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# Gibberellin 20-oxidase promotes initiation and elongation of cotton fibers by regulating gibberellin synthesis

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ABSTRACT

Cotton is the leading natural fiber, and gibberellin (GA) is a phytohormone involved in the development of cotton fibers. However, it is largely unknown how the GA content in ovules and fibers is regulated and how the endogenous GA concentration affects fiber development. To address these questions, three GA 20-oxidase homologous genes (*GhGA20ox1–3*) were cloned and the endogenous bioactive GA content in developing ovules and fibers determined by liquid chromatography-electrospray ionization-mass spectrometry. Real-time reverse transcription PCR (RT-PCR) revealed that *GhGA20ox1* expressed preferentially in elongating fibers and that the expression level varied with the endogenous GA content consistently, while *GhGA20ox2* and *GhGA20ox3* transcripts accumulated mainly in ovules. The GA accumulation kinetics as well as the *GhGA20ox* expression differed in ovules and the attached fibers, suggesting relatively independent GA regulation system in these two sites. Transgenic cotton, over-expressing *GhGA20ox1*, showed GA over-production phenotypes with increased endogenous GA levels (especially $GA_4$) in fibers and ovules. It also produced significantly more fiber initials per ovule, and fiber lengths was increased compared with the control, which demonstrates that up-regulation of the *GhGA20ox1* gene promoted fiber initiation and elongation. Our results suggest that GA 20-oxidase is involved in fiber development by regulating GA levels, and corresponding genes might be employed as target genes for the manipulation of fiber initiation and elongation in cotton.



## Introduction

Cotton is the world's most important fiber crop. Cotton fibers are single-celled hairs originating from the epidermis of the outmost integument. Mature cotton fibers are very long cells that contain more than 95% cellulose on a dry weight basis (Kim and Triplet, 2001). Due to their unique structure and rather synchronous development, cotton fibers have been employed for the investigation of many cellular and physiological processes, including carbon partitioning, cell elongation and cell wall synthesis (Kim and Triplet, 2001). The economic importance of cotton has attracted a lot of efforts to study the molecular basis of fiber development. Numerous cDNAs have been isolated from developing cotton fibers, some of which have been functionally characterized (Kim and Triplet, 2001; Li et al., 2005; Pu et al., 2008; Qin et al., 2007; Zhu et al., 2003). Recently, a large number of expressed sequence tags (EST) from cotton fibers or ovules have been produced and characterized at the transcriptional level (Shi et al., 2006; Yang et al., 2006). However, few genes have been shown to relate directly to fiber yield and quality, and the genetic basis of fiber growth and development is largely unknown.

The physiological role of plant hormones in the development of cotton fibers has been extensively studied (Basra and Saha, 1999; Luo et al., 2007; Seagull and Giavalis, 2004; Sun et al., 2005). It has been demonstrated that exogenous application of GAs promoted fiber initiation and elongation in cultured ovules and *in planta* (Basra and Saha, 1999; Gialvalis and Seagull, 2001; Seagull and Giavalis, 2004). For example, the application of exogenous $GA_3$ significantly increased the number of fibers in unfertilized ovules cultured at 1 d post anthesis (DPA) (Gialvalis and Seagull, 2001). Recently, transcriptomic analyses have revealed that GA metabolism and signaling genes, such as those encoding GA 20-oxidase (GA20ox) and DELLA proteins, are up regulated in the developing

*Abbreviations:* CaMV35S, cauliflower mosaic virus 35S promoter; DPA, day(s) post anthesis; ELISA, enzyme-linked immunosorbent assay; EST, expressed sequence tag; GA, gibberellin; GUS, β-glucuronidase; IAA, indole-3-acetic acid; IBA, indolebutyric acid; Kan, kanamycin; KT, kinetin; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; NPTII, neomycin phosphotransferase; 3′RACE, rapid amplification of the cDNA 3′ end; RT-PCR, reverse transcription PCR; SEM, scanning electron microscopy; SIM, selected ion monitoring; SPE, solid phase extraction; YADE, Y-shaped adaptor dependent extension

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fibers or in ovules undergoing fiber initiation and elongation (Shi et al., 2006; Yang et al., 2006). Aleman et al. (2008) reported the functional analysis of cotton orthologs of GA signal transduction factors GID1 and SLR1. Our previous work also revealed a GID1 homologous gene preferentially expressed in developing cotton fibers (Dong et al., 2009). These results implied that gibberellin (GA) biosynthesis and signaling are involved in the regulation of fiber development. It remains to be elucidated how endogenous GA is regulated in ovules and fibers and how GA biosynthesis affects cotton fiber development. In this investigation, we cloned three GA20ox homologous genes (*GhGA20ox1–3*) from developing fibers and ovules and found that their expression levels were consistent with the endogenous GA content in fibers and ovules. Furthermore, our results demonstrate that the up-regulation of *GhGA20ox1* can enhance GA production and promote fiber initiation and elongation in transgenic cotton.

## Materials and methods

### *Plant materials*

Cotton (*Gossypium hirsutum* L. *var.* Jimian no. 14) was cultivated in a greenhouse under natural light at 30–35 °C at day and 25–30 °C at night. The roots and hypocotyls were separated from 1-week-old seedlings. Stems and leaves were obtained from 62-month-old plants. Ovules were harvested from the bolls at various day(s) post anthesis (DPA) and fibers were striped from ovules.

### *Cloning of the cotton GA20ox homologous genes*

An *Arabidopsis* GA 20-oxidase (GenBank accession no. U20872, Xu et al., 1995) was employed as probe sequence to query the *G. hirsutum* expressed sequence tag (EST) sequences in GenBank (http://www.ncbi.nlm.nih.gov, 2006). To determine whether it contained a complete ORF, the target EST was further used to perform homology search in GenBank with BLASTX program (http://www.ncbi.nlm.nih.gov/blast).

