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ORIGINAL PAPER

Isolation and characterization of a *GAI/RGA*-like gene from *Gossypium hirsutum*

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Abstract The aim of the investigation reported here was to assess the role of gibberellin in cotton fiber development. The results of experiments in which the gibberellin (GA) biosynthesis inhibitor paclobutrazol (PAC) was tested on in vitro cultured cotton ovules revealed that GA is critical in promoting cotton fiber development. Plant responses to GA are mediated by DELLA proteins. A cotton nucleotide with high sequence homology to *Arabidopsis thaliana GAI* (*AtGAI*) was identified from the GenBank database and analyzed with the BLAST program. The full-length cDNA was cloned from upland cotton (*Gossypium hirsutum*, Gh) and sequenced. A comparison of the putative protein sequence of this cDNA with all *Arabidopsis* DELLA proteins indicated that GhRGL is a putative ortholog of AtRGL. Overexpression of this cDNA in *Arabidopsis* plants resulted in the dwarfed phenotype, and the degrees of dwarfism were related to the expression levels of *GhRGL*. The deletion of 17 amino acids, including the DELLA domain, resulted in the dominant dwarf phenotype, demonstrating that GhRGL is a functional protein that affects plant growth. Real-time quantitative PCR results showed that *GhRGL* mRNA is highly expressed in the cotton ovule at the elongation stage, suggesting that *GhRGL* may play a regulatory role in cotton fiber elongation.

Keywords *Arabidopsis* · Cotton fiber development · Cotton (*Gossypium hirsutum*) · DELLA protein · Gibberellin

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Introduction

The phytohormone gibberellin (GA) is an essential plant hormone involved in regulating many aspects of plant growth and development (Harberd et al. 1998; Richards et al. 2001). Studies on plants lacking enzymes in the GA biosynthetic pathway have shown that GA is required during many different stages of the *Arabidopsis thaliana* life cycle, such as seed germination, hypocotyl growth, internode elongation, and the development of leaves, stamens, and petals (Koornneef and van der Veen 1980; Zeevaart and Talon 1992; King et al. 2001). The GA-deficient mutant *ga1-3* is severely dwarfed, shows abnormal floral development, and requires exogenous GA for germination because the mutation blocks an early step in the GA biosynthetic pathway (Koornneef and van der Veen 1980; King et al. 2001).

The GA signaling pathway is repressed by the DELLA proteins. All of the DELLA proteins are characterized by their N-terminal conserved DELLA domain and VHYNP domain (Pysh et al. 1999; Willige et al. 2007). In the *A. thaliana* genome, there are five highly homologous DELLA protein repressors, including GA INSENSITIVE (GAI), REPRESSOR OF *ga1-3* (RGA), RGL1 (RGA-like 1), RGL2, and RGL3 (Peng et al. 1997; Silverstone et al. 1998; Richards et al. 2001; Willige et al. 2007). GAI and RGA have been proven to have overlapping functions as repressors of plant elongation growth (Willige et al. 2007). RGL1 and RGL2 play a predominant role in controlling germination and floral development, respectively (Dill and Sun 2001; King et al. 2001; Lee et al. 2002; Cheng et al. 2004; Tyler et al. 2004). All of the DELLA repressors are inactivated in response to GA by ubiquitin proteasome-dependent protein degradation (Silverstone et al. 2001; Fu et al. 2002; Thomas and Sun 2004; McGinnis et al. 2003; Sasaki et al. 2003; Willige et al. 2007). A 17-amino acid deletion, including the conserved DELLA domain, which is the mutation present in the dominant *Arabidopsis* gai-1 mutant, renders mutant gai and rga proteins insensitive to GA-induced proteolysis, and plants expressing these mutant DELLA repressors are GA-insensitive, dark-green, late-flowering dwarfs (Peng and Harberd 1997; Dill and Sun 2001; Silverstone et al. 2001; Fleck and Harberd, 2002; Itoh et al. 2002; Dill et al. 2004; Willige et al. 2007). In DELLA proteins, in addition to the conserved DELLA domain and VHYNP domain, there is also a divergent Poly S/T region at the N-terminus. At the C-terminus, DELLA proteins also share multiple conserved sequence motifs, which include a putative nuclear localization signal (NLS), two LXXLL motifs, a conserved GRAS domain, and an src homology 2 (SH2)-like domain, similar to transcription factor, STATs (Peng et al. 1997, 1999; Richards et al. 2000).

