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GhJAZ2 negatively regulates cotton fiber initiation by interacting with the R2R3-MYB transcription factor GhMYB25-like

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SUMMARY

Jasmonic acid (JA) signaling has been well studied in Arabidopsis. Most reports focus on the role of JA in biological pathways, such as stress resistance, trichome initiation and anthocyanin accumulation. The JASMONATE ZIM-DOMAIN (JAZ) protein is one of the important repressors in the JA signaling pathway. Previous studies showed that JA functions in fiber initiation and elongation, but little is known about how *JAZ* genes function in fiber development. In this study, a cotton JAZ protein (GhJAZ2) containing a highly conserved TIFY motif and a C-terminal Jas domain was identified, and its function during cotton fiber development was analysed. Gene expression analysis showed that *GhJAZ2* was preferentially expressed in the root, hypocotyl, flower and ovule 1 day before anthesis. Overexpression of *GhJAZ2* inhibited both lint and fuzz fiber initiation, and reduced the fiber length. Yeast two-hybrid assays showed that GhJAZ2 interacted with the R2R3-MYB transcription factors GhMYB25-like and GhGL1, the bHLH transcription factor GhMYC2, the WD repeat protein GhWD40 and the unknown protein GhJI1. Among these transcription factors, previous studies showed that downregulation of *GhMYB25-like* leads to a fiberless phenotype in cotton seeds. Molecular and genetic evidence showed that the GhJAZ2 protein suppresses fiber initiation in the overexpressing lines by interacting with GhMYB25-like and suppressing GhMYB25-like activity. Our results suggested that GhJAZ2 functions as a primary transcription repressor during lint and fuzz fiber initiation by interacting with GhMYB25-like, GhGL1, GhMYC2, GhWD40 and GhJI1 to regulate the JA signaling pathway.

Keywords: cotton fiber, initiation, *GhJAZ2*, jasmonic acid, lint, fuzz.

INTRODUCTION

Among fiber crops, the cotton fiber is the most important raw material for the textile industry. The cotton fiber is a single cell that differentiates from the epidermal cells of the ovule. The development process comprises five continuous but overlapping stages: initiation; elongation; transition; secondary cell wall deposition; and maturation (Haigler *et al.*, 2012). Although all cotton ovule epidermal cells have the potential ability to differentiate into fibers, only 30% of them eventually do so (Kim and Triplett, 2001). Therefore, there is potential to improve the fiber yield by regulating the number of epidermal cells that initiate the development process to fiber. Cells initiated from the epidermal layer prior to 3 days post-anthesis (DPA) have been reported to develop into lint fibers, and fuzz hairs originate from 5 to 10 DPA (Lang, 1938). However, lint fiber and fuzz fiber might share a common pathway of initiation (Wu *et al.*, 2006).

Fiber development can be affected by various internal and external factors, resulting in a complex but valuable model for studying cell differentiation. The R2R3-MYB transcription factor *GhMYB25-like* is predominantly expressed in −1 to 3 DPA ovules covered with fibers. However, a reduced expression level of *GhMYB25-like* was observed in Xu142 *fl* (fiberless mutant) compared with the wild-type Xu142. *GhMYB25-like* has previously been shown to be an important factor for fiber cell differentiation and initiation in experiments, as silencing of this gene in transgenic cotton results in the production of fiberless seeds (Walford *et al.*, 2011). Other R2R3-MYB transcription factors, such as GhMYB25, GhMYB109 and GhMYB2, are predominantly expressed during fiber initiation and early elongation stages. The overexpression of *GhMYB25* or *GhMYB2* promotes fiber initiation, whereas their downregulation suppresses fiber initiation and elongation (Machado *et al.*,

2009; Huang *et al.*, 2013). Suppression of *GhMYB109* leads to a reduction in fiber length (Pu *et al.*, 2008).

Some homologous genes from Arabidopsis have functions similar to those described in cotton fiber initiation, and cotton fiber initiation may utilize molecular mechanisms similar to those involved in trichome initiation in Arabidopsis (Wan *et al.*, 2014). The Arabidopsis *GL2* gene directly drives trichome initiation (Hung *et al.*, 1998; Szymanski *et al.*, 1998), and the R2R3-MYB/bHLH/WD40 transcription complex acts upstream of *GL2* and is a key regulator of *GL2* expression. This transcription complex is composed of MYB transcription factors (GLABRA1, GL1 and MYB family members), bHLH transcription factors (EGL3, GL3 and TT8) and WD repeat proteins (TRANSPARENT TESTA GLABRA1, TTG1; Walker *et al.*, 1999; Payne *et al.*, 2000). TRIPTYCHON (TRY) and CAPRICE (CPC) are inhibitors of hair development that are expressed in trichome cells and are transferred into neighboring cells to inhibit hair initiation in adjacent cells. The TRY protein interacts with GL1 and GL3 or EGL3 to inhibit R2R3-MYB/bHLH/WD40 transcription complex formation, thereby suppressing trichome initiation. When the cotton genes *GaDEL65*, *GaDEL61* and *GaMYB23* (which are homologous to *GL3/EGL3* or *GL1*) are transferred into Arabidopsis *gl3* or *gl1* mutants, trichome production is rescued (Wang *et al.*, 2013). The class IV HD-ZIP family transcription factor GaHOX1 (a homolog of GL2) can rescue the glabrous phenotype of the Arabidopsis mutant *gl2-2*. *GaHOX1* is highly expressed in 0 and 1 DPA ovules in *Gossypium arboreum*, but its expression in the Xu142 *fl* mutant is not detectable at the same stage (Guan *et al.*, 2008). Another class IV HD-ZIP family transcription factor, GhHOX3, controls cotton fiber elongation. Overexpression of *GhHOX3* leads to longer fibers; silencing of *GhHOX3* leads to an 80% reduction of fiber length (Shan *et al.*, 2014).

