the formation of fiber initials, and remains highly expressed until 5 d post anthesis (DPA; Lee et al., 2006). Our previous research in G. barbadense 3-79 demonstrated that the transcripts of GbPDF1 accumulate in a similar pattern, consistent with a possible role in cell fate determination as well as fiber development (Tu et al., 2007). Two protodermal factor-related transcripts were most abundant in the GH_TMO library, which was enriched in ESTs of ovules during the earliest stage of fiber development (Yang et al., 2006). AtPDF1, the homolog of GbPDF1 in Arabidopsis, is a protein involved in cell fate determination in the early stage of epidermal tissue development. The expression of AtPDF1 was exclusively limited in the L1 layer of shoot apices and was protoderm specific during embryogenesis (Abe et al., 1999). Nonetheless, the role of GhPDF1 in the cotton ovule and protodermal fiber cells remains unclear.

To unravel the role of PDF1 function in cotton fiber development, we used bioinformatic, molecular genetics, and interaction with other molecules during fiber initiation and early differentiation. Silencing GbPDF1 expression in cotton ovules around the day of anthesis revealed its role in the timing of fiber cell differentiation. A proposed functional mechanism of PDF1 in regulating the determination of fiber cell fate is discussed.

RESULTS

Isolation and Characterization of PDF1 in Cotton

GbPDF1 was isolated from a normalized cDNA library of G. barbadense 3-79 fiber (Tu et al., 2007). The full-length GbPDF1 cDNA consisted of 885 nucleotides, encoding a polypeptide of 294 amino acids.

Southern blotting of genomic DNA from diploid and tetraploid cotton showed that PDF1 is a low-copy-number gene in cotton (Supplemental Fig. S1A). Three and two PDF1-homologous genes were obtained from the genomic DNA of G. barbadense and G. hirsutum, respectively. The five sequences were classified into three types according to differences in the introns in their coding regions (Supplemental Fig. S1B). The two genes (GbPDF1.1 and GhPDF1.1) lacking introns were classified as type I. Type II contained only one sequence that was from G. barbadense (GbPDF1.2) with one 113-bp intron at the 54th amino acid. Each of these three cDNAs had an open reading frame of 885 bp. Five repeats of motif SY(S)GGGSPP were found between the two transmembrane regions. The remaining two sequences (type III) encoded proteins of 325 amino acids (GbPDF1.3) and 309 amino acids (GhPDF1.3), and each of them contained two introns. In addition, seven and nine repeats of the SY(S)GGGSPP(P) motif were found in the deduced proteins of GbPDF1.3 and GhPDF1.3, respectively. As compared with GbPDF1.1, with a putative protein sequence identical to GbPDF1, a total of nine substitution sites were present in the other four proteins (Supplemental Fig. S1C).

GbPDF1 contains novel conserved domains. Eleven GbPDF1-homologous peptides from UniGenes of Gossypium raimondii, Citrus sinensis, and others were found by database searching. The proteins show high homology, especially at the C terminus (Supplemental Fig. S2A), suggesting that this region is important for PDF1 function. The proteins most similar to GbPDF1 were from Citrus species (Ccl.2324 and Csi.5600; Supplemental Fig. S2B).

Bioinformatics analyses predicted that GbPDF1 contains two transmembrane helices (Tusnády and Simon, 2001). The first is in the N terminus (residues 9–28), and the second is in residues 193 to 210 (Supplemental Fig. S1C). To determine the subcellular localization of GbPDF1, a translational fusion between GbPDF1 and G3GFP (GbPDF1::G3GFP), under the control of the cauliflower mosaic virus (CaMV) 35S promoter, was transiently expressed in tobacco (Nicotiana benthamiana). Confocal imaging revealed that GbPDF1::G3GFP was localized to the cell surface and cytoplasm (Supplemental Fig. S3A). To examine whether GbPDF1 was localized to the cell wall, a plasmolysis experiment was performed in transformed Arabidopsis root cells. GFP signal was internalized and associated with the plasma membrane and cytoplasm, but there was no signal present around the cell wall (Supplemental Fig. S3B). These data suggest that GbPDF1 is likely to be associated with the plasma membrane, cytoplasm, or both but is not secreted to the cell wall or localized to the nucleus. This is consistent with GbPDF1 having two transmembrane domains.

Expression of GbPDF1 Is Correlated with Cotton Fiber Initiation and Early Elongation

GbPDF1 transcripts were detected in the immature ovules of G. barbadense 3-79 as early as prior to the formation of fiber initials and reached the highest level in the 5-DPA fiber (Fig. 1A). The transcript abundance sharply decreased from 5- to 25-DPA fibers, which mimicked the results of northern blotting during fiber development (Fig. 1B). In root and leaf, expression of GbPDF1 was at a very low level (Tu et al., 2007). A much higher level of GbPDF1 transcripts was found in the 5-DPA fiber-bearing ovules of Xuzhou 142 (Xu142) than in the same stage ovules of the fuzzless-lintless mutant (Xu142 fl), which lacks fibers (Fig. 1C). These results indicate that GbPDF1 is highly expressed in cotton fibers during fiber initiation and the early stages of elongation.

(Machado et al., 2009). More recently, Walford et al. (2011) reported that another MYB protein, GhMYB25-like, plays a key role in the very early stages of fiber cell differentiation, with suppression of GhMYB25-like resulting in cotton plants with fiberless seeds.
RNAi plants was investigated by quantitative reverse blotting (Supplemental Fig. S5). Positive transformants were confirmed by Southern formed lines showed normal phenotypes, and the planted to soil for seed reproduction. All the transgenic cotton lines (T0) were obtained and transgenic cotton lines were more abundant in 5-DPA ovules of Xu142 than its test, * t, P < 0.05). The accumulation of PDF1 transcript accumulation in the 5-DPA fiber cells. In the top panel, lanes were loaded with 20 μg of total RNA from ovules with fibers attached (0 DPA) or fibers (5–25 DPA). The full-length cDNA of GbPDF1 was used as the probe. The bottom panel shows ethidium bromide staining of the RNA gel used to show equal loading. C, PDF1 transcripts were more abundant in 5-DPA ovules of Xu142 than its fl mutant (Xu142 fl). The accumulation of PDF1 in the fiber-bearing ovules at 5 DPA was significantly higher than that of the fl ovules (t test, * P < 0.05).