Many DELLA proteins have also been discovered in other plants, such as height1 (Rht1) from wheat (*Triticum aestivum*), dwarf8 (d8) from maize (*Zea mays*), Slender1 (Sln1) from barley (*Hordeum vulgare*) (Gubler et al. 2002), Slr1 from rice (*Oryza sativa*), and Vvgai1 from grape (*Vitis vinifera*) (Peng et al. 1999; Ikeda et al. 2001; Boss and Thomas, 2002; Chandler et al. 2002; Muangprom and Osborn 2004). All of these DELLA proteins have the same function in repressing GA responses. The identification of GAI orthologues having the same function of repressing GA signaling in plants other than *Arabidopsis* has shown that components of the GA signal transduction pathway are conserved (King et al. 2001, Willige et al. 2007). In cotton, seven DELLA-like genes have been identified from an expressed sequence tag (EST) collection from immature cotton ovules (Yang et al. 2006; Wilkins and Arpat 2005). At least one of these, a GAI-like transcript, was present at higher levels in the immature ovules than in the other tissue samples.

Cotton fibers, which are highly elongated epidermal cells that grow from the seed integument, undergo a developmental program that includes cell fate determination, initiation, elongation, specialization and, finally, programmed cell death (Sun et al. 2004; Shi et al. 2006). The different stages of cotton fiber development are in part controlled by phytohormones (Beasley and Ting, 1974; Shi et al. 2006; Sun et al. 2004; Yang et al. 2006). Analysis of fiber development on cotton ovules grown in vitro in liquid media indicated that exogenous auxin (IAA) and GA are required for maximal fiber elongation (Beasley and Ting 1974; Shi et al. 2006; Sun et al. 2004; Yang et al. 2006). Our experiments using the GA biosynthesis inhibitor paclobutrazol (PAC) in the system of cotton ovule culture in vitro indicated that Gibberellic acid (GA_3) plays a critical role in promoting cotton fiber development.

Materials and methods

Plant materials

Cotton plants (*Gossypium hirsutum* cv. Xuzhou 142) were grown in a soil mixture in a fully automated greenhouse. *Arabidopsis* seeds (*A. thaliana* ecotype Columbia) were obtained from the Nottingham *Arabidopsis* Stock Center, and plants were grown in potting soil at 22°C under a 16/8-h (light/dark) photoperiod with light supplied by fluorescent lamps. Cotton ovule culture was carried out essentially as described by Beasley and Ting (1974). For RNA extraction, ovules at −3 to 0 days after anthesis (DPA), 3 DPA, 5 DPA, and 10 DPA were excised from bolls on the cotton plant. Fibers at 15 DPA and 25 DPA were also collected. The dissected ovules and fibers were frozen and stored in liquid nitrogen immediately after harvest (Sun et al. 2004, 2005). For in vitro ovule cultures, fresh ovules (1 DPA) picked

from the cotton plants were used. Harvested cotton ovaries were soaked in 70% ethanol for 1 min, rinsed in distilled and deionized water, and soaked again in 0.1% $HgCl_2$ solution containing 0.05% Tween-80 for 20 min (sterilization). The ovules were immediately floated on liquid media supplemented with1.0 mg/l GA_3 and 1.0 mg/l PAC in 50-ml flasks. The ovules were then incubated at 34°C in the dark. The GA_3 (99.9%) was purchased from Qianxi Chemicals; GA biosynthesis inhibitor PAC was purchased from Jiafeng (China).

Cotton and *Arabidopsis* genomic DNA and RNA isolation

Cotton and *Arabidopsis* genomic DNA samples were isolated from leaf tissues using the DNeasy Plant Mini kit (Qiagen, Valencia, CA). For cotton total RNA isolation, samples were ground to powder in liquid nitrogen and stored at −80°C. Total cotton RNA was extracted using the method of Wan and Wilkins (1994). *Arabidopsis* RNA was isolated from entire plant tissues with the RNA Plant Extraction kit (TIANGEN, China).