Plant hormones play pivotal roles in fiber development, including auxin, ethylene, gibberellic acid (GA) and jasmonic acid (JA; Qin *et al.*, 2007; Xiao *et al.*, 2010; Zhang *et al.*, 2011; Hao *et al.*, 2012; Tan *et al.*, 2012). JA is involved in multiple developmental processes, including root growth, senescence, trichome development, anthocyanin accumulation and fiber development (Staswick *et al.*, 1992; Xiao *et al.*, 2004; Qi *et al.*, 2011; Hao *et al.*, 2012; Tan *et al.*, 2012). A previous report showed that 2.5 μM JA had the most significant inhibitory effect on fiber initiation in cotton using −1 DPA ovules for culture (Tan *et al.*, 2012). On the other hand, 0.05 μM JA led to increased fiber length (Hao *et al.*, 2012). Therefore, a high concentration of JA inhibits fiber initiation and an optimal concentration promotes elongation in cultured tissues. However, the regulatory mechanism is not well understood. JA induces the expression of the bHLH transcription factor GL3 that could interact with GL1 and TTG1 to promote trichome initiation (Yoshida *et al.*, 2009). Furthermore, wounding and exogenous JA could increase the density and number of trichomes, which indicates a positive effect of JA on trichome initiation (Traw and Bergelson, 2003).

JASMONATE ZIN-DOMAIN (JAZ) is an important repressor protein in the JA signaling pathway (Chini *et al.*, 2007; Thines *et al.*, 2007). The JAZ proteins contain three conserved domains: a weak conserved NT domain in the N-terminal region (Hou *et al.*, 2010); a ZIM domain in the highly conserved TIFY domain (Vanholme *et al.*, 2007); and a Jas domain that contains 12–29 conserved amino acids in the C-terminal region (Chini *et al.*, 2007; Thines *et al.*, 2007). *JAZ* has been well studied in Arabidopsis, and is involved in anthocyanin accumulation and trichome initiation. JAZ proteins interact with the MYB and bHLH factors (MYB75, GL3 and EGL3) to inhibit downstream signal cascades, and negatively modulate anthocyanin accumulation and trichome initiation (Qi *et al.*, 2011). JAZ degradation depends on the action of the SCF^{COI1} ubiquitin ligase and the 26S proteasome (Thines *et al.*, 2007). *GhJAZ2* was first cloned from *Gossypium hirsutum* L. in our laboratory. In the current study, we characterize the function of *GhJAZ2* in fiber initiation by downregulating and overexpressing this gene in cotton. We identify GhJAZ2-interacting proteins using the yeast two-hybrid (Y2H) system, and find that the GhJAZ2 protein regulates fiber initiation by directly interacting with GhMYB25-like, GhGL1, GhMYC2, GhWD40 and GhJI1.

RESULTS

GhJAZ2 had a high expression level in the ovule at −1 DPA

In our previous studies we found that JA regulates fiber development through an unknown pathway. High levels of JA inhibit cotton fiber initiation *in vitro*, whereas an optimal level of JA enhances fiber elongation (Hao *et al.*, 2012; Tan *et al.*, 2012). We therefore analysed the JA content in cotton ovules from −3 DPA to 8 DPA in *G. hirsutum* YZ1. The JA content was highest in −1 DPA ovules, and sharply declined on the day of flowering (Figure 1a). The JA content of 0 DPA ovules showed significant differences between the wild-type (Xu142 and TM-1), the fuzzless mutants (GZnn, GZNn and n2) and the lintless-fuzzless mutants (XinWX and Xu142 *fl*; Figure 1b). The endogenous JA concentrations were higher in the wild-type compared with the mutants, suggesting that a low level of endogenous JA might be unsuitable for fiber initiation. To determine which JA level was suitable for fiber initiation, we collected −2 DPA ovules for ovule culture whose endogenous JA contents were very low (Figure 1a), and treated them with exogenous JA. The results showed that the ovules exhibited increased initiation in medium

