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A conserved RNA recognition motif (RRM) domain of *Brassica napus* FCA improves cotton fiber quality and yield by regulating cell size

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Abstract Cotton (*Gossypium hirsutum* L.) is an important crop that is used to produce both natural textile fiber and cottonseed oil. Cotton fiber is a unicellular trichome, whose length is critical to fiber quality and yield but difficult to modify. FCA was originally identified based on flowering time control in *Arabidopsis*. The function of the second RNA recognition motif (RRM) domain of *Oryza sativa* FCA in rice cell-size regulation has been previously reported, showing it to be highly conserved across dicotyledonous and monocotyledonous plants. The present study showed that the second RRM domain of *Brassica napus* FCA functioned in *Gossypium hirsutum*, leading to enlargement of multiple cell types, such as pollen, cotyledon petiole, and cotton fiber. In the resulting transgenic cotton, fiber length increased by ~10% and fiber yield per plant showed a dramatic increase, ranging from 35 to 66% greater than controls. Thus, this RRM domain may be a cell-size regulator and have great economic value in the cotton industry.

Keywords FCA · RRM · Cotton · Fiber quality · Fiber yield

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Introduction

Cotton (*Gossypium hirsutum* L.) is the dominant source of natural textile fiber and a significant oil crop, and has been a valued agricultural commodity for more than 8,000 years (Zhang et al. 2011). Cotton production is an important component of the global economy, affecting not only farmers in many countries but also consumers the world over (Lee et al. 2007). Many cotton improvement programs to enhance this crop have had the key goals of improving the yield and fiber quality (Shen et al. 2005). Cotton fiber is not part of the vascular tissue, and the appropriate botanical term for cotton fiber is

trichome, which develops from the ovule epidermis (Kim and Triplett 2001). While the majority of plant trichomes are multicellular, the cotton trichome is unicellular and, apart from its economic importance, cotton fiber provides an excellent single-celled model for studying fundamental plant biological processes (Arpat et al. 2004; Kim and Triplett 2001). Cotton fiber length affects yarn strength, evenness, and spinning efficiency (Moore 1996; De Keyser et al. 2009). As a cotton fiber cell is highly elongated, its length basically represents its size and the trichome cell size affects yield as well as quality. The average length of cotton fibers varies with genotype and appears to be under strict control (Arpat et al. 2004; Fantes 1977; Ruan et al. 2009), about which little is known. Thus, information that can shed light on this control would be useful for developing strategies for improving cotton fiber quality and yield, features which are exceedingly difficult to incorporate into a single breeding program.

FCA, which encodes a strong promoter of the transition to flowering in *Arabidopsis thaliana*, contains two RNA recognition motif (RRM) domains and a WW protein interaction domain (Macknight et al. 1997). It has been previously found that the cell size and yield of rice (*Oryza sativa*) can be increased by ectopic expression of the first RRM domain of *Os*FCA (Hong et al. 2007). The second such domain of *Os*FCA can also increase cell size (Attia et al. 2005), suggesting that these *Os*FCA-RRMs each play a role in cell-size regulation. Designated here as *Oryza sativa* cell-size RRM 1 and 2 (*Os*-csRRM1 and *Os*-csRRM2, respectively), they exhibit a high degree of evolutionary conservation in plants. For *Os*-csRRM2 in particular, significant homology has been observed in *Triticum aestivum, Hordeum vulgare*, *Lolium perenne*, *Zea mays*, *Ricinus communis*, *Vitis vinifera*, *Arabidopsis thaliana*, and *Brassica napus* (90, 90 82, 81, 76, 68, 68, and 64% identity, respectively; Online Resource 1). This conservation suggests that the RRM domain might have similar functions in different plants; we have observed that overexpression of *Bn*-csRRM2 also increases cell size in *B. napus* (unpublished). As cotton fiber length is a key factor in cotton yield and quality, the possible enhancement of this attribute was investigated in the present study through the constitutive expression of *Bn-csRRM2* in cotton plants.