Cloning DELLA Ortholog from cotton

A cotton nucleotide (GenBank accession no. AY208 992) with high sequence homology to *A. thaliana GAI* (*AtGAI*) was identified in the NCBI GenBank database with the NCBI BLAST program. Gene-specific primers (forward: 5′-ATGAAGAGAGATCATCAA GA-3′, reverse: 5′-TCATTCACTCGTACATTCT G-3′) were synthesized according to the nucleotide sequence. A nucleotide was amplified from a cotton boll cDNA library by PCR. The amplified fragments were sequenced and compared to *AtGAI*. Fragments with high sequence similarity to *AtGAI* were selected for further primer design and PCR amplification to isolate a full-length *GhRGL* cDNA from the library. The putative *GhRGL* coding sequence was also amplified and cloned from cotton genomic DNA and completely sequenced.

Overexpression of *GhRGL* and *Ghrgl* in *Arabidopsis*

The *Ghrgl* mutant allele from *G. hirsutum* cv Xuzhou 142 differs from the *GhRGL* wild-type allele in that it contains a 51-bp deletion of coding sequence of DELLA domain. Both *GhRGL* DNA and *Ghrgl* DNA were respectively inserted into *pBI121* in place of the *GusA* gene to create an expression cassette. First, *pGEM-T* containing *GhRGL* or *Ghrgl* was digested with *Spe*I and then with *Sac*I. The resulting fragments were ligated, respectively, into *pBI121* DNA, which was then digested with *Xba*I and *Sac*I. Each of the ligated plasmids was transformed into *Escherichia coli*. Both the *pBI121-GhRGL* and *pBI121-Ghrgl* plasmids were identified by restriction analysis and then transformed into *Agrobacterium tumefaciens* GV3101 (C58). *Agrobacterium tumefaciens* containing the *pBI121-GhRGL* and *pBI121-Ghrgl* constructs were selected on LB plates containing kanamycin (50 mg/l), gentamycin (25 mg/l), and rifampicin (100 mg/l). The plasmids were isolated, and the insert was sequenced to confirm the accuracy of the *GhRGL* and *Ghrgl* transgene. The *GhRGL* and *Ghrgl* constructs were introduced into *Arabidopsis* plants (*A. thaliana* ecotype Columbia) separately by the *Agrobacterium*-mediated flower infiltration transformation method (Clough and Bent 1998). The plants in pots were placed in trays and covered with plastic wrap for 16–24 h to maintain conditions of high humidity. Plants were watered from the bottom of the tray, and watering was stopped as the seeds matured. Seeds were harvested from individual plants.

Arabidopsis seeds were sterilized with 75% ethanol for 1 min and 50% chlorine bleach solution for 10 min. The seeds were then rinsed with sterile water three to five times and sown on MS solid medium (Murashige and Skoog 1962) containing antibiotics (250 mg/l of carbenicillin and cefotaxime, respectively) and 50 mg/l kanamycin. The PCR analysis with primers specific for the CaMV 35S promoter and the transgene-encoding sequence was performed to confirm the insertion of the transgene in the plants with the following: 5′-ATTCCATTGCCCAGCT-3′ (forward) and 5′-CACTACCTGACTCAGTACCA-3′ (reverse).

The expression levels of *GhRGL* transgenic plants detected by semi-quantitative reverse transcription-PCR

For the semi-quantitative RT-PCR analysis, total RNA was extracted from 30-day-old transgenic *Arabidopsis* plants with the RNA Plant Extraction kit (TIANGEN,

China). Total RNAs were treated with RNase-free DNase I (TaKaRa Biotechnology, Dalian, China) to remove residual DNA. The first strand cDNAs for each sample were generated using the cDNA Synthesis kit (TaKaRa Biotechnology). The reverse transcription (RT) product was used in semi-quantitative PCR reactions with primers for the *GhRGL* gene: 5′-GAGA AAGTTATGGGTACTGC-3′ (forward) and 5′-GA CAAGTTTCATAGAAGTGC-3′ (reverse). The semi-quantitative PCR cycling conditions consisted of a 10-min pre-denaturation at 94°C, followed by 24 cycles of 94°C (10 s), 52°C (10 s), and 72°C (30 s). An actin gene *ACT2* was used as a control in the semi-quantitative PCR with specific primers (5′-ACGTGA CCTTACTGATTACC-3′ and 5′-ACAATGTTACCG TACAGATC-3′).