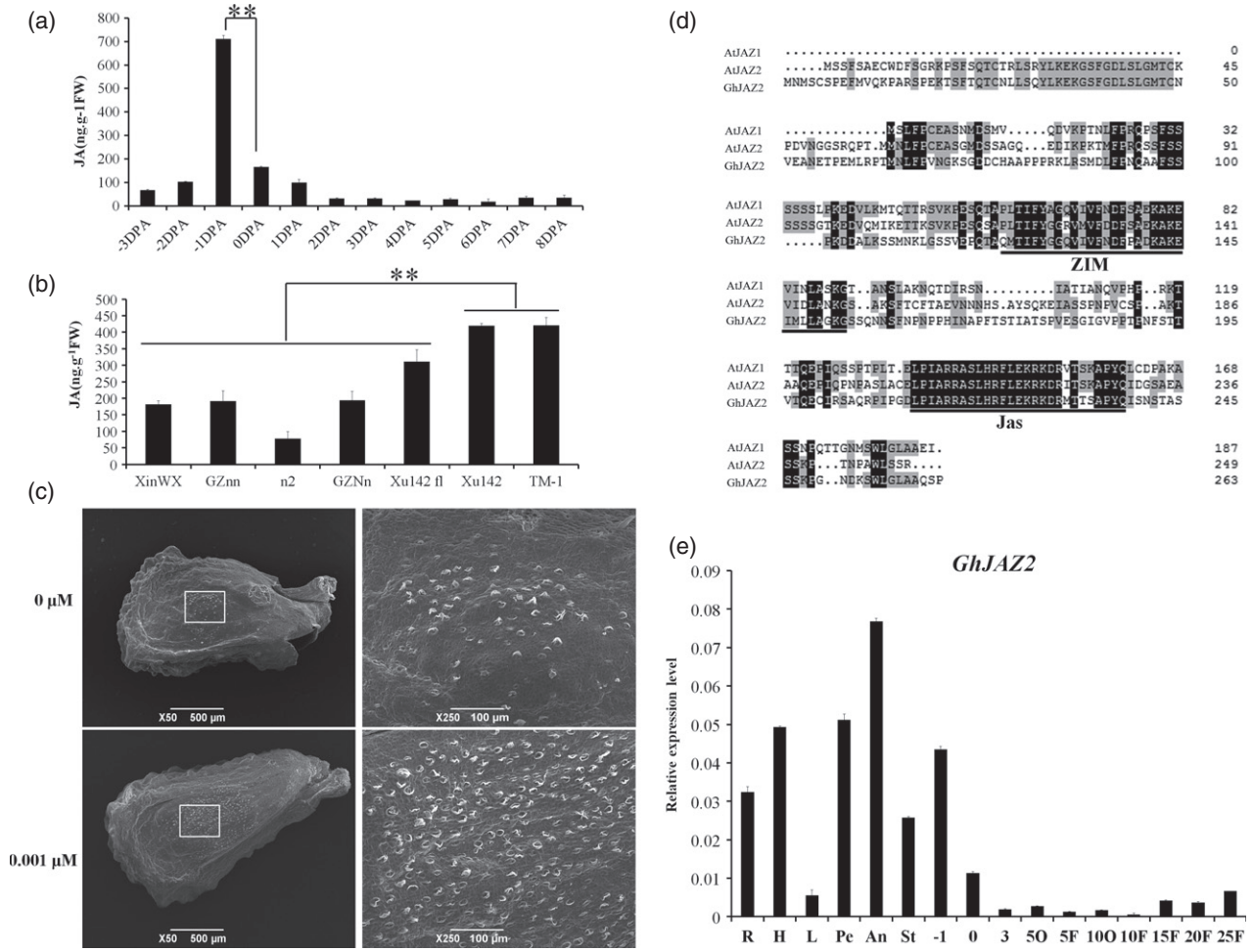

Figure 1. Jasmonic acid (JA) content and *GhJAZ2* expression are associated with fiber initiation.
(a) JA content measurement in *Gossypium hirsutum* cv. YZ1 from −3 days post-anthesis (DPA) to 8 DPA ovule-attached fibers. Three independent experiments were performed. Error bars represent the SD. Significant differences between −1 DPA and 0 DPA were calculated by Student's *t*-test analysis (***P* < 0.01).
(b) Comparison of JA contents in 0 DPA ovules between the wild-type varieties (Xu142 and TM-1), fuzzless mutants (GZnn, GZNn, and n2) and lintless-fuzzless mutants (XinWX and Xu142 *fl*). Three independent experiments were performed. Error bars represent the SD. Significant differences between the mutants and cultivated varieties were calculated by Student's *t*-test analysis (***P* < 0.01).
(c) The YZ1 −2 DPA ovules were collected from the field and treated with exogenous JA in ovule culture. Ovules were observed by scanning electron microscopy (SEM) after 2 days of culture. Scale bars: 100 μm (magnified) and 500 μm.
(d) Alignment of the amino acid sequences of *GhJAZ2*, *AtJAZ1* and *AtJAZ2*; the ZIM domain and Jas domain are shown with black bold underline.
(e) Quantitative real-time PCR analysis of *GhJAZ2* expression in various tissues of *G. hirsutum* cv. TM-1. R: root, H: hypocotyl, L: leaf, An: anther, Pe: petal, St: stigma, 0, 3: ovules attached with fibers at 0 or 3 DPA; −1, 5O and 10O: −1, 5, 10 DPA ovules without fibers; 5F, 10F, 15F, 20F and 25F: 5, 10, 15, 20 and 25 DPA fibers. *GhJAZ2* expression was calculated relative to the expression of *GhUB7*. Error bars represent the standard deviations of three independent replicates.

supplemented with 0.001 μM JA (Figure 1c). However, a higher concentration inhibited fiber initiation, which was consistent with the previous report that 2.5 μM JA inhibited fiber initiation (Tan *et al.*, 2012). These results suggest that an optimal JA concentration is important for fiber development.