PDF1-Suppressed Cotton Showed Retarded Fiber Differentiation

The sequence of GbPDF1 was identical to the PDF1 homolog isolated from G. hirsutum cv TM-1 (GbPDF1.1). To identify the function of GbPDF1, one overexpression construct and two RNA interference (RNAi) constructs targeted to the coding region or 3' untranslated region, respectively, were generated and introduced into cotton via Agrobacterium tumefaciens-mediated transformation. GbPDF1-overexpressing transgenic cotton was difficult to obtain because of poor plant regeneration (Supplemental Fig. S4). Only a few morphologically abnormal plantlets were obtained, and they failed to set seed. However, 28 independent PDF1-suppressed (RNAi) transgenic cotton lines (T0) were obtained and transplanted to soil for seed reproduction. All the transformed lines showed normal phenotypes, and the positive transformants were confirmed by Southern blotting (Supplemental Fig. S5). The expression level of PDF1 in 5-DPA fibers of T1 RNAi plants was investigated by quantitative reverse transcription (qRT)-PCR. The accumulation of PDF1 transcripts was significantly decreased in the ovules and fiber cells of the silenced plants compared with the wild-type plants (Supplemental Fig. S6A). The ovules at 1 DPA from two independent RNAi transgenic lines (U44 and C35) and a control were subjected to scanning electron microscopy. Fiber elongation in the RNAi transformants was retarded compared with both wild-type and control plants (Supplemental Fig. S6B). To further examine the effect of PDF1 silencing, we analyzed the transgenic plants of the T2 generation. The T2 progeny from RNAi line U44 were examined the following year, and the same results were obtained at 1 DPA (Fig. 2C), indicating that the delay in fiber development caused by PDF1 suppression could be stably inherited. To ascertain the time point at which the difference in fibers between the RNAi plants and the wild type appeared, ovules before anthesis were sampled for analysis. The wild-type fibers began to balloon out from the epidermal surface 12 h before flowering, whereas the PDF1-silenced fibers showed no trichome protrusion on the surface of the ovules (Fig. 2C). Therefore, the retardation occurred at the fiber cell fate determination stage, suggesting that the suppressed PDF1 was effective in the initial protrusion of the fiber cells above the ovule surface. Comparison of the mature fiber length between the PDF1-RNAi transgenic plants and control plants revealed that the reduction of PDF1 in fiber resulted in shorter fibers in the RNAi transformatants than in the wild type. The length of fiber in the T2 line U44-1 (27.03 ± 0.05 mm) was significantly shorter than that of the controls (28.86 ± 1.03 mm; t test, P < 0.05), and the fiber length of U43-5 was 28.34 ± 0.54 mm, a little shorter than the control (Fig. 2, D and E). The lint percentage of the transgenic lines was consistently lower than that for the control plants (Fig. 2F). However, other measures of agronomic performance, such as micronaire value, were not different between the transgenic lines and the control (data not shown). All the observations for RNAi plants were associated with a reduction of GbPDF1 transcript and protein (Fig. 2, A and B).

The Fiber Development Network Is Impaired Partially in the GbPDF1-Suppressed Transgenic Plants

In order to identify the mechanism of GbPDF1, its expression profiles were analyzed when several phytohormones and chemicals were added into the ovule culture medium. The GbPDF1 transcripts in the wild-type ovules increased significantly with 50 μM hydrogen peroxide (H2O2) and decreased with diphenyleneiodonium (DPI; Fig. 3A). However, the H2O2 content in the knockdowns of GbPDF1 3-DPA in planta ovules was much higher than in the wild type (Fig. 3B). The expression level of the functional ASCORBATE PEROXIDASE1 (APX1) was consistently increased in the PDF1-suppressed 3-DPA ovules and fibers to maintain H2O2 homeostasis during cotton fiber development (Li et al., 2007; Fig. 3C). Therefore, GbPDF1
Figure 2. The PDF1-suppressed T2 cotton further confirmed that fiber differentiation was later than that of controls, and the mature fiber was shorter or with a lower lint percentage than the wild type. A, Northern blotting showed the lower expression of PDF1 in the fiber-bearing ovules of RNAi transgenic plants compared with the wild type (WT). The signal of 32P-labeled PDF1 transcripts (top) and the ethidium bromide staining of the RNA gel showing equal loading (bottom) are shown. B, Protein abundance of PDF1 was decreased in the RNAi ovules according to western blotting with specific antibodies against GbPDF1. In the top panel, lanes were loaded with 50 μg of total protein extracted from 1-DPA cotton ovules and detected using PDF1-specific polyclonal antiserum. In the bottom panel, anti-histone 3 served as the loading control. C, The morphological differences of fiber cells between the wild type and PDF1-silenced T2 cotton plants. Fiber cells of the wild type ballooned out from the seed coat at about 12 h before anthesis (a–c). However, the PDF1-suppressed cotton ovules were smooth without fiber initials (d–f). The fiber cells were observed at 50× (a and i), 500× (b and d), and 1,500× (c and e). White arrows show the fiber initial.
may play a role in the regulation of antioxidation metabolism.