Materials and methods

Cloning of target genes

Total RNA was extracted from *Brassica napus* using Trizol reagent (TianGen, Beijing, China). First-strand cDNA was synthesized with a PrimeScript RT reagent Kit (TaKaRa Bio. Co., Shiga, Japan) and used for polymerase chain reaction (PCR) amplification. Two primers were designed based on the *Brassica napus FCA* sequence (GeneBank accession no. AF414188): 5′-GAGGATCCATGGGTGCGGTAGAGTT-3′ (forward) and 5′-CGTAGATCTTGTGCCACTTCCCTTG-3′ (reverse) with restriction sites *Bam*HI and *Xba*I (underlined), respectively. PCR cycling parameters were 94°C for 5 min, 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and finally 72°C for 10 min. The products were cloned into pGEM-T Vector (Promega, Madison, WI, USA), recombinant plasmid pGEM-T-Bn-csRRM2 was produced, and then sequenced for verification.

Plasmid construction

The binary vector, pBin438 (derived from pBI121) was used as the basic vector (Li et al. 1994). It carries the kanamycin resistance gene for bacterial selection and the hygromycin phosphotransferase gene for plant transformation selection. It contains a 35S-35S promoter (a variant of the cauliflower mosaic virus 35S promoter with higher transcriptional activity) to drive transgene expression (Kay et al. 1987). The *Bn-csRRM2* fragment was digested with *Bam*HI/*Xba*I from plasmid pGEM-T-Bn-csRRM2 and ligated into the corresponding sites of pBin438 to result in the binary vectors pBin438-Bn-csRRM2 used for cotton transformation.

Transformation and regeneration of transgenic cotton plants

Bn-csRRM2 was introduced into cotton tissue by particle bombardment (Finer and McMullen 1990; Altpeter et al. 2005). In brief, surface-sterilized *G. hirsutum* cv. CCRI 12 seeds were husked and germinated in Murashige and Skoog (MS) medium. After 2–3 days, the meristems of hypocotyls were exposed by microscopic dissection, incubated in MS

medium at 15°C in the dark overnight, and subjected to particle bombardment using a Biolistic PDS 1000/He System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's instructions. The meristems were then allowed to germinate and were grown for 3–7 days before transfer to a selection medium containing 70 mg/L kanamycin. The explants were subcultured every 2 weeks and kanamycin concentrations gradually elevated to 140 mg/L; all controls died at 100 mg/L. The surviving plantlets were grafted to CCRI 12 plants and the regenerated, primary, transformed plants designated as T_0 plants; seeds from self-fertilization of T_0 plants were used to raise T_1 and subsequent progeny.

Analysis of transgenic plants by PCR and Southern blotting

T_0 plant leaves were treated with 1,500 mg/L kanamycin and 18 of 31 plants appeared positive for transformation. The presence of *Bn-csRRM2* in these plants was determined by PCR analysis carried out using a PCR Screening Kit (TaKaRa Bio Co., Dalian, China), a forward primer (5′-AGTCGTGGATGC GGGTTTGTTA-3′), and a reverse primer (5′-GCA AGGCGATTAAGTTGGGTAA-3′). PCR conditions involved denaturing at 95°C for 5 min followed by 32 cycles of 40 s at 95°C for denaturation, 40 s at 58°C for annealing, 45 s at 72°C for elongation, and finally incubation at 72°C for 5 min. The expected PCR product size was about 210 bp.

The transgenic status of the selected plants was confirmed by Southern blot analyses showing positive reactions from the PCR. A DIG High Primer DNA Labeling and Detection Starter Kit I (Roche Applied Science, USA) was used for the labeling and hybridization process, following the standard protocol. Here, *Hin*dIII digested cotton genomic DNA (30 μg) and digoxigenin-labeled DNA Marker (Lambda DNA/*Eco*RI + *Hin*dIII Marker, Fermentas International, Inc., China) were electrophoresed in 1% agarose gel and transferred onto a Hybond-N$^+$ nylon membrane (Amersham Biosciences, Buckinghamshire, UK) according to the standard protocol; the probes for Southern hybridization were fragments of the *Bn-csRRM2* coding sequences.