Because JAZ is an inhibitor of the JA pathway, we speculated that JAZ might be involved in fiber development through the regulation of JA homeostasis. Our earlier data really showed that *JAZ* was highly expressed in −1 and 0 DPA ovules (Tan *et al.*, 2012). Therefore, we cloned a *JAZ* gene (*GhJAZ2*) based on sequence information (ES802380) from NCBI. *GhJAZ2* is 1056 bp in length with an open reading frame of 792 bp, and the GhJAZ2 protein contains a predicted 263 amino acids. GhJAZ2 contained ZIM and Jas domains (the characteristic domains of a JAZ protein) based on an alignment with Arabidopsis JAZ protein sequences. Phylogenetic analysis showed that GhJAZ2 has homology with AtJAZ1 and AtJAZ2. GhJAZ2 shows 33.21% identity with AtJAZ1 and 43.28% identity with AtJAZ2; therefore, we named this protein GhJAZ2 (Figure 1d). The expression pattern of *GhJAZ2* in different tissues was analysed by qRT-PCR. *GhJAZ2* had high expression levels in the root, hypocotyl, flower and −1

DPA ovule, but low expression levels in ovules with attached fibers and fibers at different developmental stages (Figure 1e).

Overexpression of *GhJAZ2* resulted in less fuzz and shorter fibers

To test the function of *GhJAZ2*, *GhJAZ2* overexpression and downregulation constructs were developed and transformed into cotton via *Agrobacterium tumefaciens*-mediated transformation. We found that the fuzz was reduced when *GhJAZ2* was overexpressed in the transgenic lines compared with the wild-type (Figure 2a). To confirm whether this phenotype was derived from the overexpression of *GhJAZ2*, a total of 29 *CaMV 35S::GhJAZ2* overexpression and *CaMV 35S::iGhJAZ2* (RNAi) T1 lines were analysed. After analysis by Southern and Northern blotting or qRT-PCR analysis, four transgenic overexpression lines (OE-9, OE-39, OE-92 and OE-31) and 3 RNAi lines (Ri-1, Ri-3 and Ri-13) with altered expression levels, and one RNAi line (Ri-7) showing no expression level change (acting as a negative control) were selected for analysis (Figure S1). The *GhJAZ2* expression levels in the ovules/fibers of the transgenic lines were examined in the T3 generation using gene-specific primers (Table S1).

The results show that the *GhJAZ2* transcription levels were upregulated in the −1 DPA ovules in the overexpression lines (two- to fivefold) and in the 5 DPA ovules with attached fibers (17- to 100-fold) compared with the wild-type and negative control (Figure 2b). The *GhJAZ2* expression levels in the −1 DPA ovules were reduced by approximately 50% in the three RNAi transgenic cotton lines compared with the wild-type and negative control line Ri-7, but the expression level in 5 DPA ovules/fibers was not obviously changed, possibly reflecting the very low expression levels.

A sensitivity analysis to JA, comparing transgenic lines and wild-type, was conducted in seedling and ovules. The seedlings of OE lines were less sensitive to JA and RNAi lines more sensitive compared with controls (Figures 2b and S2a). An ovule culture system was also used to characterize the response of ovules from transgenic lines and wild-type to JA. Fiber length was inhibited when the ovules were cultured in BT medium with 0.05 μM JA (Figure S2c). Fiber length from YZ1 (WT) ovules was only 75% of the control after JA treatment; 95.4% and 82.3% of controls, respectively, in the two OE lines; and 17% and 44.6% of controls, respectively, in the two RNAi lines (Figure S2d). Therefore, cultured ovules exhibit a similar JA response to seedlings. Western blotting showed that GhJAZ2 protein accumulated in the three OE lines (Figure 2c).

To investigate the phenotypes of the transgenic lines, they were planted in the field. It was found that overexpressing *GhJAZ2* led to earlier flowering (about 7 days earlier than wild-type). All transgenic cotton lines were also grown in a controlled environment greenhouse for further evaluation. Greenhouse-grown overexpression plants similarly exhibited earlier flowering (Figure 2d), and the seeds of the OE lines were smaller compared with wild-type. The seed indices (weight of 100 seeds) of the four OE lines were 8.08 ± 1.99 g (OE-9), 7.88 ± 1.53 g (OE-39), 8.97 ± 2.20 g (OE-92) and 9.28 ± 1.60 g (OE-31); all indices were significantly smaller than the wild-type seed index (12.85 ± 0.50 g). There was generally no significant change in the seed indices for the RNAi lines (10.47 ± 1.32 g, 10.87 ± 1.72 g and 12.37 ± 0.52 g for Ri-3, Ri-13 and Ri-7, respectively), with the exception of line Ri-1 (8.66 ± 0.25 g; Figure 2a; Table 1). In the OE lines, the fuzz was significantly reduced, the lint fibers were shorter and the lint indices were decreased compared with the controls; these results were confirmed by one more year of performance tests in the greenhouse (Table 1). In the greenhouse, the fiber lengths of the four overexpression lines were 26.01 ± 0.18 mm, 25.93 ± 0.30 mm, 25.83 ± 0.45 mm and 27.10 ± 0.51 mm; these lengths were much shorter than the lengths of the two controls (29.95 ± 0.33 mm for WT and 29.99 ± 0.30 mm for the negative control; Figure 2e and f). The fiber lengths were not changed significantly in the *GhJAZ2*-silenced lines (30.06 ± 0.26 mm, 29.43 ± 0.37 mm and 27.82 ± 0.32 mm). Therefore, overexpressing *GhJAZ2* in cotton resulted in less fuzz fiber, shorter lint fibers and smaller seeds, while suppression of *GhJAZ2* expression had no significant effect.