H$_2$O$_2$ concentration and GhAPX1 transcription can be promoted by exogenous ethylene treatments in cotton ovules (Li et al., 2007). Three ethylene synthesis-related genes (GhACO1, GhACO2, and GhACO3; Shi et al., 2006) active during fiber development were selected for a comparative analysis between the transgenic and wild-type fiber and ovules at 0 DPA using qRT-PCR. GbPDF1 suppression led to a substantial reduction of all three GhACO genes (Fig. 3D). These data biosynthesis is an important biochemical reaction downstream of ethylene signaling that promotes fiber elongation (Pang et al., 2010). We selected several known pectin-related genes, UER1, UGP1, and UGD1 (Pang et al., 2010), and two putative glycosyltransferases (GT8 and GT8-like) for analysis in 0-DPA ovules. GT8 was preferentially expressed in ovules at the day of anthesis, but GT8-like showed no significant expression difference during fiber development (Supplemental Fig. S7B). The transcripts of UER1, UGP1, and UGD1 were unchanged in the GbPDF1-silenced plants; however, the GT8 and GT8-like transcripts were clearly decreased by half or more (Fig. 3D). In addition, our results revealed that reduced GbPDF1 expression led to a substantial decrease of SUT1 (TC192320) and two putative sugar transporters (TC259819 and TC257931; Fig. 3D), which indicated that GbPDF1 potentially influences the supply of carbon during fiber development.

Interacting Proteins of GbPDF1 in Yeast and in Planta

Identifying the proteins interacting with GbPDF1 would improve our understanding of the role of GbPDF1 during cotton fiber differentiation. The full-length GbPDF1 fused with the GAL4-DNA-binding domain was used to screen a yeast two-hybrid library, using the cDNAs from cotton ovules and fibers to construct prey vectors. Thirteen candidate GbPDF1-interacting proteins were identified by screening 1.18 × 10$^6$ colonies (Supplemental Table S1). The open reading frame of each candidate was fused with the activation domain for retransformation.

For further analyses, new baits containing specific fragments of GbPDF1 were constructed, designated as ΔN (residues 31–294), ΔNM (residues 182–294), and ΔC (residues 1–182; Fig. 4A). Three putative proteins were obtained after being retransformed into the bait strains that contained full-length or domain-deleted GbPDF1 fragments; these proteins were named PPIP1 (for putative GbPDF1 interaction protein), PPIP2, and PPIP3, respectively (Fig. 4B; Table I). These three genes could be detected in fiber and ovules (Supplemental Fig. S7, A and B).

As shown in Table I, PPIP1 encodes a ubiquitin protein with a ubiquitin-like domain in the N terminus followed by a ubiquitin-associated domain. However, no interaction was detected between the truncated GbPDF1 fragments and PPIP1. PPIP2, a protein containing an ankyrin repeat (AKR) with high homology to NtTIP2 (Fridborg et al., 2003), interacted only with the full-length GbPDF1. A GRAM (for glucosyltransferases, Rab-like GTPase activators, and myotubularins) domain was found in PPIP3. Interestingly, PPIP3 could not bind with GbPDF1 but could bind to the fragment lacking the N domain (residues 1–30). All of these results were confirmed by β-galactosidase assay (Supplemental Fig. S8). The GbPDF1 protein could not form a dimer in yeast, because no interaction was found between GbPDF1 and the four baits (Fig. 4B). Therefore, it was less likely that GbPDF1 works as a homodimer.

The bimolecular fluorescence complementation (Waadt et al., 2008) and transient gene expression (Yoo et al., 2007) systems verified the protein interactions in planta. The corresponding constructs (VenusN: GbPDF1 with VenusC:PPIP1/2 and VenusN:GbPDF1 ΔN with VenusC:PPIP3, respectively) were cotransformed into protoplasts, and strong yellow fluorescence was expressed in the plasma membrane and cytoplasm (Fig. 4C). These results suggest that GbPDF1 (GbPDF1 ΔN) interacts with PPIP1, PPIP2, or PPIP3 in living plant cells. Additionally, in the RNAi transgenic cotton ovule, PPIP1 and PPIP2 expression levels were decreased (Fig. 4D).

The 236-bp Promoter Fragment Was Sufficient to Drive GUS Expression in the Cotton Ovule and Fiber

Two GbPDF1 gene promoters, differing in the lengths of the 5' flanking regions (PGbPDF1-1, 1,679 bp; PGbPDF1-2, 1,285 bp), were cloned from G. barbadense 3-79 by genome walking. The two promoters shared a nearly identical 390-bp sequence upstream of the translational initiation codon. The promoter sequences were classified into four distinct regions according to the locations of known cis-elements (Fig. 5A). The regions from −391 to −1,679 bp in the sequence of PGbPDF1-1 and from −391 to −1,285 bp in PGbPDF1-2
were designated as regions D and D’, respectively. The common 390-bp fragment comprised regions A (from −1 to −128), B (−129 to −236), and C (−237 to −390). Region A contained only the elements for basal transcription. A putative TATA box (from −95 to −88) that provided the binding site for RNA polymerase was present in the two promoters. The transcriptional start site of GbPDF1 was found at position −63 from the initial ATG according to the TSSP prediction result (Fig. 5A). Region B contained a binding site (CARGCW8-GAT) for the MADS-domain protein AGAMOUS-like15 (Tang and Perry, 2003), and three cis-elements were identified that were predicted to bind with homeodo-

Figure 3. The knockdowns of GbPDF1 impaired the fiber development gene network. A, qRT-PCR analysis of PDF1 transcripts from wild-type ovules after 2 d of control (CK), H$_2$O$_2$, or DPI treatment. RNA samples from ovules cultured for the same period of time without the addition of H$_2$O$_2$ or DPI were used as controls. B, Higher concentrations of H$_2$O$_2$ accumulated in GbPDF1-suppressed cotton ovules at 3 DPA. FW, Fresh weight. C and D, Expression analysis of several fiber-related genes in ovules from GbPDF1-suppressed cotton lines and the control at 3 or 0 DPA. The y axis shows the relative expression level normalized to the wild type (WT).
DPA (Fig. 5B). GUS activity was found in the balloononed fibers, the inner integument, and some cells of the outer integument adjacent to the fibers (Fig. 5B). GUS expression was also visible in the reproductive tissues such as anther and style, but the GUS activity was much less than that of fiber-bearing ovules (Fig. 5B; Supplemental Fig. S9). However, the signals were barely detectable in vegetative organs such as leaf, bract, and stem.

