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Functional analysis of *Gossypium hirsutum cellulose synthase catalytic subunit 4* promoter in transgenic *Arabidopsis* and cotton tissues

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ABSTRACT

Gossypium hirsutum cellulose synthase catalytic subunit 4 (*GhCesA4*) plays an important role in cellulose biosynthesis during cotton fiber development. The transcript levels of *GhCesA4* are significantly up-regulated as secondary cell wall cellulose is produced in developing cotton fibers. To understand the molecular mechanisms involved in transcriptional regulation of *GhCesA4*, β-glucuronidase (GUS) activity regulated by a *GhCesA4* promoter (−2574/+56) or progressively deleted promoters were determined in both cotton tissues and transgenic *Arabidopsis*. The spatial regulation of *GhCesA4* expression was similar between cotton tissues and transgenic *Arabidopsis*. GUS activity regulated by the *GhCesA4* promoter (−2574/+56) was found in trichomes and root vascular tissues in both cotton and transgenic *Arabidopsis*. The −2574/−1824 region was responsible for up-regulation of *GhCesA4* expression in trichomes and root vascular tissues in transgenic *Arabidopsis*. The −1824/−1355 region negatively regulated *GhCesA4* expression in most *Arabidopsis* vascular tissues. For vascular expression in stems and leaves, the −898/−693 region was required. The −693/−320 region of the *GhCesA4* promoter was necessary for basal expression of *GhCesA4* in cotton roots as well as *Arabidopsis* roots. Exogenous phytohormonal treatments on transgenic *Arabidopsis* revealed that phytohormones may be involved in the differential regulation of *GhCesA4* during cotton fiber development.

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1. Introduction

Cellulose, the most abundant biopolymer in nature, organizes into microfibrils in plant cell walls, providing strength and flexibility to plant tissues. Cellulose is synthesized by a plasma membrane associated, multisubunit enzyme called cellulose synthase [1]. The first plant cellulose synthase catalytic subunits (CesAs) were identified by comparing cotton fiber ESTs with bacterial cellulose synthase [2]. Extensive searches for *CesA* genes and mutant phenotypes in a model plant, *Arabidopsis* revealed that at least 10 different *cellulose synthase catalytic subunits* (*AtCesAs*) exist [3]. Three genes, *AtCesA1*, *AtCesA3*, and *AtCesA6* are expressed during primary cell wall (PCW) biosynthesis in roots and hypocotyls [4–6]. Another set of three genes, *AtCesA4*, *AtCesA7*, and *AtCesA8* are expressed during secondary cell wall (SCW) biosynthesis in *Arabidopsis* xylem cells [7–9]. *GhCesA1* [2] and *GhCesA4* [10] isolated from cotton fibers are orthologs of *AtCesA8* [9] involved in SCW cellulose biosynthesis in *Arabidopsis* [11]. The sequence comparison of *GhCesA1* (U58283) and *GhCesA4* (AF413210) with two BACs containing homologous *GhCesA1* genes showed that *GhCesA1* and *GhCesA4* are homologous genes of the D and A subgenomes of allotetraploid *Gossypium hirsutum*, respectively [12]. Northern blot analyses showed that *GhCesA1*, *2*, and *4* are specifically expressed in fiber tissues [2,10]. During cotton fiber development, transcript levels of *GhCesA1*, *2*, and *4* are significantly up-regulated at the transition from PCW to SCW biosynthesis [2,10].

Cotton (*G. hirsutum* L.) fibers are unicellular trichomes that differentiate from epidermal cells of developing cotton ovules [13]. Cotton fiber development is divided into four overlapping stages, (1) initiation, (2) PCW biosynthesis for fiber elongation, (3) SCW biosynthesis for cellulose production, and (4) maturation [14]. Fiber initiation starts a day before up to a day or two after anthesis, and the initials enter into the elongation phase immediately. During the PCW stage, a thin PCW is deposited in elongating fibers and cotton fibers elongate up to 3–6 cm for 2–3 weeks. The SCW stage initiates approximately 14–16 days post-anthesis (DPA), overlapping the final PCW stage. Mature fibers exhibit thickened SCW composed of nearly pure cellulose. At the transition from PCW to SCW biosynthesis in cotton fiber, synthesis of other cell wall polymers ceases and the rate of cellulose synthesis in cotton fibers are estimated to increase nearly 100-fold *in vivo* [15].