Determination of fiber growth parameters

Mature bolls of each plant were collected and counted and, for weight determination, the lint and seeds from each plant's bolls were separated and weighed. Raw cotton parameters, such as fiber length, strength, uniformity, and micronaire (cotton fineness) value, were assessed using a High Volume Instrument HFT 9000 (Premier, India) according to the USDA mode (Ibrahim 2010; Wang et al. 2010).

Microarray

Transgenic and wild-type cotton plants were grown under the same conditions and the leaves of day 25 and 45 plants harvested for microarray analysis. RNA samples were isolated from three replicates using Trizol (Invitrogen, USA) as described by the manufacturer. Microarray analyses were carried out using an Agilent Cotton Gene Expression Microarray (G2519F-022523, Agilent Technologies, USA), scanned on an Agilent Technologies Scanner (G2505C), and data collected using Agilent Feature Extraction software (version 10.7.1.1). Comparisons were made between transgenic samples and their corresponding wild-type samples and all microarray data were deposited in a public database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26445). Genes with two-fold differences in expression and supported by a probability of $\leq$0.05 were considered significant. Data was analyzed using GeneSpring GX Software (Agilent Technologies) and Gene Ontology Enrichment Analysis Software Toolkit (http://omicslab.genetics.ac.cn/GOEAST/php/agilent.php).

Results

Expression of *Bn-csRRM2* in cotton

To investigate the potential effect of *Bn-csRRM2* in cotton, pBin438-Bn-csRRM2 was introduced into the shoot-tip meristem tissues of the elite cotton variety CCRI 12 by particle bombardment and, based on kanamycin resistance and PCR analysis for the presence of *Bn-csRRM2*, 12 independently transformed plantlets were obtained. Four of these plants (L008–L011) were randomly selected for further

study and Southern blot hybridization indicated that L008–L011 had different *Bn-csRRM2* copy numbers and insertion sites, confirming them as arising from independent transformation events (Fig. 1). Seeds of L008–L011 (T_0) were collected separately for further experiments.

Expression of *Bn-csRRM2* increased plant size

The T_1 plants of L008–L011 displayed visible increases in plant size (L008, Fig. 2e). Statistical analysis of the T_2 and T_3 generations of these four transgenic lines showed that kanamycin-resistant and *Bn-csRRM2* PCR-positive plants (109.8 ± 6.8 cm, $n = 14$) were significantly larger than control CCRI 12 plants (82.2 ± 5.5 cm, $n = 5$, $P < 0.01$), whereas the *Bn-csRRM2* PCR-negative plants without kanamycin-resistance (83.4 ± 7.2 cm, $n = 8$) were similar to control CCRI 12 ($P = 0.75$, Online Resource 2). These data indicated that enlarged plant size indeed resulted from *Bn-csRRM2* ectopic expression.

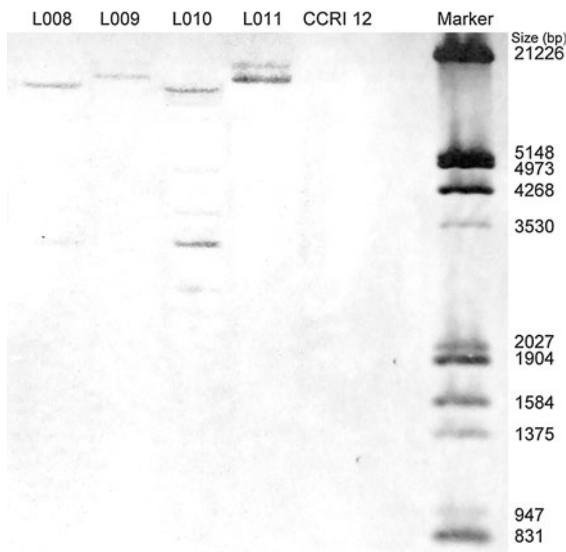

Fig. 1 Southern blot hybridization of *Bn-csRRM2* in cotton. Binary vector pBin438-Bn-csRRM2 was introduced into cotton by particle bombardment; 12 independently transformed plantlets were obtained by kanamycin-resistant screening and PCR analysis for *Bn-csRRM2* presence; four plants (L008–L011) were randomly selected for Southern blot hybridization (fragment of *Bn-csRRM2* probe used to hybridize genomic DNA); the result indicated that L008–L011 had different *Bn-csRRM2* copy numbers and insertion sites, confirming them to be from independent transformation events