To define the core regulatory region responsible for the transcriptional regulation of GbPDF1, three progressive 5′ deletions of PGbPDF1-1 fused with GUS were also introduced into cotton. Region A alone could not activate GUS in the ovules or other tissues (Fig. 5C). However, no significant difference in GUS expression pattern was observed in the transgenic cotton containing ΔC and ΔD as compared with the full-length promoter. Accordingly, the minimal region necessary for the expression of PDF1 in cotton was confined within the 236-bp fragment (regions A and B).

In addition, we also determined GUS protein activity in the ovule and fiber at different developmental stages. The highest GUS activity was in the ovule on the day of anthesis, even though the transgenic cotton carried different lengths of promoter fragments. However, the promoter activity decreased gradually during fiber development, and a very low accumulation of expression was detected in the 15-DPA fiber cells, except in the lines that carried the ΔD construct (Fig. 5D). Clearly, region B was important for the basal transcription of GbPDF1 but was not responsible for its high accumulation in the fibers, according to the different GUS activity between plants that carried ΔCD and ΔD.

**Mutational Analysis within Region B Revealed That HDZIP2ATATHB2 Was the Key Regulatory Element**

We deleted region B to generate the constructs ΔB, ΔAT9 (HDZIP2ATATHB2), ΔL1 (L1 box and CARGCW8-GAT), and ΔHD (BIHD1OS). To elucidate the role of the putative regulatory sequences, T1 and T2 generations of Arabidopsis transformants with PGbPDF1::GUS were used for histochemical analyses. GUS activity was detected in the vegetative shoot meristem, cotyledons of a

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**Figure 4.** The native or truncated GbPDF1 interacted with PPIPs in yeast and in planta. A, Structure of the GbPDF1 protein and schematic diagrams of truncated fragments for yeast two-hybrid analysis. GbPDF1 was divided into three fragments as N (amino acids 1–30), M (amino acids 31–180), and C (amino acids 181–294). ΔN was the GbPDF1 protein with the N fragment deleted, ΔNM was the protein with only the C fragment, and ΔC was the protein without fragment C. The native and different fragments were fused with the DNA-binding domain of GAL4 as bait for yeast two-hybrid analysis. The plasmid of pDEST32 was empty. B, PPIP1 and PPIP2 interacted with GbPDF1, but PPIP3 bound with the N-domain-deleted protein in yeast. The GAL4-activation domain (AD)-fused interaction partners were retransformed into the yeast strains carrying different baits. The pDEST32 plasmid was empty. Transformants were assayed for growth on 20 mM 3-amino-1,2,4-triazole (3-AT) synthetic complete medium without Leu, Trp, and His (SC-L-T-H). ORF, Open reading frame. C, The interactions were confirmed by transient expression in planta. Fluorescence signal could be detected in the transformed protoplasts, except for the negative control. D, PPIP1 and PPIP2 were down-regulated in the RNAi transgenic cotton plants compared with the wild type.
5-d-old seedling, and the cutting site of older leaves. After flowering, the styles, stamens, and immature siliques were stained (Fig. 6A).

As expected, the region B-deleted promoter was unable to drive the reporter gene. GUS activity in the plants that carried ΔL1 and ΔHD showed a similar pattern as those containing the full-length promoter. However, GUS activity was undetectable in the inflorescence and immature siliques of transgenic plants carrying the construct without HDZIP2ATATHB2 (5'-TAATAATTA-3', Fig. 6B).

To confirm if there were any proteins binding with HDZIP2ATATHB2, several probes were designed for gel-shift assays with the nuclear extracts of the reproductive tissues of Arabidopsis and fiber-bearing cotton ovules. The first probe was HDZIP2ATATHB2 itself; the second and the third ones were P1 (from -229 to -180) and P2 (from -179 to -130); in addition, SP1 (5'-TAAT-3') and SP2 (5'-AATA-3') split the HDZIP2ATATHB2 element. No matter whether the nuclear extracts were from Arabidopsis or cotton, the 32P-labeled HDZIP2ATATHB2 was retained during the separation on polyacrylamide gels (Fig. 7, A and B). The competitive probes unrelated to HDZIP2ATATHB2 did not efficiently reduce the intensity of the band shifts compared with HDZIP2ATATHB2 itself as the competitor (Fig. 7C). These results showed that the observed HDZIP2ATATHB2-binding activity was sequence specific. However, no interaction was found between P1 or P2 and the nuclear extract from Arabidopsis (Supplemental Fig. S10, A and B), which agreed with the GUS staining result. The loss activity of probes binding with cotton nuclear extracts was observed once the HDZIP2ATATHB2 was split (Supplemental Fig. S10C). These data together suggest the direct involvement of the HDZIP2ATATHB2 motif in regulating the basal expression of GbPDF1 in Arabidopsis as well as in cotton fiber.

**DISCUSSION**

**GbPDF1 Contained Conserved Domains**

GbPDF1 is a Pro- and Gly-rich protein with two putative transmembrane helices and several repeating motifs. The motif SY(S)GGGSP found in the cotton PDF1 proteins was novel, and the repeat number was variable within the cotton homologs (Supplemental Fig. S1C). Domains conserved among the PDF1 proteins were assumed to be critical for their functions. First, we showed that the N terminus was important for the interaction between GbPDF1 and PPIPs, because the interaction patterns changed when the N region was deleted. Second, the highly homologous C terminus suggested its potential role in the protein’s function. The loss-of-function interaction between GbPDF1 and PPIPs after fragment C was deleted in yeast proved this deduction. Notably, the C region alone was not sufficient, because the PPIPs could not interact with the GbPDF1 fragment without the N and M domains (Figs. 4, A and B).