To obtain the upstream sequences of the putative initiation ATGs, the Y-shaped adaptor dependent extension (YADE) method was used to amplify 5′ flanking sequences of target ESTs as previously described (Xiao et al., 2007). With the primers annealing to the upstream sequences of the putative initiation ATGs, the cDNAs were amplified from 10-DPA fibers or ovules using a 3′-full-length RACE kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Finally, the genomic sequences were amplified with primers compassing the putative ORFs. The primers are presented in Supplemental Table 1. All the amplified fragments were cloned into a TA-cloning vector pGEM-T easy (Promega, USA), and sequenced in Invitrogen (Shanghai, China).

### *RNA blot analysis and real-time reverse-transcription PCR*

RNAs were extracted from cotton tissues as described previously (Luo et al., 2003). Approximately, 30 μg of total RNA was separated in 1.5% agarose gels with 2.2 M formaldehyde and transferred to nylon membranes using standard protocols. The *GhGA20ox1* cDNA was labeled with $\alpha$-$^{32}$P using the ready-to-go DNA labeling beads (Amersham Biosciences, Shanghai, China). Hybridization and signal detection were performed as described previously (Luo et al., 2003).

For real-time reverse transcription PCR (RT-PCR), a First-Strand cDNA Synthesis Kit (MBI FERMENTAS, ON, Canada) was used to synthesize first-strand cDNAs from total RNAs according to the manufacturer's instructions. The real-time PCR reactions were performed on an iCycler iQ$^R$ multicolor real-time PCR detection system with SYB Green supermix (Bio-Rad, CA, USA). Gene-specific primers (Supplemental Table 1) were employed to amplify different target genes. The *histone3* gene was amplified as RNA standard (Zhu et al., 2003). The amplicon sizes were designed to be around 250 bp to ensure comparable amplification efficiencies for different genes.

### *Vector construction and cotton transformation*

Plant expression vector p5 containing the expression cassette of the β-glucuronidase (*GUS*) and neomycin phosphotransferase (*NPTII*) genes was used as master vector to construct the *GhGA20ox1* over-expression vector as described (Xiao et al., 2006a). The p5-GhGA20ox1 construct (Fig. 4A) was mobilized into *Agrobacterium tumefaciens LBA4404* and the resulting *Agrobacterium* strain was used for hypocotyl infection to produce transgenic cottons according to a method reported previously (Luo et al., 2007). The regenerated plantlets were further confirmed by GUS-staining of leaf tissues, and the GUS-positive plantlets were transplanted in pots and grown in the greenhouse.

### *Analysis of transformed cottons*

$T_0$ seeds of representative *GhGA20ox1*-overexpressing plants were sown to produce $T_1$ populations. The null-segregants were identified from $T_1$ seedlings by GUS-staining of leaves. To select homozygous transgenic plants from GUS-positive $T_1$ plants, immature embryos (around 20 DPA) were stained with GUS solution to determine whether there was any segregation in the $T_2$ embryos. The seeds from null-segregants and homozygous $T_1$ plants were sown to produce $T_2$ lines, by which phenotypes of transgenic lines and null-segregants were compared. All the transgenic cottons and corresponding null-segregants were grown in pots in the greenhouse.

To compare stem growth rate, each 10 nodes with visible unexpanded leaves were marked in null-segregant and transgenic line, and length of the internode just up the marked node was measured every 3 d for 6 times. The fiber initials per ovule were counted at 2 and 3 DPA using the method as described (Gialvalis and Seagull, 2001; Seagull and Giavalis, 2004). The length of immature fiber was measured at 5 DPA with a ruler after soaking in 95 °C water for 5 min (Sun et al., 2005). All the seed and fiber traits were determined using mature bolls harvested in the same period. The length of mature fibers was gauged on the seed after the fiber was stretched by combing. In all cases, the significance of divergence between averages was determined by *t* test.

### *Microscopic analysis*

For microscopic observation, the 0-DPA bolls were gathered at 9:00–10:00 a.m. (at anthesis) or 5:00–6:00 p.m. (8 h after anthesis), and the minus-1-DPA and 1-DPA bolls were collected at 5:00–6:00 p.m. 1 d before and after flowering, respectively. The ovule samples were fixed in formaldehyde-acetic acid for 24 h and dehydrated in a *tert*-butyl alcohol series. The dehydrated ovules were imbedded in paraffin and sectioned using an RM2235 microtome (Leitz, Germany). Sections (10-μm thick) were mounted and stained with Safranine O and Light Green, and examined using a BX41TF light microscope (Olympus, Japan). For scanning electron microscopy (SEM), samples were prepared as described (Sun et al., 2005), and analyzed with a Hitachi S-3000N SEM (Hitachi, Japan).

*Quantitative analysis of GAs by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS)*

Approximately 1 g ovules or 2 g fibers were used to extract GAs. After harvest, tissues were immediately immersed in liquid $N_2$ and the fresh weight was recorded. Materials were then ground to fine powder in liquid $N_2$ with a mortar and pestle, homogenized with 25 mL 80% (v/v) aqueous methanol and 20 ng each of deuterated GAs ([17,17-$^2H_2$] $GA_1$, [17,17-$^2H_2$] $GA_4$, [17,17-$^2H_2$] $GA_9$, [17,17-$^2H_2$] $GA_{12}$, [17,17-$^2H_2$] $GA_{20}$ and [17,17-$^2H_2$] $GA_{53}$) were added as internal standards. The deuterated standards were purchased from Prof. L.N. Mander (Australian National University). The homogenates were transferred into 50-mL tubes and extracted overnight at −20 °C. Extracts were centrifuged and the supernatants evaporated *in vacuo*. The resulting aqueous phase was adjusted to pH 2.5 using 3 M $H_2SO_4$ and partitioned to acidic ethyl acetate. The ethyl acetate phase was then partitioned by 10 mM Tris (adjusted to pH 8.0 using 1 M KOH). The resultant aqueous phase was subjected to Bond Elut diethylaminopropyl solid phase extraction (SPE) column (Dikma Technologies, China). After washing with 10 mM Tris (pH 8.0), the bound GAs were eluted with 0.2 M formic acid and directly applied to a Sep-Pak tC18 cartridge. GAs were eluted again with 80% (v/v) methanol after washing with 10% (v/v) methanol. Finally, elutions were evaporated to dry, resuspended in 25 μL 10% (v/v) methanol, and 5 μL purified GAs were subjected to LC-ESI-MS analysis.