GhRGL expression pattern analysis in cotton and the expression levels of *GhRGL* in transgenic *Arabidopsis* plants detected by real-time quantitative PCR

For detecting the expression levels of *GhRGL* in transgenic *Arabidopsis* plants, we selected an actin gene (*Atact2*) as an endogenous control; for the analysis of the *GhRGL* expression pattern in cotton, we selected a *Histone 3* gene of *G. hirsutum* (*Ghhis3*) as an endogenous control. The primers and probes of *Atact*, *Ghhis3*, and *GhRGL* gene were designed by the on-line ProbeFinder ver. 2.40 program (https://www.roche-applied-science.com/sis/rtpcr/upl/acenter.jsp?id = 030000), Probe no. 31, 61, and 25 from the Universal ProbeLibrary of Roche (Indianapolis, IN) were selected for *Atact2, Ghhis3*, and *GhRGL*, respectively. Both forward and reverse primers for *Atact2* (5′-GGAGAAGCTCTCCTTTGTTGC-3′ and 5′-GCTGGTCTTTGAGGTTTCCA-3′) were also from the Universal ProbeLibrary of Roche. Primers for *Ghhis3* were 5′-TTCCAGAGGCTTGTTCGTG-3′ and 5′-TTCCAGAGGCTTG TTCGTG-3′, and *GhRGL* 5′-CAGCGGCGATGGGTATAG-3′ and 5′-CTTGTA TGCCACCCAAGC AT-3′. The first strand cDNAs for each sample were generated using the PrimeScript RT reagent kit (TaKaRa Biotechnology). cDNAs from three biological samples were used for analysis, and all reactions were run in triplicate using a Stratagene Mx3000P QPCR System (Stratagene, La Jolla, CA). Reactions with no template and with only the RNA before RT were run as controls. Real-time RT-PCR was performed in a 25-μl reaction mixture containing 12.5 μl FastStart Universal Probe Master (ROX) (Roche), 0.25 μl hydrolysis probe (25 μM), 0.9 μl forward primer (50 μM), 0.9 μl reverse primer (50 μM), 9.45 μl ddH_2O, and 2 μl of template cDNA (5 ng/μl). The PCR conditions were 10-min pre-denaturation at 94°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. The MxPro QPCR software ver. 3.00 (Stratagene) was used for data collection and analysis. Quantification results were expressed in terms of the cycle threshold (CT) value determined according to the manually adjusted baseline. Relative gene expressions in samples were determined using a method previously described (Zhang et al. 2007). Briefly, differences between the CT values of the target genes and control genes were calculated as $\Delta CT = CT^{target} - CT^{control}$, and the expression levels of target genes relative to control genes were determined as $2^{-\Delta CT}$. The PCR was repeated three times, and the average values of $2^{-\Delta CT}$ were used to determine the difference in expression between samples and control (Zhang et al. 2007).

Results

GA plays key roles in cotton fiber elongation

Auxin and GA have long been known to play important roles in plant cell expansion or elongation (Phinney 1984; Evans 1985; Crozier et al. 2000; Shi et al. 2006), with Beasley and Ting (1974) being the first to report that the addition of IAA and GA_3 to cotton ovule culture in vitro promoted the elongation of fibers on the ovule wall. We found that the presence of 1.0 mg/l GA_3 alone in the ovule culture medium caused a significant increase in fiber length (Fig. 1). In contrast, the addition of 1.0 mg/l of a GA biosynthesis inhibitor, PAC, eliminated fibers from most parts of the ovule. The addition of both GA_3 and PAC to the cultured ovules partially restored fiber development (Fig. 1), indicating that the inhibition of fibers by PAC is at least partially due to its inhibition of GA biosynthesis.