**The Potential Role of GbPDF1 in Cotton Fiber Development**

Previous studies suggested that PDF1 plays a critical role in the early stage of the development of epidermal tissues under the regulation of AtPDF2 and ATML1 (Abe et al., 1999, 2003). An intact epidermis is crucial for certain key processes in plant development, shoot growth, and plant defense (Javelle et al., 2011). For instance, the double L1-specific gene mutant pdf2-1 atml1-1 resulted in a disorganized shoot apex during embryogenesis and had problems in leaf initiation and development (Abe et al., 1999, 2003). Trichomes are the specialized structures that differentiate from epidermal cells (Ishida et al., 2008). Cotton fibers are trichomes, but they are derived from individual cells of the epidermal layer of the seed coat. Studies have also shown that cotton fibers share a similar model of cell fate determination with the leaf trichomes in Arabidopsis (Wang et al., 2004; Lee et al., 2007).

In our studies, we found that the transcripts of *GbPDF1* accumulated predominantly in the fibers, especially at the early stage, suggesting its potential role in fiber development (Fig. 1, A and B). PDF1-silenced cotton showed retarded fiber initiation and had shorter fibers or lower lint percentage, and a field trial lasting for more years will be done to confirm the traits (Fig. 2; Supplemental Fig. S6). Crucially, scan-
ning electron micrographs of the RNAi-transformed cotton ovules indicated that the delay in fiber development occurred before the outgrowth of fiber cells specialized from seed epidermal cells (Fig. 2C; Supplemental Fig. S6B). These results provided evidence for the role of PDF1 in regulating the initial protrusion of the fiber cells above the ovule surface, which is in agreement with the report that PDF1 may play important roles in the differentiation of fiber initials in the ovule protodermal layer (Yang et al., 2006). The timing of fiber initiation has a great impact on the number of lint fibers, because the later initials always develop as fuzz fibers (Lee et al., 2006). Both cotton fiber yield and quality are greatly improved by increasing the number.

**Figure 5.** Diagram of the GbPDF1 promoter, and expression patterns of GUS driven by the native and truncated promoter in transgenic cotton plants. A, The structure of the GbPDF1 promoter, PGbPDF1-1. Regions A, B, C, and D ranged from −1 to −128, −129 to −236, −237 to −390, and −391 to −1,679 bp, respectively. The transcription start site was at −63 bp upstream of ATG. The TATA box was located from −95 to −88 bp. The predicted cis-regulatory elements in region B are labeled in detail. B, PGbPDF1 preferentially drives GUS expression in the cotton ovule and fiber. Staining was observed on the surface of the ovules and fiber at −1 DPA (a), 2 DPA (b), and 5 DPA (c). Reproductive tissues such as flower (g), anther (h), and filament (i) were stained. No GUS activity was detected in bud (f), stem (j), leaf (k), or bract (l) of transgenic cotton. GUS was expressed moderately in the cotyledons and root tip of the 2-d-old seedlings (m). A high level of GUS activity was located in the fiber cells and inner integument according to longitudinal sections of the stained 1-DPA ovules carrying PGbPDF1-1::GUS viewed with a light microscope at 100× (d) and 400× (e). f, Fiber cell; ii, inner integument; n, nucellus; oi, outer integument. Black arrows show the fiber initials. C, Comparison of GUS expression in transformed cotton among different constructs. The plants carrying the ΔBCD vector showed no GUS expression in anther and 2-DPA ovules. The ΔCD- and ΔD-carrying plants showed a similar expression pattern to the native promoters PGbPDF1-1 and PGbPDF1-2. D, The deleted promoters resulted in different accumulation patterns of GUS. GUS activity in the ovules and fibers of transformed cotton was determined. Plants carrying each of the four constructs showed the most accumulation of GUS in 0-DPA ovules. Cotton with ΔD retained a high level of GUS from 0 to 15 DPA, while GUS in the samples with the other three constructs decreased. Error bars represent SD of three replicates. 4-MU, 4-Methylumbelliferone.
of lint fibers, which is the result of increasing auxin levels in the epidermis of cotton ovules at the fiber initiation stage that transforms more fuzz cells into lint cells (Zhang et al., 2011). The shorter fibers and the decrease of fiber lint percentage in the PDF1-RNAi transgenic lines were consistent with the delay of fiber initiation.

Three important genes for ethylene biogenesis were reduced compared with the wild type. Previous studies demonstrated that ethylene and H$_2$O$_2$ interact to regulate cotton fiber elongation, and they can be induced by each other (Li et al., 2007; Qin et al., 2008). However, the increase in H$_2$O$_2$ content is accompanied by down-regulation of the three ACO genes in the PDF1-RNAi cotton (Fig. 3, B and D). The expression levels of the three ethylene-responsive genes (UIER1, UGP1, and UGD1; Pang et al., 2010) showed no differences between wild-type and transgenic ovules (Fig. 3D), proving that no more ethylene was induced by the high accumulation of H$_2$O$_2$ in the knockdowns of GbPDF1. So, it is likely that GbPDF1 plays a role in the feedback regulatory mechanism from H$_2$O$_2$ to ethylene biosynthesis in modulating cotton fiber development.