***GhJAZ2* suppresses both fuzz and lint fiber initiation**

According to previous reports, fuzz fibers start to initiate from the ovule surface at 5 DPA and begin to elongate at 6 DPA (Lang, 1938; Du *et al.*, 2013). To investigate the fuzz numbers of the transgenic and wild-type lines, we sectioned 6 DPA ovules to count the fuzz cells. Vertical sections of 6 DPA ovules revealed that the fuzz numbers of the OE lines were 12.3 ± 5.0 (OE-9) and 11.6 ± 2.6 (OE-39), much lower than the controls (28.3 ± 2.9 for WT and 24.8 ± 3.0 for Ri-7). The fuzz numbers in the RNAi lines were similar to the controls (Figure 3a and b).

It is possible that lint and fuzz fibers might share a common developmental pathway during initiation (Wu *et al.*, 2006), and so we speculated that GhJAZ2 may also play a role during early fiber development. We determined the numbers of initial lint fibers in the OE lines using scanning electron microscopy (SEM), and found that they were significantly less than in the controls and RNAi lines (Figure 3c and d). We also obtained OE transgenic Arabidopsis lines and found fewer trichomes on the leaves of the OE lines than the WT line (Figure S3a),

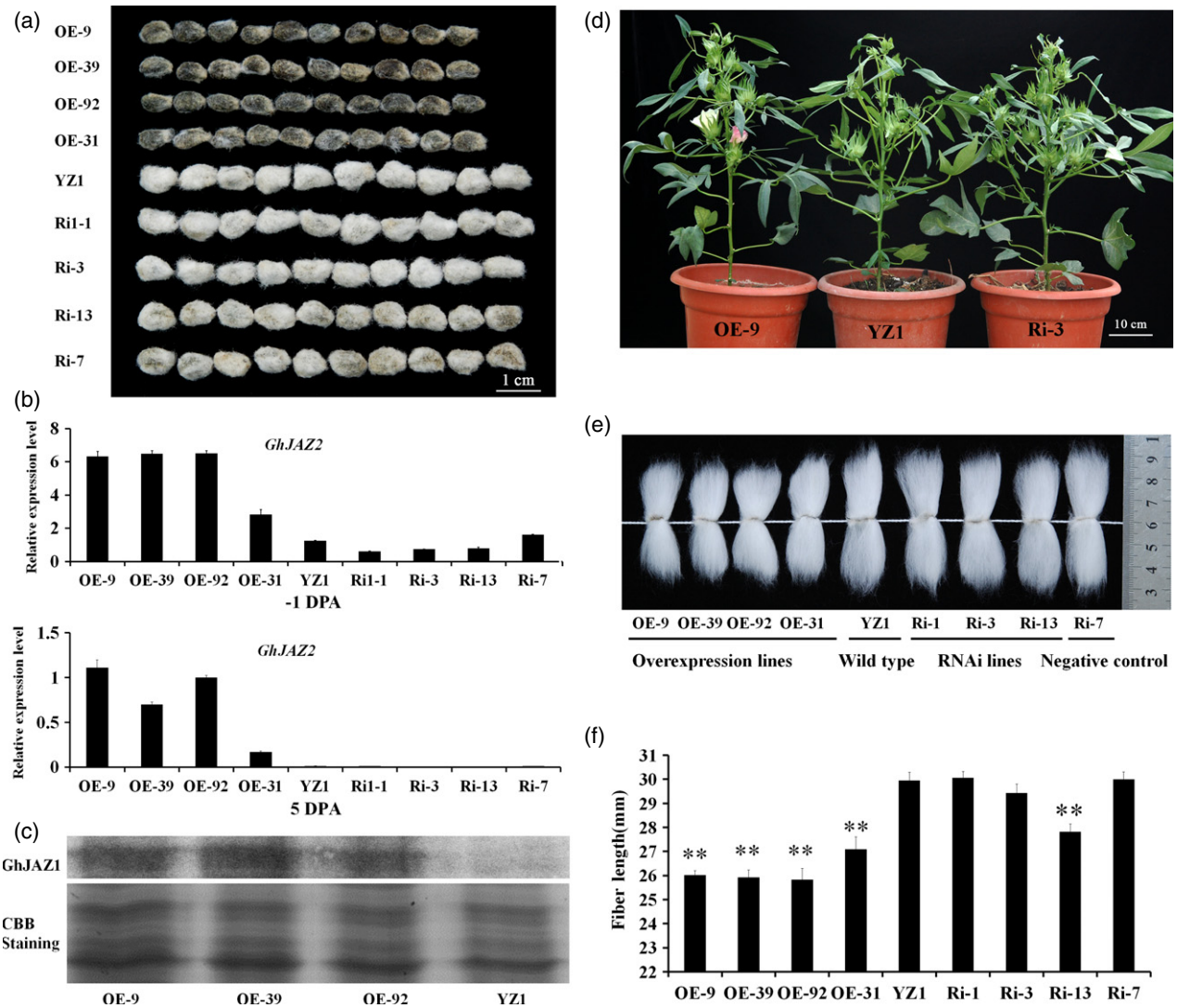

Figure 2. Overexpression of *GhJAZ2* results in less fuzz and shorter lint.
(a) The fuzz fiber numbers of the overexpression lines were fewer than those of the wild-type and the RNAi lines. Scale bar: 1 cm.
(b) Quantitative RT-PCR analysis of *GhJAZ2* expression in −1 and 5 days post-anthesis (DPA) ovules of the transgenic lines and controls (YZ1: the wild-type and Ri-7: the negative transgenic control). *GhJAZ2* expression was calculated relative to *GhUB7* expression.
(c) Western blotting analysis to identify the protein level in leaves of the GhJAZ2 overexpression lines and YZ1. CBB staining: Coomassie Brilliant Blue staining.
(d) Upregulation of *GhJAZ2* resulted in early flowering in the overexpression lines (OE-9). The RNAi lines (Ri-3) showed no obvious morphology change compared with the wild-type (YZ1). Scale bar: 10 cm.