Bn-csRRM2 induced increased organ size

Expression of *Bn-csRRM2* in cotton did not alter the general organ morphology, but led to significant enlargement of multiple organs, including flowers, ovaries, cystigenic valves, bolls, sepals, cotyledons, leaves, stems (Fig. 2), and seeds. Cotton seed enlargement resulted in significant increases in average seed weight, ranging 6–24% greater than controls (Fig. 3a). Increases in boll size were associated with significant increases in lint weight/boll, ranging 9–40% greater than controls (Fig. 3b). At the same time, the bolls/plant also showed a marked increase, ranging 12–34% greater than controls (Fig. 3c). Consequently, the increase in lint yield/plant was significant, ranging 35–66% greater than controls (Fig. 3d).

Bn-csRRM2 induced increased cell size

A change in organ size can reflect an alteration in the size or number of cells, or both. In transgenic rice which constitutively overexpressed *Os*-csRRM2, histological analysis showed that the increase in organ size resulted from the increase in cell size (Hong et al. 2007). In our study, expression of *Bn-csRRM2* also led to changes at the cellular level. The cotyledon petiole cells of the transgenic plants were noticeably larger than the same cell type in control plants (Fig. 4d, e). Similarly, cells of other organs were clearly larger than controls (data not shown); the most obvious was increased cotton fiber cell size. Photoelectric measurement showed that the cotton fiber length increased considerably (Table 1; Fig. 4a), and was associated with increased fiber strength (Table 1). Longer fiber length and higher strength are both highly desirable attributes.

Pollen is the flower structure which transports the male gamete to the ovule and within the exine of a binucleate pollen grain are a generative nucleus and a large vegetative cell. The latter accumulates abundant stored metabolites required for rapid pollen tube extension, while the diminutive generative cell is enclosed by vegetative cell cytoplasm and contains relatively few organelles and stored metabolites (Bedinger 1992). Thus, pollen size fairly reflects cell size. To investigate whether *Bn-csRRM2* increased pollen size, pollen cross-sectional areas were determined by the Scanning Image Pixels Method (Ohto