The pectin biosynthesis of the PDF1-suppressed cotton fibers and ovules might be disturbed because of the down-regulation of GT8 and GT8-like together with the reduced carbon source (Fig. 3D). Pectin is a major polysaccharide component of the primary cell wall (Gou et al., 2007); it is important for fiber elongation and is regulated by ethylene signaling (Pang et al., 2010). Cotton GT8, which encodes a putative glycosyltransferase, might be involved in the synthesis of the pectic polysaccharides (Scheible and Pauly, 2004). The cotton GT8 is highly expressed in the 0-DPA ovules (Supplemental Fig. S7B) and shares 78% identities and 88% positives (number and fraction of residues for which the alignment scores have positive values; http://searchlauncher.bcm.tmc.edu/help/BLASToutput.html) at the amino acid level with AtGATL1, which plays a potential role in the synthesis of the structure to which the reducing end xylan oligosaccharide is attached (e.g. a specific fraction of pectin; Mohnen, 2008; Kong et al., 2011). Cotton GT8 may function similarly to AtGATL1, owing to the largely conserved roles of GATL genes between different species (Kong et al., 2011).

Our yeast two-hybrid data suggest that the interaction partners of GbPDF1 may involve cellular signaling or metabolism. PPI1 is a well-conserved ubiquitin protein that functions in the regulation of protein degradation (Rothenberg and Monteiro, 2010). Dsk2p, a homolog of PPI1 in Saccharomyces cerevisiae, is an adaptor that facilitates the delivery of polyubiquitin...
chains or proteins to the proteasome (Funakoshi et al., 2002). One homolog of PPIP2 is Arabidopsis ANKYRIN REPEAT-CONTAINING PROTEIN2A (AKR2A), which functions as a molecular chaperone for proteins that are targeted to membranes of different organelles in plant cells (Shen et al., 2010). Decreased AKR2A expression leads to reduced steady-state levels of peroxisomal membrane-bound APX3 and reduced targeting of APX3 to peroxisomes in plant cells, indicating that AKR2A plays an important role in controlling antioxidation metabolism (Shen et al., 2010). The GRAM domain in PPIP3 has been reported to be involved in membrane-associated processes such as intracellular protein or lipid-binding signaling pathways (Doerks et al., 2000). Oku et al. (2003) found that GRAM was essential for the fungal UDP-Glc:sterol glucosyltransferase in peroxisome degradation. The expression levels of PPIP1 and PPIP2 were downregulated in PDF1-silenced cotton ovules, which suggested that the expression of these genes was also influenced by GbPDF1, although the mechanism of signal transduction could not be elucidated in this study.

A proposed model could be constructed according to the results above. PDF1 may influence ethylene and H$_2$O$_2$ production in cotton ovules, which would lead to a rate-limiting step in pectin biosynthesis and ultimately result in retarded fiber initiation and shorter fibers. GbPDF1 is synthesized on free ribosomes and moved to the right destination with the help of PPIP2. It then functions with PPIP3, and PPIP1 responds to the degradation of GbPDF1.

The Basal Expression of GbPDF1 Is Regulated via a cis-Element, HDZIP2ATATHB2, in the Promoter

According to the expression profile of 5’ deletions of PGbPDF1-1, we found that regions A and B were sufficient for the expression of GbPDF1 in cotton. Region B was thought to be critical for the basal expression of GUS, which was confirmed by GUS being undetectable in the plants carrying the ΔB construct (Fig. 6B). The expression pattern of PGbPDF1-1::GUS in Arabidopsis was similar to that driven by the promoter of AtPDF1 (Fig. 6A; Abe et al., 1999). The transcription of AtPDF1 was mainly regulated through the DNA-protein interaction between a pair of functionally redundant HD-ZIP class IV transcriptional factors (AtPDF2 and ATML1) and the L1 box. Previous studies showed that the L1 box was an 8-bp motif required not only for epidermis-specific gene expression in Arabidopsis but also for the expression of a fiber-specific gene, GaRDL1 (Abe et al., 2001; Wang et al., 2004). Two HD-ZIP class IV family members in cotton, GhHOX3 and GbML1, were found to interact with the L1 box (Wang et al., 2004; Zhang et al., 2010). Unexpectedly, the promoter fragment without L1 was able to drive GUS expression in the flowers and siliques (Fig. 6B). Moreover, the binding site for OsBIHD1 might not be the important element for PGbPDF1-1, because there was no significant difference between the transformed plants carrying ΔHD and the full-length GUS-fused plants. Ultimately, the plants with ΔAT9 abolished the ability of transcriptional activation completely. In addition, HDZIP2ATATHB2 could bind to proteins from 5-DPA fiber-bearing ovules in vitro specifically (Fig. 7). These observations suggest that HDZIP2ATATHB2 plays a critical role in the basal transcriptional control of PDF1 in cotton. However, region B as well as HDZIP2ATATHB2 is not responsible for the high accumulation of PDF1 transcripts in fibers, because very low GUS activity can be detected, except in ovules at 0 DPA in ΔCD-carrying cotton (Fig. 5D). Based on these results, we proposed that interaction between HDZIP2ATATHB2 and the binding protein was also activated in the β mutant to keep a moderate expression level in ovules. The positive regulatory elements involved in increasing the transcriptional level of PDF1 might be located in region C, according to the GUS activity comparison between ΔCD and ΔD. In addition, the observed GUS activity in the cotton ovules and fibers at different developmental stages suggests that region D (D’), containing cis-elements was responsible...
for preserving the specificity of GbPDF1 transcripts (Fig. 5D).

HDZIP2ATATHB2 is a 9-bp pseudopalindromic motif interacting with HD-ZIP class II proteins (Ogishii et al., 2001). The Arabidopsis genome contains 10 genes belonging to the HD-ZIP II family, including ATHB2 and HAT2 (Ciarbelli et al., 2008). In Arabidopsis, overexpression of ATHB2 and HAT2 resulted in longer hypocotyls and petioles, respectively, but smaller and fewer leaves, with reduced formation of lateral roots. These phenotypes were regulated by auxin redistribution (Steindler et al., 1999; Sawa et al., 2002). These findings could help us to understand the role of the HD-ZIP II transcription factors in cotton fiber elongation through the interaction with HDZIP2ATATHB2. Future research should attempt to identify the target proteins that interact with the elements as well as their roles in cotton fiber differentiation.