GhRGL protein has conserved motifs of DELLA proteins

In *Arabidopsis*, there are five DELLA proteins that are negative regulators of GA signaling, including

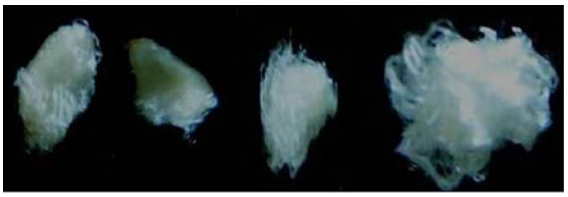

Fig. 1 Effect of exogenously applied gibberellic acid (GA_3) and the GA_3 biosynthesis inhibitor paclobutrazol (*PAC*) on cotton ovule development in vitro. Cotton ovules were isolated at 1 day after anthesis (*DPA*) and cultured for 10 days with and without 1.0 mg/l GA_3 and 1.0 mg/l PAC

GAI, RGA, GL1, GL2, and GL3 (Peng et al. 1997; Silverstone et al. 1998; Richards et al. 2001; Willige et al. 2007). The sequences of all of these genes are similar. According to BLAST search results, DELLA proteins from all plant species share a high degree of homology. We used the known DELLA proteins as queries to BLAST search GenBank and found a single cotton nucleotide (AY208992) with a strong sequence similarity to *AtGAI*. This 2041-bp nucleotide, which shares 59.5% identity to the overlapping region of *AtGAI*, is from a *G. hirsutum* L. cDNA library.

Based on the sequence of the nucleotide, we designed specific primers to amplify corresponding cDNAs from a cDNA library derived from cotton (*G. hirsutum* L.) boll mRNA. The resulting cDNA has a 1724-bp open reading frame (ORF) that encodes a derived polypeptide of 548 amino acids, as determined by Vector NTI software analysis. The coding sequence was also amplified and cloned from cotton genomic DNA. Our sequencing analysis results showed that the coding gene has no introns. The BLAST search revealed that the fragments have a high sequence similarity to *AtGAI*. We conclude that the cDNA is a putative ortholog of *AtGAI*.

The complete ORF of *GhRGL* has 63.5% nucleotide sequence identity to *AtGAI*. The amino acid sequence of GhRGL also has a high identity to all DELLA proteins in *Arabidopsis*, including GAI, RGA, RGL1, RGL2, and RGL3, showing identities of 60.5, 59.8, 63.1, 61.8, and 58.1%, respectively (Figs. 2, 3). The genes coding for DELLA proteins belong to a larger group of genes known as the GRAS family (Pysh et al. 1999). The DELLA proteins share a high homology with other GRAS family proteins at the C-terminus but not at the N-terminus. The *GhRGL* cloned in our study has a highly conserved DELLA domain, a VHYNP domain, and a divergent Poly S/T region that are characteristic of DELLA protein. This protein also shares multiple conserved sequence motifs which are inside the GRAS domain, including a putative nuclear localization signal (NLS) and two Leu zipper motifs.

Real-time PCR results suggest important roles for *GhRGL* in cotton fiber elongation

During cotton fiber development, protodermal cells of ovules undergo fiber initiation (−3 to 0 DPA), elongation (5–15 DPA), secondary cell-wall biosynthesis (15–25 DPA), and maturation (25–45 DPA), leading to mature fibers (Basra and Malik 1984; Tiwari and Wilkins 1995; Wilkins and Jernstedt 1999; Kim and Triplett 2001; Yang et al. 2006). In order to understand the expression pattern of the *GhRGL* gene during all stages of cotton fiber development, we employed the sensitive real-time PCR technique using gene-specific primers to quantify the transcript levels of *GhRGL* during the different stages of cotton fiber development. Total RNA samples isolated from different stages of cotton ovules and fibers were used for analysis, and six samples were selected for analyzing *GhRGL* gene expression pattern in cotton fiber development, including ovules at −3 to 0, 3, 5, 10, and 15 DPA, respectively, and fibers at 25 DPA. The *GhRGL* gene was expressed in all of the selected cotton samples (Fig. 4). Higher levels of *GhRGL* mRNA were detected in 5- and 10-DPA ovules, and the highest levels of *GhRGL* mRNA were detected in 10-DPA ovules. These results demonstrate that the *GhRGL* gene was expressed at the highest levels during the fiber elongation stages, suggesting that *GhRGL* has important roles in cotton fiber elongation.