Although most genes involved in fiber elongation and cellulose biosynthesis in developing cotton fibers are transcriptionally regulated [2,10,11,13–15], difficulties in regenerating transgenic cotton

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have impeded the study of transcriptional regulation of cotton fiber genes. To circumvent the lengthy and labor intensive tissue culture procedures for constructing multiple transgenic lines of cotton plants, most functional analyses of cotton promoters have been studied in transgenic tobacco or *Arabidopsis* [16–22]. Since cotton fibers are seed trichomes, numerous cotton fiber specific promoters were studied in leaf trichomes of heterologous transgenic plants despite the limited understanding of potential similarities for transcriptional regulation between seed trichomes and leaf trichomes. Analyses of cotton fiber specific promoters using heterologous transgenic plants led to the identification of MYB and L1 as promoter motifs for trichome specific expression [18], and an AT-rich motif for repressing gene expression in non-fiber tissues [20]. In spite of these advances, whether the developmental and transcriptional regulation of cotton genes can similarly occur in heterologous transgenic plants is unknown. Therefore, cotton fiber specific promoter motifs identified from heterologous transgenic plants must be further verified in cotton tissues.

Although spatial regulations of cotton promoters involved in PCW biosynthesis during fiber development have been extensively studied using transgenic tobacco or *Arabidopsis* [16–21], comparatively less is known about cotton promoter activity involved in SCW biosynthesis during fiber development. A recent promoter activity assay of *GhCesA4*, a gene involved in SCW biosynthesis of developing cotton fibers, showed that *GhCesA4* was preferentially expressed in vascular tissues and induced by a synthetic auxin, NAA when a GUS reporter regulated by a short version (−1407/+106) of the *GhCesA4* promoter named P1482 was analyzed in transgenic tobacco [22].

To understand transcriptional regulation of SCW cellulose biosynthesis in cotton fibers, our group has also studied *GhCesA4* promoter activity using a longer version (−2574/+56) of the *GhCesA4* promoter (AF413210) isolated from *G. hirsutum* DPL90 [10,13]. In our study, we evaluated *GhCesA4* promoter activity by monitoring GUS expression in cotton tissues as well as transgenic *Arabidopsis* transformed stably or transiently regulated by the *GhCesA4* promoter (−2574/+56) or progressively smaller promoters. Consistent with the results reported by Wu et al. [22], we found that GUS activity regulated by one of the progressively deleted *GhCesA4* promoters (−1.355/+56), a size similar to P1482 (−1407/+106), was mainly detected in vascular tissues in both cotton tissues and transgenic *Arabidopsis*. Furthermore, we report here that one upstream region (−1.824/−1355) of the *GhCesA4* promoter is involved in down-regulating *GhCesA4* expression in vascular tissues and another upstream region (−2574/−1824) is involved in up-regulating *GhCesA4* expression in trichomes and roots. For basal expression of *GhCesA4* in both transgenic *Arabidopsis* and cotton roots, one downstream region (−693/−320) was required. We also show that several phytohormones differentially regulated *GhCesA4* promoter activity in various tissues at different developmental stages of transgenic *Arabidopsis*. In contrast to the previous report [22], our study shows by using the longer version of *GhCesA4* promoter (−2574/+56) that *GhCesA4* promoter activity was down-regulated by NAA in transgenic *Arabidopsis*.