**CONCLUSION**

In summary, our findings indicate that GbPDF1 is required for the fiber cell initiation process, possibly by keeping the normal concentrations of H2O2 and ethylene. According to promoter-truncated analysis, the 236-bp promoter fragment was sufficient for the gene expression in cotton. Moreover, we show that the regulatory element HDZIP2ATATHB2 supplied a binding site for the basic transcriptional machinery of GbPDF1.

**MATERIALS AND METHODS**

**Plant Materials**

The cotton plants Gossypium barbadense 3-79 and Gossypium hirsutum Xu142, Xu142 fl, and YZ1 used in these experiments were cultivated in the field in Wuhan, China, using normal farming practices. The bolls were tagged on the day of anthesis. The fiber cells at different developmental stages were removed carefully from ovules and immediately immersed in liquid nitrogen. Tissues such as petals and ovules were harvested from plants in the field and stored in liquid nitrogen. Roots and leaves were collected from 15-d-old seedlings cultured in a growth chamber. All frozen materials were preserved at −70°C. Arabidopsis (Arabidopsis thaliana) plants were grown in long-day conditions (16 h of light, 8 h of dark) under white fluorescent light at 20°C.

**Cotton Ovule Culture and Treatment with H2O2 or NADPH Oxidase Inhibitor**

Cotton ovules from wild-type plants were collected immediately after flower opening. They were sterilized and then placed in liquid BT medium supplemented with 50 μM H2O2 or 2 μM of the NADPH oxidase inhibitor DPI (Sigma; Li et al., 2010). The samples were collected for RNA extraction after 48 h of culture.

**Southern and Northern Blotting and qRT-PCR**

The methods of genomic DNA isolation and Southern blotting were according to Li et al. (2010). The probe for PDF1 gene detection was the small fragment digested from the cDNA sequence inserted in pSPORT with HindIII (Tu et al., 2007). For the detection of transgenic cotton plants, the probe was the NPTII fragment, and genomic DNA was digested with HindIII.

To determine the expression level of PDF1 in wild-type and transgenic cotton, RNA was isolated from the collected tissues using the method described by Zhu et al. (2005). Twenty micrograms of total RNA per lane was transferred onto nylon membranes. The blots were hybridized with 32P-labeled full-length GbPDF1. Hybridization and detection of the signal on the filter were as reported previously (Tu et al., 2007). The first cDNA strand was synthesized, and qRT-PCR was performed as described by Muniis et al. (2010). The expression level of UBQ7 was used as the internal control to standardize the RNA samples for each reaction. Error bars indicate the SD of three sample replicates. The primers used in the study are listed in Supplemental Table S2.

**Gene Cloning and Sequence Analysis**

To obtain the genomic sequences of the PDF1 gene in TM-1 and 3-79, gene-specific primers (PDF1-F/R) were designed to amplify the full-length open reading frame using the genomic DNA as template. All the fragments were sequenced after being linked to T-vectors. The accession numbers of these genes can be found in Supplemental Table S3. The conserved domains of proteins were searched in the National Center for Biotechnology Information database (Marchler-Bauer et al., 2009). Transmembrane regions of GbPDF1 were predicted using the Web-based program HMMTOP (http://www.enzim.hu/hmmtop/).

**Plasmid Construction and Plant Transformation**

To construct the overexpression vector, a pair of primers (OE-PDF1-F/R) with added XbaI and SacI restriction sites, respectively, was used to amplify GbPDF1. The amplified product was ligated into pCAMBIA 2300S vector without a stop codon was constructed to pGWB451 through recombination reaction (Nagawa et al., 2007). The resulting construct consisted of GbPDF1 fused to the N terminus of G3GFP under the control of the CaMV 35S promoter. The construct was transferred into Agrobacterium tumefaciens strain AGL101 by electroporation for future Arabidopsis transformation (Clough and Bent, 1998) or transient expression in tobacco (Nicotiana benthamiana) via agroinfiltration (Sainsbury and Lomonossoff, 2008). Green fluorescence of GbPDF1::G3GFP was detected in the root cells of 10-d-old Arabidopsis after plasmolysis with 4% (w/v) NaCl for 10 min as well as in the epidermal cell layer of tobacco leaves. Images were observed using a Leica Microsystems TCS SP2 AOBS confocal microscope.

**Subcellular Location of GbPDF1 in Arabidopsis Root Cells and Tobacco**

The full-length cDNA of GbPDF1 without a stop codon was constructed to pGWB451 through recombination reaction (Nagawa et al., 2007). The resulting construct consisted of GbPDF1 fused to the N terminus of G3GFP under the control of the CaMV 35S promoter. The construct was transferred into Agrobacterium tumefaciens strain GV3101 by electroporation for future Arabidopsis transformation (Clough and Bent, 1998) or transient expression in tobacco (Nicotiana benthamiana) via agroinfiltration (Sainsbury and Lomonossoff, 2008). Green fluorescence of GbPDF1::G3GFP was detected in the root cells of 10-d-old Arabidopsis after plasmolysis with 4% (w/v) NaCl for 10 min as well as in the epidermal cell layer of tobacco leaves. Images were observed using a Leica Microsystems TCS SP2 AOBS confocal microscope.
For further analysis of the cis-elements located within region B, the whole region B and regions spanning from −186 to −178, −166 to −159, and −153 to −149 bp were deleted from PGbPDF1-1 to produce ΔB, ΔAT9, ΔL1, and ΔHD promoters, respectively. The upstream region of the deleted sites was amplified with HindIII (forward primer)-PshI (reverse primer) and cloned to the pUC19 vector after being digested, then the PshI-XbaI-containing downstream region of the deleted elements was inserted at the corresponding site. The modified promoters were fused with GUS on the base of pBII121.