Overexpression of *GhRGL* and *Ghrgl* in *Arabidopsis*

In order to determine whether *GhRGL* encodes a functional DELLA protein, we separately introduced two recombinant genes, *GhRGL* and *Ghrgl* (17 amino acid deletion from *GhRGL*), placed under the control of a CaMV 35S promoter into *Arabidopsis* plants. Transgenic T1 seedlings were selected on kanamycin-containing media, and the phenotypes of the two

Fig. 2 Amino acid sequence alignment comparing DELLA proteins GA INSENSITIVE (*GAI*), REPRESSOR OF *ga1-3* (*RGA*), RGA-like 1 (*RGL1*), RGL2, and RGL3 in *Arabidopsis* and *Gossypium hirsutum* (Gh)RGL in cotton

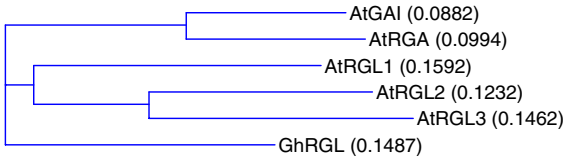

Fig. 3 Phylogenetic analysis of GhRGL proteins of cotton with DELLA proteins of *Arabidopsis*

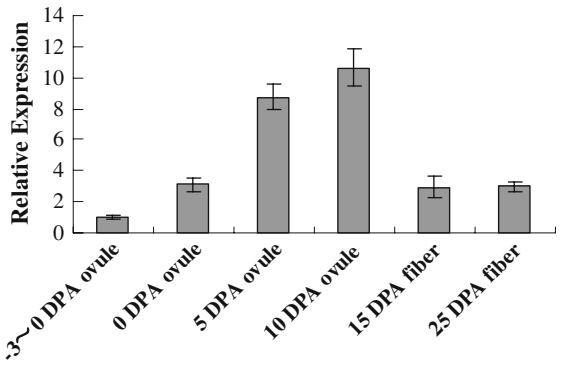

Fig. 4 *GhRGL* expression levels in cotton plants. *GhRGL* mRNA levels were assayed in total RNA samples using the TaqMan quantitative real-time PCR technique with *GhHIS3* as a reference. The sample with the lowest ratio of *GhRGL* mRNA to *GhHIS3* signal (−3 to 0 DPA ovule) was set at 1, and the values for the other samples are presented as a fold increase relative to that value. Values are means ± standard deviation of three independent assays

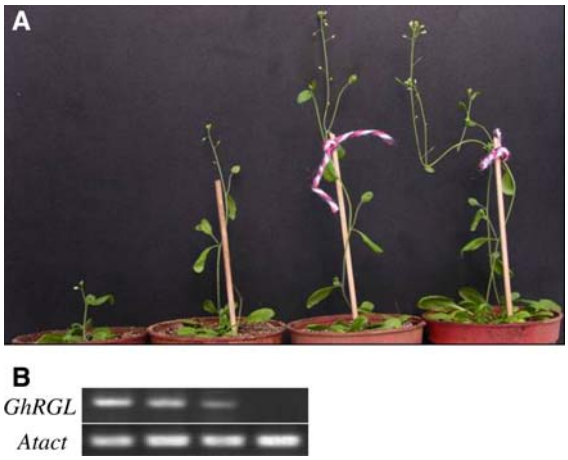

Fig. 5 Overexpression of *GhRGL* gene in *Arabidopsis* led to the dwarf phenotype. **a** Plants from three independent transgenic lines homozygous for a *35S:GhRGL* transgene showing different degrees of dwarfism compared with a wild-type (*WT*) plant on the *right*. Transgenic lines (from *left* to *right*) are *GhRGL19*, *GhRGL11* and *GhRGL7*. **b** Overexpression of *GhRGL* transgenic plants from three independent transgenic lines shown in (**a**) detected by semi-quantitative RT-PCR