(e, f) The fiber length of the overexpression lines is less than the fiber length of the RNAi lines and wild-type. The mature fiber length was measured in the T_3 overexpression lines, RNAi lines and controls. The fiber length was averaged from 60 ovules; the error bars represent the SD. Significant differences between the wild-type lines and transgenic lines were calculated by Student's *t*-test analysis (**$P < 0.01$).

which confirmed that *GhJAZ2* negatively regulated fiber/trichome initiation.

GhJAZ2 negatively regulated fiber initiation via interacting with GhMYB25-like

To evaluate the mechanism underlying the effect of JAZ on fiber initiation, we used the Y2H system to screen for interacting proteins. *GhJAZ2* was fused into the pGBKT7 vector to construct a bait plasmid encoding a fusion protein with the DNA-binding domain of GAL4. The fusion plasmid that had no autoactivation in Y2H was used to screen the −2 to 4 DPA ovule/fiber cDNA library to identify GhJAZ2-interacting proteins (Figure S3b). Approximately 80 positive clones were identified. After sequencing the clones and removing the redundant sequences (the WD40-repeat protein was the most redundant gene), we found that putative interacting proteins that included PP2C, tublins, a Ca^{2+}-dependent kinase, transcription factors amongst others (Table S3). Among the interacting factors, we focused on transcription factors such as the R2R3-MYB

Table 1 The lint index, seed index and lint percentage of the overexpression lines, RNAi lines and control lines

	2013			2014		
	Lint index (g)	Seed index (g)	Lint percentage (%)	Lint index (g)	Seed index (g)	Lint percentage (%)
OE-9	6.42 ± 0.43*	8.08 ± 1.99**	0.45 ± 0.04**	5.55 ± 0.21**	6.97 ± 0.18**	0.44 ± 0.00
OE-39	6.34 ± 0.32*	7.88 ± 1.53**	0.45 ± 0.03**	4.84 ± 0.14**	6.02 ± 0.10**	0.45 ± 0.00
OE-92	6.51 ± 0.60*	8.97 ± 2.20**	0.43 ± 0.04*	5.56 ± 0.13**	7.25 ± 0.11**	0.43 ± 0.01
OE-31	6.97 ± 0.30	9.28 ± 1.60**	0.43 ± 0.03*	5.49 ± 0.39**	7.17 ± 0.58**	0.43 ± 0.00
YZ1	7.63 ± 0.68	12.85 ± 0.50	0.37 ± 0.02	6.94 ± 0.41	8.69 ± 0.35	0.44 ± 0.01
Ri-1	6.14 ± 0.47**	8.66 ± 0.25**	0.41 ± 0.01	6.62 ± 0.38	8.67 ± 0.26	0.43 ± 0.01
Ri-3	8.01 ± 0.06	10.47 ± 1.32	0.41 ± 0.03	4.94 ± 0.44**	7.37 ± 0.60**	0.40 ± 0.01**
Ri-13	7.57 ± 0.20	10.87 ± 1.72	0.41 ± 0.03	5.37 ± 0.16**	7.95 ± 0.34	0.40 ± 0.01**
Ri-7	8.20 ± 0.44	12.37 ± 0.52	0.40 ± 0.01	6.35 ± 0.72	8.34 ± 0.20	0.43 ± 0.02