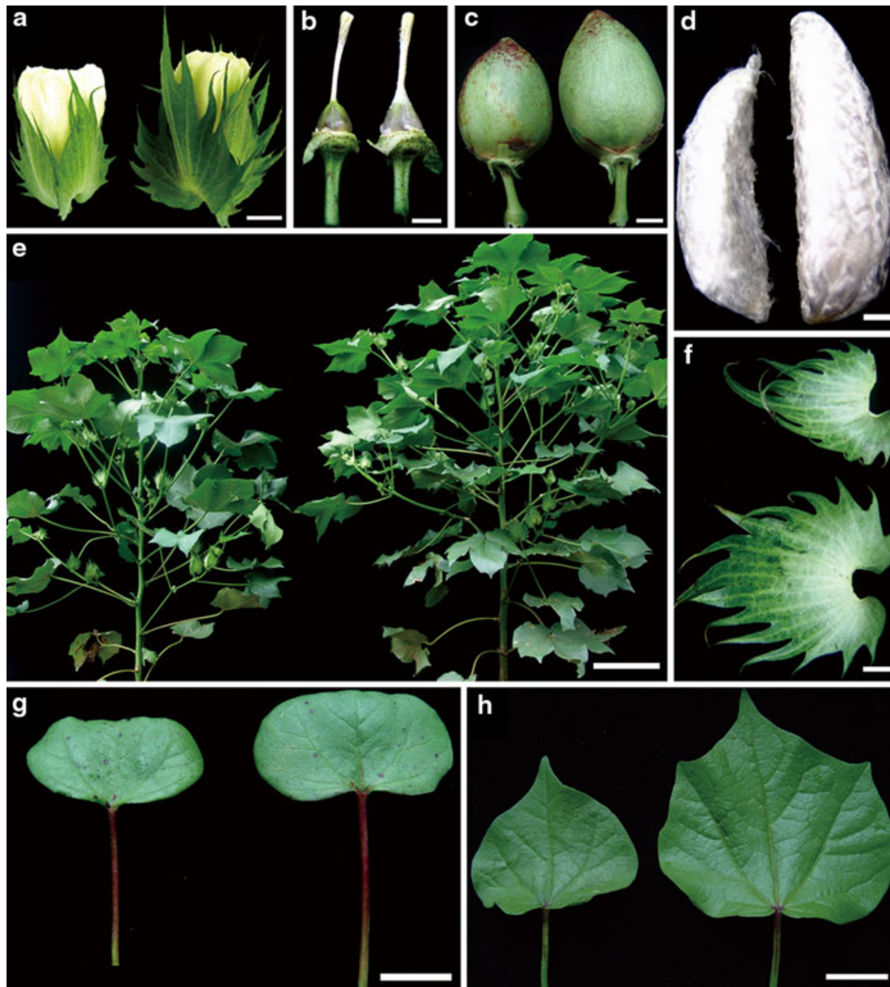

Fig. 2 *Bn-csRRM2* affected sizes of plants and multiple cotton organs. Controls on *left* or *top* of each panel; **a** flower, 1 day post-anthesis (DPA), *bar* = 1 cm; **b** ovary, 1 DPA, *bar* = 5 mm; **c** boll, *bar* = 1 cm; **d** cystigenic valve, *bar* = 5 mm; **e** whole plant, full-bloom stage, *bar* = 20 cm; **f** sepal, 1 DPA, *bar* = 2 cm; **g** cotyledon, fully expanded, *bar* = 1 cm; **h** leaf, 1st leaf pair photographed when 2nd leaf pair emerged, *bar* = 5 cm

et al. 2005). Three each of transgenic cotton and control CCRI 12 plants were analyzed, with pollen grains from anthers deposited onto a glass slide and observed immediately by light microscopy. The cross-sectional areas of at least 30 pollen grains were measured and averaged for each plant. Statistical analysis showed that the transgenic pollen was significantly larger than control pollen ($P < 0.01$, Online Resource 3, Figs. 4b, c).

Taken together, these results demonstrated that the increase in transgenic organ size was due in part to increased cell size. In most crop production, high yield is the most important goal and sources of yield include cell numbers, size, and mass (Frary et al. 2000). The present observations that constitutive expression of *csRRM2* enhanced the sizes of multiple cell types could have high practical value.

Discussion

Many reports on FCA have related to its roles in floral development (Macknight et al. 1997, 2002). It negatively regulates its own expression by promoting cleavage and polyadenylation of its pre-mRNA, which causes the production of a truncated, inactive transcript at the expense of the full-length *FCA* mRNA, thus limiting the expression of active FCA