2. Materials and methods

2.1. Plant materials and growth conditions

Cotton plants (*G. hirsutum* L. TM-1) were grown in the field at the USDA, ARS, Southern Regional Research Center, New Orleans. Developing bolls were collected by 9 am at 4-day intervals from 8 through 24 DPA and fibers were immediately harvested and frozen in liquid nitrogen. Fully grown leaves (15 cm in diameter), expanding young leaves (5 cm in diameter), hypocotyls and roots were harvested from 1 or 6-week-old plants grown in a greenhouse at 25–32 °C. All tissues were frozen in liquid nitrogen, and stored at −80 °C. *Arabidopsis* plants were grown at 23 °C under 16 h light/8 h dark photoperiod.

2.2. Functional analysis of GhCesA4 promoter (−2574/+56) in various cotton tissues

The longest *GhCesA4* promoter (−2574/+56) fused to a *GUS* reporter was constructed using the pCAMBIA vector 1391z [23] and named pCes1. For transient expression, one micron gold particles coated with the pCes1 construct were bombarded into various cotton tissues using a Biolistic Particle Delivery System (1000/He) according to the described method [10]. For stable transformation, the pCes1 construct was introduced into *Agrobacterium rhizogenes* ATCC #15834 and subsequently inoculated on cotyledon leaves of *G. hirsutum*. Cotton hairy roots were developed and cultured according to the described method [24]. Transgenic cotton roots containing pCes1 were screened on HRIM medium [25] with 50 mg/L hygromycin. Localization of GUS activity was carried out using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) [26]. To prevent diffusion of the GUS product during staining, 0.5 mM potassium ferri/ferrocyanide was added to the histochemical staining buffer.

2.3. RNA extraction and quantitative RT-PCR

Total RNA was extracted from cotton fibers and other tissues at different developmental stages using a Plant Total RNA Kit and DNase I (Sigma, St. Louis, MO). First strand complementary DNA was synthesized using 1 μg of total RNA by priming with random hexamers at 48 °C for 30 min followed by inactivation of MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA) at 95° C for 10 min. Quantitative reverse transcription-PCR (qRT-PCR) was performed using the SYBR® Green PCR Master Mix with a specific primer set for *GhCesA4* (5′-CCTTGCCTTGGACTACCCTGTA-3′/5′-CTTTCTTGCAAAGTCGGCTGTT-3′; amplicon size, 109 bp). The transcript levels of *GhCesA4* were normalized with respect to *18S ribosomal RNA* (5′-CGTCCCTGCCCTTTGTACA-3′/5′-AACACTTCACCGGACCATTCA-3′; amplicon size, 63 bp). A total of six qRT-PCR reactions were performed at each time point for cotton tissues representing two biological replications and three technical replications. Statistical analyses and construction of graphs were performed using Prism version 3.00 software (GraphPad Software, Inc., San Diego, CA).

2.4. Promoter deletion assays in transgenic Arabidopsis and cotton tissues

The progressively shorter versions of the *GhCesA4* promoter were PCR-amplified, cloned into pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA) and confirmed by DNA sequencing. All *GhCesA4* promoters were fused to the *GUS* reporter using the pCAMBIA vector 1391z and named orderly according to the length of the promoter sequence: pCes1 (−2574/+56), pCes2 (−1824/+56), pCes3 (−1355/+56), pCes4 (−898/+56), pCes5 (−693/+56), pCes6 (−320/+56), and pCes7 (−174/+56). The promoterless pCAMBIA 1391z was used as a negative control, pCes8. All constructs were introduced into *Arabidopsis* through *Agrobacterium tumefaciens* strain GV3101 using a floral dip method [27] or into cotton hairy roots through *Agrobacterium rhizogenes* ATCC #15834 using a tissue culture method [25]. Transgenic *Arabidopsis* and transgenic cotton hairy roots were selected on media containing 50 mg/mL of hygromycin. Average quantitative GUS activity controlled by each 5′ deleted *GhCesA4* promoter was determined from soluble pro-