*Indicates significant differences ($P < 0.05$); **indicates significant differences ($P < 0.01$) among the different lines according to Student's *t*-test analysis. Values are the mean ± standard deviation of assays for three repetitions of each line.

transcription factors GhMYB25-like (GenBank accession XM_134084), GhGL1 (GenBank accession XM_012582325), the bHLH factor GhMYC2 (GenBank accession XM_012583866), the WD40-repeat protein GhWD40 (GenBank accession XM_012610860) and an unknown protein GhJI1 (GenBank accession XM_012583490) that had a lower expression level in the lintless-fuzzless mutant Xu142 *fl* before −2 DPA (Figure 4a and c). To date, only GhMYB25-like has been reported amongst these transcription factors to impact fiber development. Specifically, GhMYB25-like acts as an essential transcription factor in cotton fiber initiation, and GhMYB25-like silencing results in fiberless seeds (Walford *et al.*, 2011).

We used bimolecular fluorescence complementation (BiFC) assays (Waadt *et al.*, 2008) to confirm the interactions between GhJAZ2 and GhMYB25-like, GhGL1, GhMYC2, GhWD40 and GhJI1 in protoplasts of *Nicotiana benthamiana*. When GhJAZ2 and the transcription factor GhMYB25-like were co-expressed in the *N. benthamiana* protoplast, strong YFP fluorescence was detected. We also found that GhJAZ2 interacted with GhGL1, GhMYC2, GhWD40 and GhJI1 (Figure 4b).

To investigate a potential role for these transcription factors during cotton fiber initiation, the expression profiles of *GhJAZ2*, *GhMYB25-like*, *GhGL1*, *GhMYC2*, *GhWD40* and *GhJI1* were investigated in Xu142 and Xu142 *fl* ovules/fibers from −3 DPA to 5 DPA (Figures 4c and S3c). *GhMYB25-like* had the highest expression level in the −1 DPA ovule, which was similar to the *GhJAZ2* expression pattern, but with much lower expression levels in the Xu142 *fl* ovule, as previously reported (Walford *et al.*, 2011). *GhMYC2*, *GhWD40* and *GhJI1* had high expression levels during the early initiation stage. The expression level of *GhJI1* was higher in the −3 DPA and −2 DPA ovules of Xu142 compared with Xu142 *fl*. The expression levels of *GhWD40* and *GhGL1* showed the same tendency in the −1 DPA ovules in Xu142 compared with Xu142 *fl*. The *GhMYC2* ovule expression level did not show obvious differences between Xu142 and Xu142 *fl* (Figure 4c). *GaHOX1* (of *G. arboreum*) has been reported to act downstream of *GhMYB25-like* and regulate fiber initiation and elongation (Guan *et al.*, 2008). Therefore, we examined *GhHOX1* (a homoeolog of *GaHOX1*) expression in the transgenic lines. Its expression levels in three OE lines (OE-9, OE-39 and OE-92) were lower than those in the controls and RNAi lines, which is consistent with the reduced fiber initiation in the OE lines compared with the controls and RNAi lines (Figure 4c). We therefore conclude that the low expression level of *GhHOX1* lead to less fiber initiation in OE lines.

GhJAZ2 interacted with GhMYB25-like

A *N. benthamiana* protoplast transient expression system was used to explore the interaction between GhJAZ2 and GhMYB25-like, based on the interaction between the DNA-binding domain of GAL4 (GAL4DB) and the binding sites of GAL4(5X)-TATA-LUC (firefly luciferase gene; Ohta *et al.*, 2001). *GhMYB25-like* was fused with the DNA-binding sites GAL4DB driven by the 35S promoter. *GhJAZ2* was fused with the 35S promoter to serve as effectors. The *LUC* gene under control of five upstream GAL4 DNA-binding sites [GAL4(5X)-TATA-LUC] was used as a reporter. The Renilla LUC (Renilla luciferase) gene driven by AtUbiquitin 3 promoter was used as an internal control (Figure 5a). The LUC/Renilla LUC ratio of GAL4DB-GhMYB25-like was found to be increased compared with the GAL4DB, which indicates that GhMYB25-like acts as a transcriptional activator (Figure 5b). Co-expression of GhJAZ2 and GhMYB25-like also significantly reduced the LUC/Renilla LUC ratio. As expected, GhJAZ2 repressed the transcriptional activity of GhMYB25-like in *N. benthamiana* protoplasts (Figure 5b).

In order to detect further the interaction between GhJAZ2 and GhMYB25-like and characterize the function of GhMYB25-like *in vivo*, the *GhMYB25-like* gene driven by *CaMV 35S* promoter was overexpressed in cotton (overexpression lines M25L-19, M25L-22) and gene expression