The LC-ESI-MS analysis was performed on a LC-MS 2010A system (Shimadzu, Japan) with an Xterra RP18 column ($2.1 \times 150$ mm$^2$, 3.5 μm, Waters, USA). A gradient of methanol: 0.1% (v/v) ammonium hydroxide (0.028% $NH_3$ in water, adjusted to pH 8.5 with acetic acid) was used to elute GAs at a flow-rate of 0.15 mL/min. At 0–2 min, the methanol concentration (v/v) rose from 10% to 50%, then after 7-min flow at 50%, elevated to 100% in 5 min, and remained at 100% for 25 min. Negative ions were monitored in a selected ion monitoring (SIM) mode. The amounts of $GA_1$, $GA_4$, $GA_9$, $GA_{12}$, $GA_{20}$ and $GA_{53}$ were calculated from the peak area ratio at typical retention time determined with deuterated GA standards (with *m/z* of 349/347 at 13.04 min, 333/331 at 19.72 min, 317/315 at 22.39 min, 333/331 at 24.98 min, 333/331 at 14.57 min and 349/347 at 14.85 min, respectively).

## Results

*GA levels in cotton ovules and fibers*

We determined endogenous GA levels in developing fibers (5–15 DPA) and ovules (0–15 DPA) of wild-type cotton Jimian no. 14. It was found that $GA_1$ was the major bioactive GA in fibers and ovules, while $GA_4$ was very low or not detectable (data are not shown). In fibers, $GA_1$ reached the highest level at 10 DPA (Fig. 1A), whereas in ovules, the content increased continuously from 0 to 15 DPA (Fig. 1B).

*Cloning of cotton GA20ox homologous genes*

Three cotton GA20ox homologous genes were cloned from developing fibers and ovules, by searching homologous ESTs in GenBank (http://www.ncbi.nlm.nih.gov/Blast, 2006), extending upstream sequences by Y-shaped adaptor dependent extension (YADE) and finally amplifying cDNA by rapid amplification of the cDNA 3′ end (3′-RACE) method (Supplemental Table 1). The genes were named *GhGA20ox1–3*, and their genomic sequences were deposited under GenBank accession nos. FJ623272, FJ623273 and FJ623274, respectively. Comparing cDNA with genomic DNA sequences indicated that each of the three genes contained two introns in its coding region. The intron positions were conserved, but the intron length varied dramatically in these GA20ox genes (Supplemental Fig. 1A).

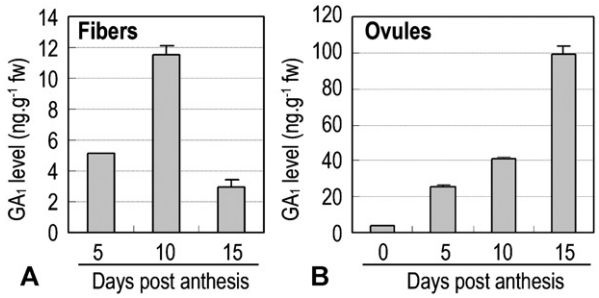


**Fig. 1.** $GA_1$ levels in developing cotton fibers (A) and ovules (B).

The cDNAs of *GhGA20ox1–3* consisted of ORFs of 1164, 1140 and 1137 bp, encoding proteins of 387, 379 and 378 aa, respectively. BLAST analysis showed that deduced proteins of these cDNAs were homologous to the GA20oxs from other plants (Fig. 2, Supplemental Fig. 1B) with identities of 48–78% at the amino acid level. These proteins also shared significant homology (with identity of 20–30%) with other plant 2-oxoglutarate-dependent dioxygenases, e.g. GA 3-oxidase, GA 2-oxidase, GA 7-oxidases, flavonol synthases, flavanone 3-hydroxylases and aminocyclopropane carboxylic acid (ACC) oxidases (Fig. 2; Supplemental Fig. 1B). Multiple sequence alignment of GhGA20oxs and its homologous proteins revealed that cotton GhGA20ox proteins contained the conserved regions typical of 2-oxoglutarate-dependent dioxygenases, including the Fe-binding sites (His-250, Asp-252 and His-306 in GhGA20ox1), and the amino acid residues interacting with 2-oxoglutarate (Arg-316 and Ser-318 in GhGA20ox1; Fig. 2; Hedden and Phillips, 2000).

*Expression patterns of GhGA20oxs*

Real-time reverse transcription PCR (RT-PCR) was employed to detect GhGA20oxs transcript levels in various cotton tissues (Fig. 3A). *GhGA20ox1* was preferentially expressed in the fibers, while *GhGA20ox2* and *GhGA20ox3* transcripts mainly accumulated in the developing ovules. In elongating fibers, the GA20ox transcript level (mainly *GhGA20ox1*) reached its peak at 10 DPA (Fig. 3B), showing a kinetic consistent with $GA_1$ content in fibers (Figs. 1 and 3B). On the contrary, *GhGA20ox2* and *GhGA20ox3* were the predominant GA20ox transcripts in developing ovules, which continuously increased from 5 to 15 DPA as did the $GA_1$ level in ovules (Figs. 1 and 3B).

*Over-expression of GhGA20ox1 in cotton*

An over-expression construct of *GhGA20ox1* was generated (Fig. 4A) and transferred into cotton by *Agrobacterium*-mediated transformation. Thirteen *GhGA20ox1*-overexpressing plants (designated as G1–13) were obtained. RNA blot analysis was used to examine *GhGA20ox1* transcript level in leaves. Significant bands were detected with the *GhGA20ox1* cDNA probe in the transformants, while no hybrid signal was found in the β-glucuronidase (GUS)-negative null-segregants under the same conditions (Fig. 4D).