transgenic *Arabidopsis* lines were examined. In transgenic lines expressing the *35S:GhRGL* transgene, approximately 37% exhibited a dwarf phenotype (Fig. 5). The degree of dwarfism was different among different transgenic lines and ranged from severely dwarfed to mildly dwarfed and wild-type phenotypes (Fig. 5). We also examined the relationship between transgene expression and severity of the dwarf phenotype in these lines. Based on the results of the semi-quantitative RT-PCR, the transgene transcript was relatively abundant in lines that exhibited a severely dwarfed phenotype (e.g., RGL19) and less abundant in lines with taller stature (e.g., GAI11). Thus, there seemed to be a correlation between the severity of the dwarfing phenotype and the level of transgene expression. To confirm the results of semi-quantitative RT-PCR, we employed the sensitive real-time PCR technique using gene-specific primers to quantify transcript levels of *GhRGL* in different transgenic lines in the T3 generation. The height of six plants from six independent transgenic lines (T3-RGL1 to T3-RGL6) and of wild-type plants was measured after 30 days of growth (Fig. 6c). TaqMan assays were used to quantify the expression of *GhRGL* mRNA in these lines. The expression level of *GhRGL* mRNA was found to be negatively correlated with the height of the transgenic plants (Fig. 6a, b). The expression level of the *GhRGL* transgene in the shortest transgenic plant line (T3-RGL1) was nearly fivefold that in T3-RGL6 plants that showed an almost wild-type phenotype. Plant lines with intermediate heights showed intermediate levels of *GhRGL* expression.

All transgenic lines expressing *35S:Ghrgl* showed dwarf phenotypes (Fig. 7). These results indicate that *Ghrgl* can have a dominant effect over the endogenous DELLA protein coding gene in causing dwarfism.

Discussion

Bioactive GAs are important phytohormone that affect many aspects of plant development (King et al. 2001; Richards et al. 2001). The effect of GA on cotton fiber development has been documented in

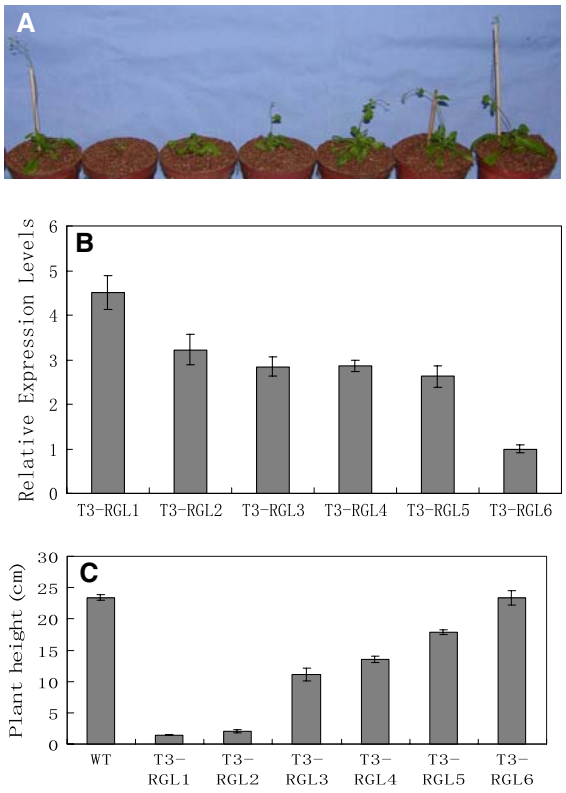

Fig. 6 *GhRGL* expression levels detected by quantitative (Q)PCR were negatively correlated with the height of *GhRGL* transgenic *Arabidopsis* plants. **a** Images of wild-type and third generation plants from six independent transgenic lines (T3-RGL1 to T3-RGL6). **b** *GhRGL* mRNA levels of the transgenic plants in **a** detected by QPCR. **c** The height of six independent transgenic *Arabidopsis* lines (T3-*RGL1* to T3-*RGL6*) and of a wild-type plant

several studies (Beasley and Ting 1974; Sun et al. 2004; Yang et al. 2006; Shi et al. 2006). Davidonis (1990) reported that GA caused cell elongation in vitro in cotton ovule culture using both auxin-dependent and auxin-independent lines, although the elongation was more pronounced in the former. Exogenous application of IAA and GA to flower buds in planta and unfertilized ovules in vitro was found to produced an increased number of fibers (Gialvalis and Seagull 2001; Yang et al. 2006). Gokani and Thaker (2002) analyzed the effect of fiber length and dry weight in relation to endogenous GA levels and found that GA was one of the more important factors that determine fiber length. In our study, the addition of GA alone (without other plant hormones) to the culture medium promoted fiber elongation on fertilized ovules cultured in vitro, confirming earlier results. To further examine the GA dependence of cotton fiber development, we used a GA inhibitor, PAC, to block GA synthesis in vivo. The presence of PAC almost completely abolished fiber development, indicating that GA is required in fiber development. To the best of our knowledge, this is the first report of the effect of PAC on cotton fiber development. To test the possibility that this was through some pathways not mediated by GA, we added GA to the PAC treatment (combination treatment); our results showed that GA overcame the effect of PAC. Taking all these results together, it is clear that GA both promotes and is required for cotton fiber development.