The overexpression vector, RNAi vectors, PGbPDF1–GUS, ΔB, ΔB, ΔBCD, ΔACD, ΔD, and PGbPDF1–GFP were introduced into cotton (YZ1) plants by A. tumefaciens-mediated (EHA105 and LBA4404) transformation as described by Jin et al. (2006) and Li et al. (2010). After regeneration, transformed cotton lines were checked by PCR (V-RNAi-F/R for RNAi constructs and V-gus-F/R for GUS-fused vectors) and Southern blotting. PGbPDF1–GUS, PGbPDF1–GUS, ΔB, ΔAT9, ΔL1, ΔHD, and pBII121 were transformed to Arabidopsis ecotype Columbia plants by the floral dip method (Clough and Bent, 1998). The transgenic Arabidopsis was selected on the base of pBII121.

GUS Assay of the Transgenic Plant Tissues

Histochemical analysis of GUS activity was performed according to the protocol reported by Sessions et al. (1999). Tissues were prefixed in prechilled 80% (v/v) acetone for 30 min and infiltrated into staining solution (0.9 g L−1 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid, 50 ms sodium phosphate buffer [pH 7.0], 20% [v/v] methanol, and 100 mg L−1 chloromycetin) under vacuum for 15 min and then moved to 37°C for 1 to 12 h. The chlorophyll was decolorized at 37°C with 75% to 95% (v/v) ethanol and fixed in 50% ethanol, 5% acetic acid, and 3.7% formaldehyde. Photographs were captured with an anatomy microscope (Leica Microsystems) or a Nikon D40 camera. The stained samples were cut into 8-μm-thick sections and examined using the protocol reported by Zhu et al. (2008). The quantitative analyses of GUS activity was expressed as pmol 4-methylumbelliferone mg−1 protein min−1, according to Cai et al. (2008). Fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 455 nm using the Infinite 200 PRO multimode reader (Tecan).

Electrophoretic Mobility Shift Assay

The isolation and quantification of nuclear extract from reproductive tissues of Arabidopsis and 5-DPA fiber-bearing cotton ovules was described previously (Qiu et al., 2007). Gel mobility shift assay was applied using the protocol reported by Dai et al. (2007). The synthetic DNA probes were generated by mixing the two complementary oligonucleotides and labeled with 32P-dCTP using the Klenow fragment (New England Biolabs). DNA-binding reactions were performed in a 30-μL mixture. The mixture contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM MgCl2, 5% (v/v) glycerol, 3.5 μg of poly(dI-dC)-poly(dI-dC) (Sigma), 10 μg of the nuclear extract, and 1 μg of the probes. After incubation at room temperature for 20 min, the mixture was separated on 12% polyacrylamide gels in Tris-Gly buffer (25 mM Tris, 2 mM EDTA, and 380 mM Gly).

Quantification of H2O2 Levels

The fiber-bearing ovules (0.5 g fresh weight) at 3 DPA from wild-type and PDF1-silenced plants were sampled for H2O2 measurement. H2O2 content was determined according to the production of H2TiO3 using TiCl4 as the substrate (Li et al., 2007). The concentration was expressed as μM g−1 fresh ovules.

Bimolecular Fluorescence Complementation

The native and N domain (residues 1–30) deleted GbPDF1 was cloned into the Spel-KpnI site of pYVENe(R) vector (Wada et al., 2008) to obtain VenusN leica systems laser-scanning microscope.

Yeast Two-Hybrid Screening

The yeast two-hybrid assay was performed using the ProQuest Two-Hybrid System (Invitrogen). The bait vectors with full-length or partial cDNA of GbPDF1 fused to the GAL4-DNA-binding domain in the pDEST22 vector were generated. A prey library of cotton ovule and fiber (0-DPA ovule; 5-, 10-, and 15-DPA fiber) was constructed by fusing cDNAs with the GAL4 activation domain in the pDEST22 vector according to the manufacturer’s instructions. The yeast strain Mal2V03 carrying the full-length GbPDF1 bait vector was transformed with the plasmid DNA of the prey cDNA library. The transformants were selected on synthetic complete selection medium containing 20 μM 3-amino-1,2,4-triazole (Sigma) but lacking Leu, Trp, and His. The positive clones were isolated and retransformed to different bait strains to test their interaction, using empty vector as the control.

Supplemental Data

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

Supplemental Figure S1. Genetic characterization of PDF1 genes in cotton.

Supplemental Figure S2. Conserved C terminus was found among the PDF1 proteins from different sources of plants by sequence alignment.

Supplemental Figure S3. Subcellular localization of GbPDF1 through the transient and stable expression of the GbPDF1::G3GFP reporter construct.

Supplemental Figure S4. Fast-proliferated nonembryogenic callus with GbPDF1 overexpression.

Supplemental Figure S5. Southern blotting of transformed cotton plants.

Supplemental Figure S6. Reduced expression of PDF1 in T1 transformed cotton resulted in retarded initiation.

Supplemental Figure S7. Expression patterns of GTS, GTS-like, and PPIPs during fiber development.

Supplemental Figure S8. The control transformants for yeast two-hybrid and β-Gal assay results.

Supplemental Figure S9. The GUS activity in anther and style driven by the GbPDF1 promoters.

Supplemental Figure S10. Electrophoretic mobility shift assay results showed no interaction between PI or P2 and extracts from Arabidopsis.
Supplemental Table S1. The proteins identified by yeast two-hybrid library screening.

Supplemental Table S2. Oligonucleotides used in this study.

Supplemental Table S3. Accession numbers of the sequences in this article.

Supplemental Table S4. The number of transgenic plants obtained in this study.

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