The null-segregants showed no detectable phenotypic difference from the non-transformed cottons, and were used as control to evaluate the phenotypic alterations in *GhGA20ox1*-overexpressing cottons. All the transformants showed GA-overproduction phenotypes, such as pale green leaves (Fig. 4B), slender plants (Fig. 4C, Supplemental Fig. 2A and F), faster internode growth

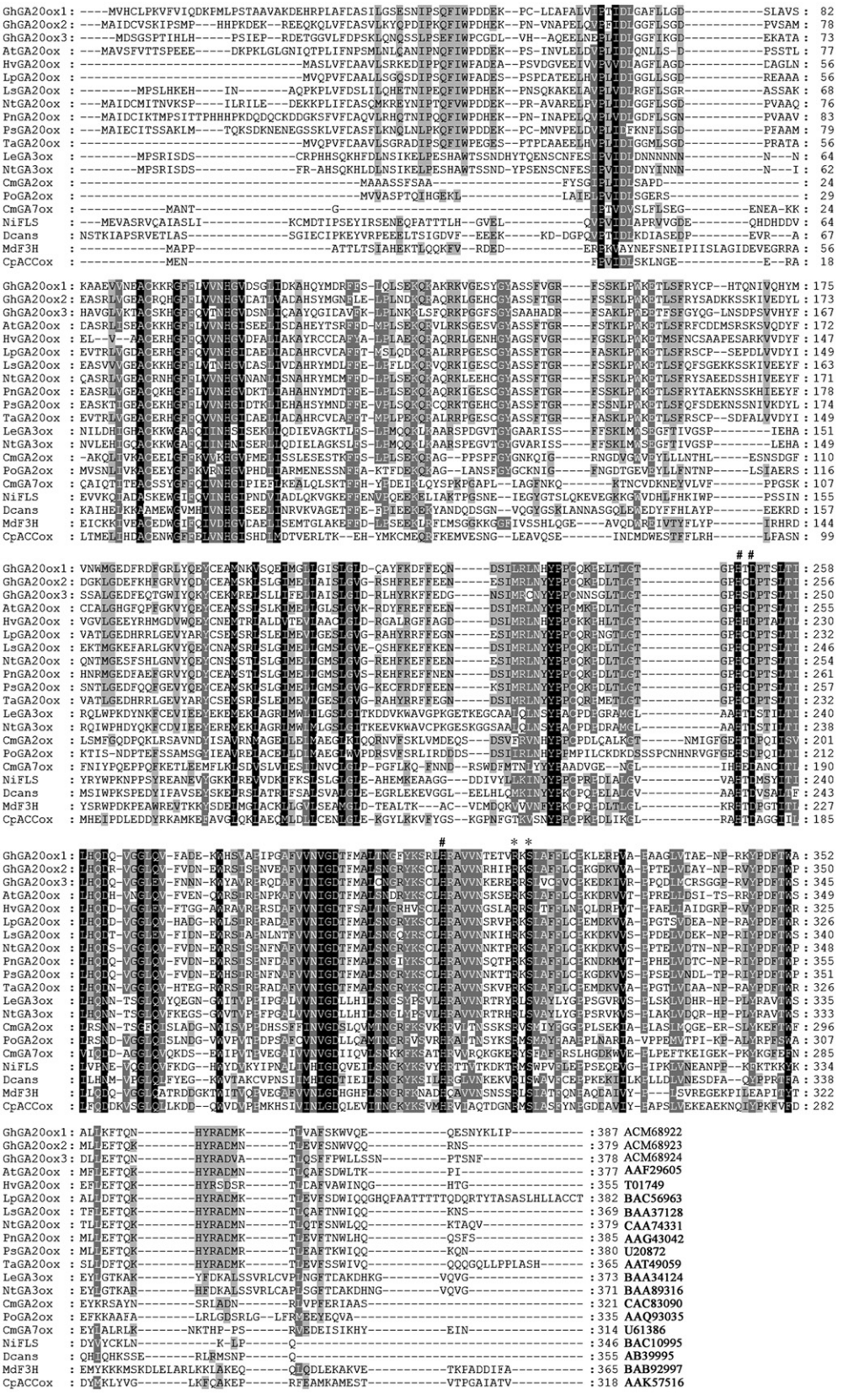

**Fig. 2.** Alignment of the deduced GhGA20ox proteins with their homologous proteins. The proteins aligned include 11 GA20oxs, 2 GA3oxs, 2 GA2oxs and 1 each GA7ox, flavonol synthase (FLS), flavanone 3-hydroxylase (F3H) and amino-cyclopropane carboxylic acid oxidase (ACCox). The regions conserved in all aligned proteins are boxed in black and the amino acids conserved in more than 60% sequences are shaded in gray. The symbol (#) indicates Fe-binding sites typical of 2-oxoglutarate-dependent dioxygenases, and the asterisks (∗) the putative 2-oxoglutarate-interacting amino acids. Dashes are introduced for the maximum sequence homology. The sequence position is marked by numbers on the right. GenBank accession nos. are listed at the end. Species names are abbreviated as follows: At, *Arabidopsis thaliana*; Cm, *Cucurbita maxima*; Cp, *Carica papaya*; Dc, *Dianthus caryophyllus*; Hv, *Hordeum vulgare*; Le, *Lycopersicon esculentum*; Lp, *Lolium perenne*; Ls, *Lactuca sativa*; Md, *Malus x domestica*; Ni, *Nierembergia* sp. NB17; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Pn, *Populus nigra*; Po, *Populus alba x Populus tremuloides*; Ps, *Pisum sativum*; Ta, *Triticum aestivum*.