Given that cotton fiber development is GA dependent and that DELLA proteins are known to be involved in GA signaling, we then looked at whether the cotton DELLA protein is involved in fiber development. We cloned a DELLA-like gene *GhRGL* from cotton. Bioinformatics analysis of the predicted polypeptide, GhRGL, revealed its high homology to DELLA proteins in *Arabidopsis*. This sequence

Fig. 7 Overexpression of *Ghrgl* gene led to a dwarf phenotype in *Arabidopsis*. **a** Wild type and five independent lines of transgenic plants over expressing *Ghrgl*. **b** RT-PCR of *Ghrgl* from samples taken from plants in (**a**)

homology and the presence of all of the important DELLA and GRAS function domains in GhRGL made it highly likely that *GhRGL* is a DELLA-type GA signal regulator in cotton.

To further demonstrate the role of *GhRGL* as a functional *GAI*-type DELLA gene, wild-type and mutant versions of *GhRGL* were overexpressed in *Arabidopsis*. Previous studies have shown that overexpression of wild-type *AtGAI* in Populus (Busov et al. 2006) produced wild-type plants, while dwarf transgenic plants were observed in rice (Fu et al. 2001) and tobacco (Hynes et al. 2003). Overexpression of *GhRGL* in *Arabidopsis* in our study produced plants with various degrees of dwarfism, which is in accordance with the results of *AtGAI* overexpression in rice and tobacco and suggests that *GhRGL* is functionally equivalent to *AtGAI*. Among the *Arabidopsis* transgenic lines overexpressing *GhRGL*, the degree of elongation was negatively correlated to the levels of *GhRGL* expression (Fig. 5). The level of *GhRGL* transcripts in the dwarf lines may have surpassed the capacity of endogenous GA to release the suppressing effect of *GhRGL* on plant height growth. In general, the higher the *GhRGL* transcript level, the more growth inhibition by *GhRGL*.

Mutations in the critical DELLA domain of *AtGAI* can render the resultant gai protein GA-insensitive, thus creating severe dwarf phenotypes in the plants that overexpress the mutant *gai* transcripts (Fu et al. 2001; Hynes et al. 2003; Busov et al. 2006). Ectopic expression of *Ghrgl* with a deletion in the DELLA domain of *GhRGL* in *Arabidopsis* resulted in severe dwarfs in most of the transgenic lines, further demonstrating that *GhRGL* is a functional equivalent of *AtGAI*.

To study the *GhRGL* gene expression pattern in cotton, we employed the sensitive real-time PCR technique using gene-specific primers to quantify transcript levels of *GhRGL* during different stages of cotton fiber development. The results showed that *GhRGL* had a higher level of expression in ovules at the fiber elongation stage, i.e, at 5 and 10 DPA, with the highest levels at 10 DPA. This result is in agreement with transcript profiles in immature cotton ovules obtained by Yang et al. (2006) using another DELLA protein-encoding gene in cotton. We also used HPLC methods to analyze the GA concentration in different tissues in cotton, and the results indicted a peak GA concentration in ovules at 5 and 10 DPA (data did not show). *GhRGL* represents a cotton DELLA protein that represses specific growth and development activities, and GA is thought to deactivate *GhRGL*-type DELLA proteins so that the repressing effect of *GhRGL* can be released. It is therefore puzzling that both the levels of *GhRGL* and GA increased in the ovules at the early to middle stages of cotton fiber development. One suggestion, based on results in *Arabidopsis*, is that certain levels of GAI are not subjected to GA regulation and therefore continue to suppress some of the GA promoted growth even in the presence of GA (Fleck and Harberd 2002). However, our data suggests that *GhRGL* is subjected to GA regulation because the mutant version of *GhRGL* with deletions in the GA-interacting DELLA domain, *Ghrgl*, was much more effective in producing dwarf phenotypes. Why the levels of a supposedly effector, *GhRGL*, and that of its supposedly repressor, GA, increase at the same development stages and in the same tissue remains to be determined.

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