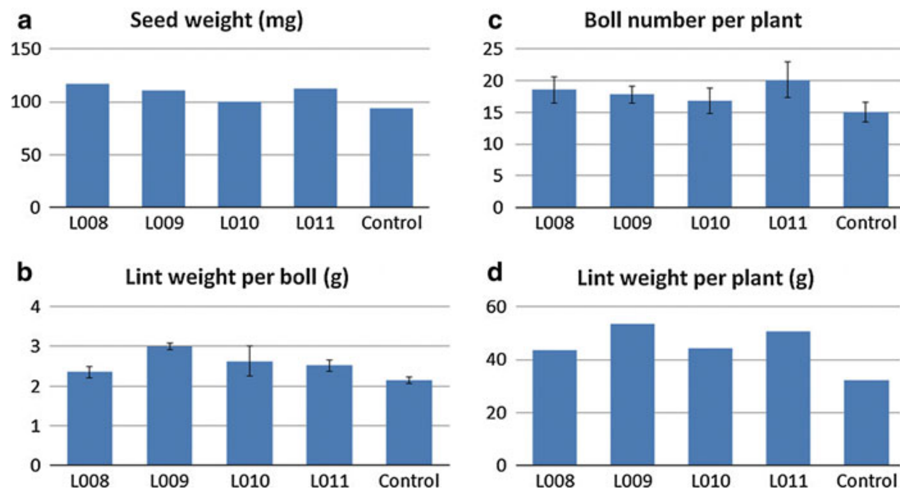

Fig. 3 *Bn-csRRM2* increased lint yield and seed size. **a** Average weight of 100 randomly selected seeds: L008, 117.0 mg; L009, 111.1 mg; L010, 100.0 mg; L011, 112.4 mg; and control CCRI 12, 94.3 mg. **b** For each *line*, five plants for lint weight/boll; means ± SD: L008, 2.35 ± 0.14 g; L009, 3.00 ± 0.08 g; L010, 2.63 ± 0.37 g; L011, 2.52 ± 0.14 g; and control, 2.15 ± 0.08 g. **c** For each *line*, 10 plants for bolls/plant; means ± SD: L008, 18.60 ± 2.07; L009, 17.80 ± 1.32; L010, 16.80 ± 2.04; L011, 20.10 ± 2.81; and control, 15.00 ± 1.56. **d** Lint weight/plant calculated by lint weight/boll times bolls/plant: L008, 43.71 g; L009, 53.40 g; L010, 44.18 g; L011, 50.65 g; and control, 32.25 g

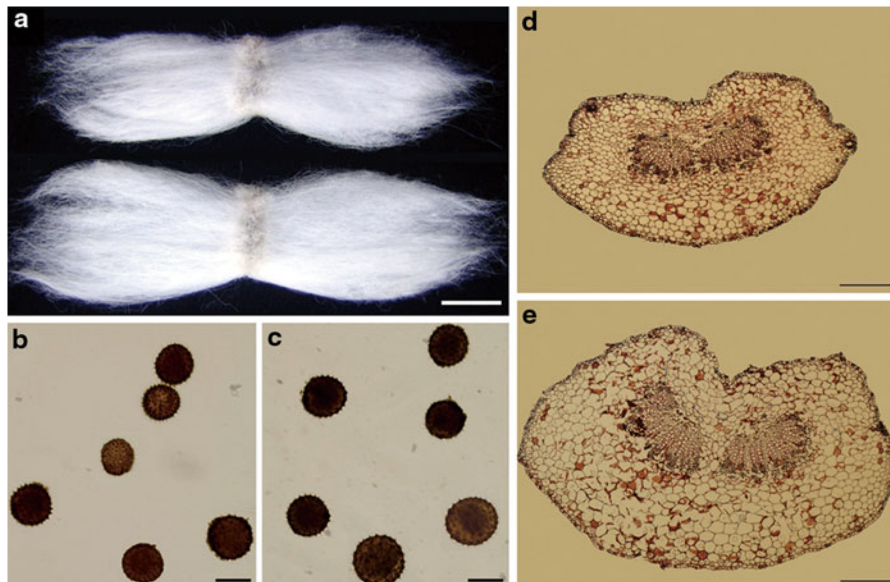

Fig. 4 *Bn-csRRM2* affected cotton cell size. **a** Fiber length of transgenic (*bottom*) was significantly longer than control CCRI 12 (*top*), *bar* = 8 mm; transgenic pollen grain (**c**) was bigger than control (**b**), *bars* = 100 µm; cross-sections of cotyledon petiole (cut from basal part of fully expanded cotyledons) showed transgenic cells (**e**) larger than control cells (**d**), *bars* = 320 µm

protein (Quesada et al. 2003). The overexpression of csRRM2 may affect FCA autoregulation. As the manipulation of FCA did not result in similar phenotypes, cell-size regulation should be a new function of csRRM2 (Macknight et al. 1997; Furner et al. 1996).