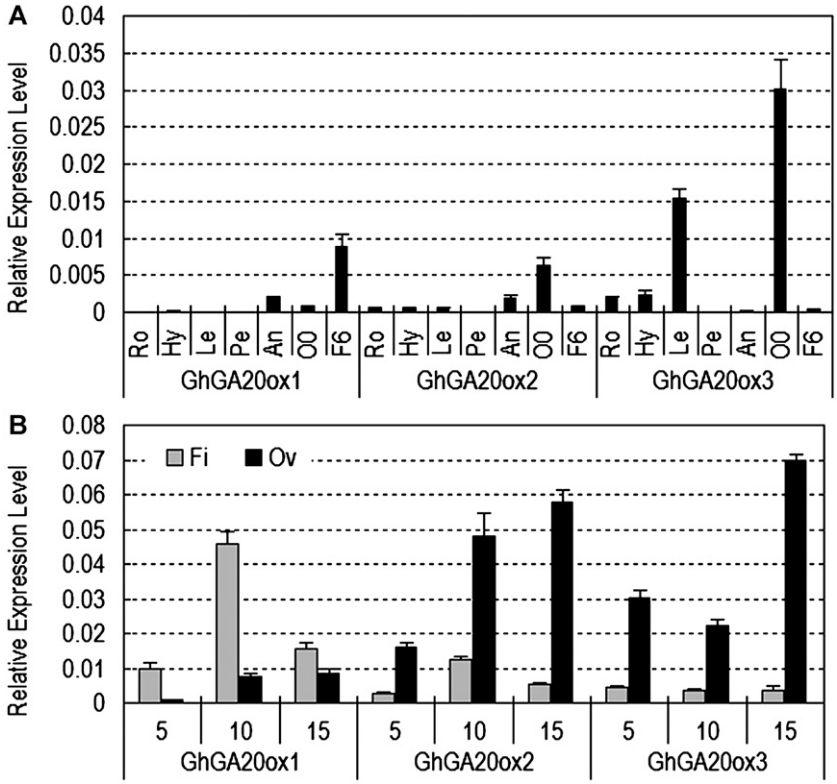


**Fig. 3.** Real-time RT-PCR analysis of the expression of *GhGA20oxs*: (A) Expression pattern of *GhGA20oxs* in various organs and tissues, including roots (Ro), hypocotyls (Hy), leaves (Le), petals (Pe), 0-DPA ovules ($O_0$) and 6-DPA fibers ($F_6$). (B) Transcript levels of *GhGA20oxs* in developing fibers (Fi) and ovules (Ov) of 5, 10 and 15 DPA.

(Fig. 4E), reduced flower (Fig. 4F and Supplemental Fig. 2B–D) and boll size (Fig. 4G). Finally, all the GA-overproduction phenotypes co-segregated with the GUS activity and, thus, the transgene (cauliflower mosaic virus 35S promoter (*CaMV35S*):*GhGA20ox1*) in the $T_1$ generation.

### *Effects of GhGA20ox1 over-expression on fiber initiation and elongation*

*GhGA20ox1*-overexpressing seeds contained more fibers than the null-segregants (Fig. 5A), but the transgenic cotton had significantly less seeds and seed fiber weight per boll (Supplemental Fig. 2G and H). Also, *GhGA20ox1*-overexpressing cotton produced significantly more fiber initials in a single ovule at 2 and 3 DPA when the long fibers (lint) initiated (Fig. 5B). As shown in Fig. 5C and D, the *GhGA20ox1*-overexpressing fibers were significantly longer than those of the control both at maturation and the early stage of fiber elongation (5 DPA). Differences in fiber length and initial number among the *GhGA20ox1*-overexpressing lines (G2, G5 and G12) were not significant ($p > 0.18$).

To confirm this observation, we further investigated the fiber development in the 0- and 1-DPA ovules of *GhGA20ox1*-overexpressing plants by light microscopy and scanning electron microscopy (SEM). The fiber initials emerged at anthesis in all cotton ovules investigated (Figs. 6 and 7). At anthesis, *GhGA20ox1*-overexpressing plants produced more fiber initials than the control, especially in the chalazal cap and the micropylar region (compare Fig. 6A and B). We used the longitudinal ovule sections of 8 h post anthesis to quantify the proportion of fiber initials in epidermal cells. As shown in Fig. 7C, the percentage of fiber initials in the epidermal cells was significantly ($p = 2 \times 10^{-9}$) elevated in *GhGA20ox1*-overexpressing ovules compared with the control. From 0 to 1 DPA, both the fiber initials of the control and *GhGA20ox1*-overexpressing cotton elongated rapidly. Nevertheless, the fiber initials in *GhGA20ox1*-overexpressing ovules were larger or longer than those of the control at the same developmental stage (Figs. 6 and 7). Although the fiber initial diameter at 8 h post anthesis varied from 6 to 16 μM both in the transgenic ovules and the control, the *GhGA20ox1*-overexpressing ovules contained more large fiber initials (Fig. 6E) and the average fiber initial diameter was significantly greater ($p = 0.0009$; Fig. 6F). Furthermore, the average length of fiber initials in the *GhGA20ox1*-overexpressing cotton reached 0.36 mM at 32 h post anthesis, significantly longer than that of the control (0.27 mM; Fig. 7F). These results are consistent with the observations on developing and mature fibers mentioned above.

### *GA biosynthesis in the GhGA20ox1-overexpressing ovules and fibers*

To elucidate how *GhGA20ox1* over-expression influenced GA biosynthesis in cotton, we determine GA levels in fast-elongating fibers at 10 DPA and in 0-DPA ovules (Fig. 8). In the non-13-hydroxylation pathway, intermediate $GA_9$ and bioactive $GA_4$ were both undetectable in wild-type ovules and fibers, but in *GhGA20ox1*-overexpressing ovules and fibers, $GA_4$ accumulated to a level comparable to $GA_1$. For the 13-hydroxlated pathway, the level of GA20ox substrate ($GA_{53}$) in transgenic fibers and ovules decreased in comparison to the control, and the direct product ($GA_{20}$) showed a slightly increased level in the transgenic plants. However, the $GA_1$ level decreased in transgenic fibers but increased in transgenic ovules, presumably due to the low level of GA20ox substrate ($GA_{53}$) in fibers (Fig. 8B and C). The total amount of bioactive GAs ($GA_1$ and $GA_4$) was significantly elevated in *GhGA20ox1*-overexpressing ovules and fibers (Fig. 8B and C).

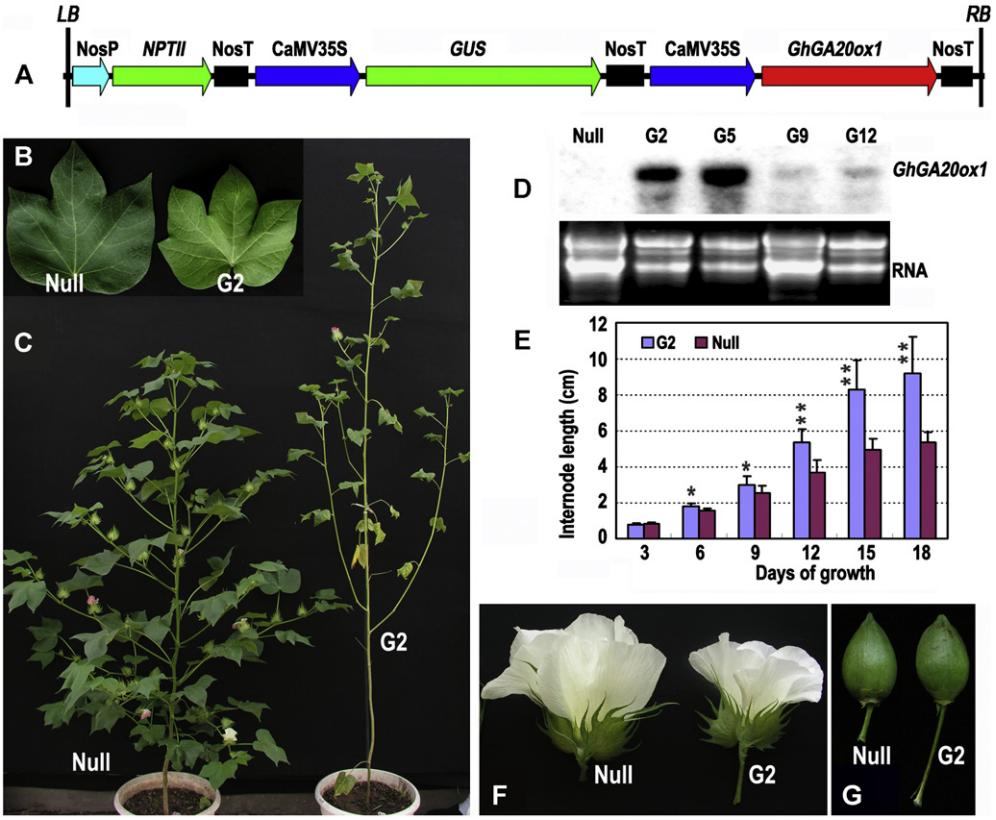


**Fig. 4.** Over-expression of *GhGA20ox1* in cotton: (A) Schematic representation of the over-expression vector used for cotton transformation. NosP and NosT, the promoter and terminator region of *A. tumefaciens* nopaline synthase gene, respectively; *NPTII*, the neomycin phosphotransferase gene; *GUS*, the β-glucuronidase gene; CaMV35S, cauliflower mosaic virus 35S promoter. (D) RNA blot analysis of *GhGA20ox1* expression in the leaves of transgenic cotton and null-segregant. (E) Time course of internode elongation in the *GhGA20ox1*-overexpressing cotton (G2) and the null-segregant. Asterisk (*) and double asterisks (**) represent significant differences at $p=0.05$ and $p=0.01$ compared with the null-segregant, respectively. (B, C, F and G) Expanded leaves (B), 2-month-old whole plants (C), 0-DPA flowers (F) and 25-DPA bolls (G) of *GhGA20ox1*-overexpressing cotton (G2) and the null-segregant. These pictures showed representative phenotypic variations observed in at least 10 plants grown in pots.

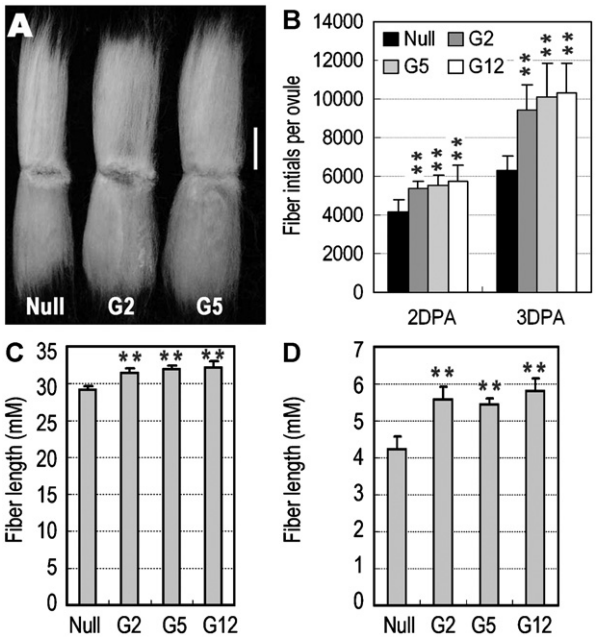


**Fig. 5.** Effect of *GhGA20ox1* over-expression on fiber development: (A) Mature fibers on seeds; bar=1 cm. (B) Number of fiber initials per ovule at 2 and 3 DPA. (C and D) Fiber length at 5 DPA and maturation. Double asterisks (**) in B, C and D indicate significant differences ($p < 0.01$) compared with the null-segregant.

## Discussion

GA 20-oxidases belong to a large superfamily of 2-oxoglutarate-dependent dioxygenases (Hedden and Phillips, 2000). Except for a pumpkin GA20ox, which is involved in the synthesis of inactive $GA_{25}$ and $GA_{17}$ (Curtis et al., 2000), all investigated GA20oxs promoted the biosynthesis of active GAs (Hedden and Phillips, 2000). *GhGA20ox1* encodes a homologous protein of GA 20-oxidases and preferentially expresses in elongating fibers. *GhGA20ox1* over-expression enhanced the biosynthesis of bioactive GAs in transgenic cotton and caused GA-overproduction phenotypes. When *GhGA20ox1* was expressed ectopically in tobacco, *GhGA20ox1*-overexpressing plants also exhibited GA-overproduction phenotypes and elevated $GA_{4+7}$ contents (Xiao et al., 2006a). These results collectively demonstrated that *GhGA20ox1* encoded a functional GA 20-oxidase which promoted active GA biosynthesis in cotton fibers.