RRM domains are abundant in all life kingdoms (Lorkovic and Barta 2002) and are known to

Table 1 *Bn-csRRM2* improved fiber quality

Genotype	Fiber length (mm)*	Length uniformity (%)	Micronaire value	Fiber strength (cN/tex)
L008	30.80 ± 0.85[A]	85.34 ± 1.07	3.46 ± 0.63	28.29 ± 3.16
L009	30.78 ± 0.72[A]	86.76 ± 2.26	3.59 ± 0.55	27.64 ± 1.61
L010	30.68 ± 0.81[A]	84.88 ± 0.93	3.66 ± 0.33	29.14 ± 2.47
L011	30.27 ± 0.69[A]	84.92 ± 1.29	4.20 ± 0.46	29.98 ± 1.54
Control	27.90 ± 0.48[B]	84.38 ± 1.34	4.18 ± 0.43	26.48 ± 1.38

Plants grown concurrently under identical conditions and used for statistical analysis ($n = 5$); similar results obtained in independent experiment performed at another site; means ± SD

* Fiber length values different at $P = 0.01$ significance level labeled with different letters

recognize and bind sequence-specific RNA or DNA (Burd and Dreyfuss 1994; Maris et al. 2005). By associating with different protein domain types, RRM domains can modulate their RNA-binding affinity and specificity, thus diversifying their biological function (Maris et al. 2005; Eulalio et al. 2009). Moreover, besides nucleic acid binding, they participate in diverse protein–protein interactions (Trzcińska-Daneluti et al. 2007). FCA possesses two RRM domains, which have been reported to be involved in polyadenylation site selection (Simpson et al. 2003), chromatin silencing of single and low-copy genes, and interaction with the canonical small interfering RNA-directed DNA methylation pathway for regulating common targets (Baurle et al. 2007; Baurle and Dean 2008). In the present study, *Bn*-csRRM2 was observed to be involved in cell-size regulation. For multicellular plants, average cell size not only varies with genotype but also varies between different tissues within the same genotype, which appears to suggest that there is specificity in cell-size regulation for individual cell types (Yi et al. 2010). Thus, the finding here that the sizes of several cell types were similarly increased by ectopic *Bn-csRRM2* expression in *G. hirsutum* was unexpected and suggested that csRRM2 may be a conserved and ancient cell-size regulator and that this regulation may exist at two levels in higher plants, one at the individual cell type level and another at a global level, affecting most cell types.

Eukaryotic RRM proteins are known to modulate gene expression by participating in different post-transcriptional events (Maris et al. 2005). Agilent Cotton Gene Expression Microarray was used to identify the genes differentially expressed in these *Bn-csRRM2* cotton plants. Comparisons were made between transgenic cotton and control CCRI 12 at day 25 and 45. The results showed that 67 and 277 genes, respectively, were significantly regulated (Online Resource 4 and 5). Gene ontology (GO) classification revealed that cytosolic large ribosomal subunit (GO: 0022625) was the top-represented GO category ($P = 0.02$) in the 45-day group. Furthermore, 16 genes showed regulation in both developmental stages and were considered to be closely related to the *Bn-csRRM2*-induced phenotypes (Online Resource 6), but the involvement of these in common genes in cell-size regulation await future experimentation. Among these genes, two 60S ribosomal protein genes (ES800645 and ES843981), whose amino acid sequences are strongly similar (94.6 and 94.2% identity, respectively) to *Arabidopsis thaliana* 60S ribosomal protein L35a-3 (TAIR: AT1G74270) and L23a-1 (TAIR: AT2G39460), were both downregulated in transgenic cotton. It has been reported that deletion of genes encoding 60S subunit proteins or processing factors or treatment with a small molecule, which all inhibit 60S subunit biogenesis, are each sufficient to significantly increase replicative life span in yeast (Steffen et al. 2008). The downregulation of the two 60S ribosomal protein genes might also extend the cell cycle. At the same time, ES816367, whose amino acid sequence is moderately similar (79.8% identity) to *Arabidopsis thaliana DEHYDROASCORBATE REDUCTASE 2* (*DHAR2*; TAIR: AT1G75270), was also downregulated. It has been reported that ascorbate stimulates cell cycle activity in competent cells, while the oxidised form, dehydroascorbate, blocks normal cell cycle progression (Potters et al. 2002), indicating that

the redox state of the ascorbate–dehydroascorbate pair is a specific regulator of cell division (De Pinto et al. 1999). Downregulation of DHAR2 might increase the level of dehydroascorbate, which, in turn, blocks cell cycle progression. These lines of evidence suggested that *Bn-csRRM2* overexpression might inhibit cell cycle progression and, consequently, result in the increase in cell size.

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