In an attempt to confirm *GhGA20ox1* function, we transformed cotton using a CaMV35S-driven *GhGA20ox1*RNAi construct generated from a genomic *GhGA20ox1* sequence by direct amplification of intron-containing hairpin RNA (DA-ihpRNA; Xiao et al., 2006b; Supplemental Fig. 3A). Although the *GhGA20ox1* transcript level was dramatically decreased in fibers (Supplemental Fig. 3B), no significant variation was found in GA content, plant growth and fiber development in transgenic plants in comparison with the control (Supplemental Fig. 3C–E). This implies that other redundant GA20ox gene(s) expressed in fibers complemented the suppression of *GhGA20ox1* in RNAi transgenic cotton.

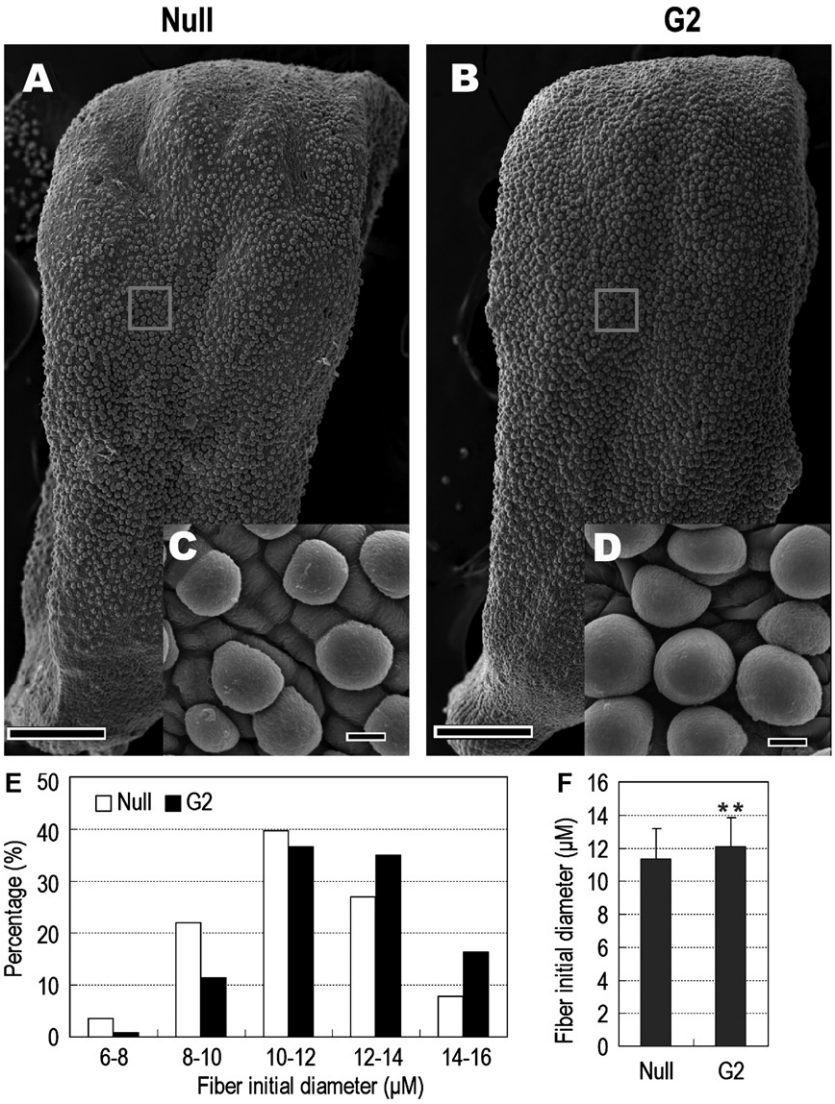


**Fig. 6.** SEM analysis of fiber initiation at anthesis. Ovules at 0 DPA from the null-segregant (A and C) and the *GhGA20ox1*-overexpressing line G2 (B and D) are compared. C and D are the magnified illustrations of the boxed regions in A and B, respectively. Bars = 160 μm in A and B; bar = 5 μm in C and D. (E) Distribution of fiber initial diameter. Data were obtained from ca. 250 fiber initials from the indicated regions in A and B of 10 ovules each. (F) Average fiber initial diameter of the null-segregant and the *GhGA20ox1*-overexpressing line G2; "**" indicates significant difference ($p = 0.0009$).

Consequently, we found another GA20ox homologous EST (accession no. ES801247; http://www.ncbi.nlm.nih.gov, 2008) preferentially expressed in developing fibers (Supplemental Fig. 4). In addition, *GhGA20ox2* and 3 are primarily expressed in developing ovules, and their expression patterns are consistent with the changes of endogenous GA concentrations in developing ovules. From these results, we concluded that there might be multiple biologically functional GA20ox genes involved in GA biosynthesis in developing fibers and ovules.

GA functions in fiber development have been studied by application of exogenous GAs *in vitro* or *in planta* (Basra and Saha, 1999; Gialvalis and Seagull, 2001; Seagull and Giavalis, 2004). When added to *in vitro* cultured ovules, $GA_3$ could dramatically increase the number of fiber initials per ovule (Gialvalis and Seagull, 2001) and promote fiber elongation (Davidonis, 1993). When applied before anthesis, $GA_3$ also promoted fiber initiation *in planta* (Seagull and Giavalis, 2004). Recently, transcriptomic analyses demonstrated that the number of expressed genes related to GA biosynthesis and that signaling increases in ovules of minus-3-3 DPA, when fiber initiation and early elongation occurred (Yang et al., 2006). Coinciding with previous observations, our data show that *GhGA20ox1* over-expression promoted fiber initiation and fiber elongation by regulating bioactive GA biosynthesis in ovules and fibers. Our results provided genetic evidence that GAs play important roles in fiber initiation and elongation.

Although it is known that GAs are involved in fiber development, little is known about how endogenous GA levels are regulated in fibers. Our observations revealed that fiber and ovule exhibited different kinetics of GA accumulation and differentially expressed GA20ox genes. This indicates that cotton fibers have a GA homeostatic system independent of ovules. Given that bioactive GA levels in developing fibers changed consistently with the expression of the fiber-specific *GhGA20ox1* gene, we propose that *de novo* biosynthesis is essential in regulating GA concentrations in fibers. Notably, GA levels in developing ovules were much higher than in the attached fibers. However, our data do not exclude the possible influx of GAs from ovules to fibers.

Seagull and Giavalis (2004) showed that exogenous application of $GA_3$ or indole-3-acetic acid (IAA) *in planta* significantly

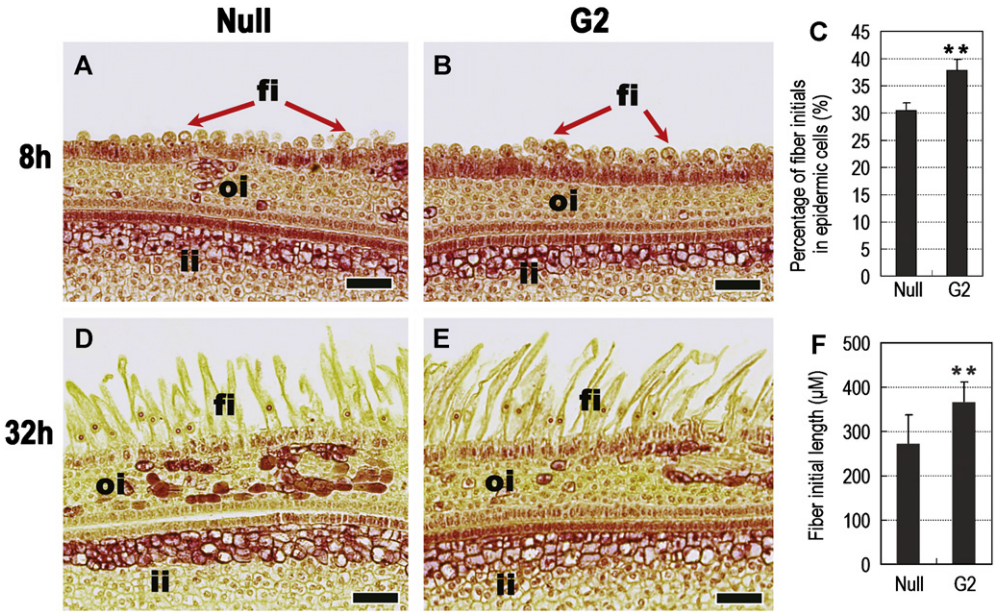


**Fig. 7.** Light microscopy of fiber initiation and elongation at 8 and 32 h post anthesis. (A, B, D and E) Ovules from null-segregant and *GhGA20ox1*-overexpressing line (G2). (C) The percentages of fiber initials in outmost epidermal cells of the ovules at 8 h post anthesis. Data were from 10 each (null and G2) longitudinal sections containing micropylar end and chalazal cap. (F) Fiber initial length at 32 h post anthesis. Data are from ca. 50 complete fiber initials in the middle region of 10 s each. Double asterisks (**) in C and F indicate significant differences ($P < 0.01$) compared with the null-segregant. The fiber cells (fi), inner integuments (ii) and outer integuments (oi) are indicated. Bars=50 μm.

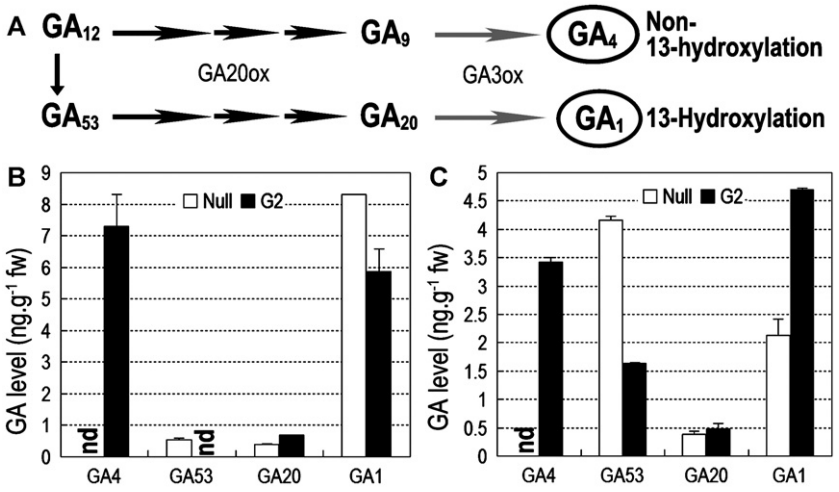


**Fig. 8.** GA biosynthesis in *GhGA20ox1*-overexpressing cotton and null-segregant: (A) The final steps in GA biosynthesis. (B) GA levels in 10-DPA fibers of *GhGA20ox1*-overexpressing cotton (G2) and the null-segregant. (C) GA levels in 0-DPA ovules of *GhGA20ox1*-overexpressing cotton (G2) and the null-segregant (nd, not detected).

increases the fiber number per ovule, implying a strategy to improve fiber yield by manipulating levels of GA and/or IAA in flowers or squares. However, it is practically infeasible to increase phytohormone contents in cotton flowers or bolls by exogenous application in the field due to the long period of the flowering time and the intensive labor involved. Transgenic manipulation of endogenous hormone contents may be advantageous in terms of cost-effectiveness, high efficacy and reduced side effects in comparison to the exogenous application (Li et al., 2003). Here we have provided a demonstration that up-regulation of *GhGA20ox1* promoted fiber initiation and elongation, both of which may affect the cotton yield and/or fiber quality (Calhoun and Bowman, 1999; Kohel, 1999). These data suggest that the *GhGA20ox1* gene could be exploited to improve cotton yield and/or quality by manipulating GA biosynthesis in cotton ovules or fibers. Yet, the constitutive expression of *GhGA20ox1* may lead to several negative side effects, such as overgrown plants (Fig. 4C), reduced boll size (Fig. 4G and Supplemental Fig. 2E), and decreased mature seeds and seed fiber weight per boll (Supplemental Fig. 2G and H). A logical step will be to regulate the GA contents in ovules and/or young fibers via tissue-specific rather than constitutive expression of the *GhGA20ox1* gene.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jplph.2010.01.003.

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