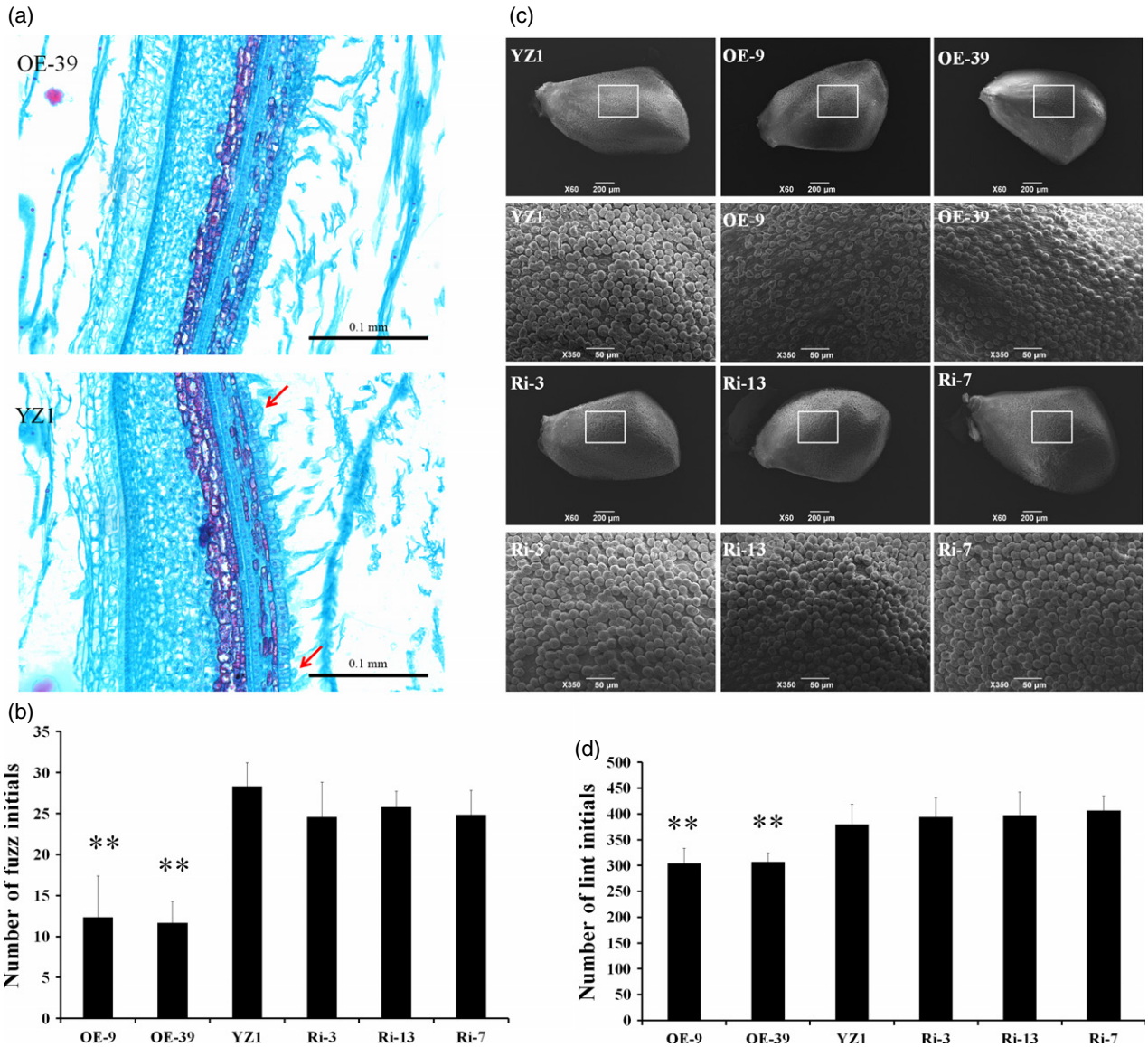

Figure 3. Detection of fuzz and lint initiation by microscopy.
(a) Cross-section of 6 days post-anthesis (DPA) ovules from the overexpression (OE-39) and wild-type (YZ1) lines; red arrows indicate the fuzz initiation. Only the sections from precisely the middle part of the ovules were observed. Scale bar: 0.1 mm.
(b) The number of fuzz initiation events was calculated from more than 40 ovules from the cross-section of each line; the *y*-axis indicates the fuzz number per ovule circumference.
(c) The scanning electron microscopy (SEM) photographs of 0 DPA ovules of the overexpression lines, RNAi lines and controls. All ovules were taken from the same position in the boll. Scale bars: 50 μm (magnified) and 200 μm.
(d) The number of lint initiation events in the rectangular areas on the ovules of the transgenic lines and controls are shown as the white rectangles indicated in (c). More than 40 ovules were counted. Error bars represent the standard deviations (SD). Asterisks indicate a significant difference between the transgenic lines and wild-type as determined by Student's *t*-test analysis (**$P < 0.01$).

levels were determined (Figure 5c). *GhJAZ2* overexpressing lines were also crossed into *GhMYB25-like* overexpressing lines, and the expression levels in the F_1 plants, their transgenic lines and in wild-type were determined (Figure 5c and d). The number of initials in these lines was quantitatively compared using SEM. The number of initial lint fibers in *GhMYB25-like* overexpression lines was significantly increased, but the initials of the F_1 plants and the *GhJAZ2* overexpression lines show no statistically significant differences (Figure 5e and f). These results show that GhJAZ2 can interact with GhMYB25-like and inhibit its transcriptional activation.

The JAZ family may be redundant in fiber initiation

A study in Arabidopsis has shown that the JAZ family contained 12 members with functional redundancy (Thines

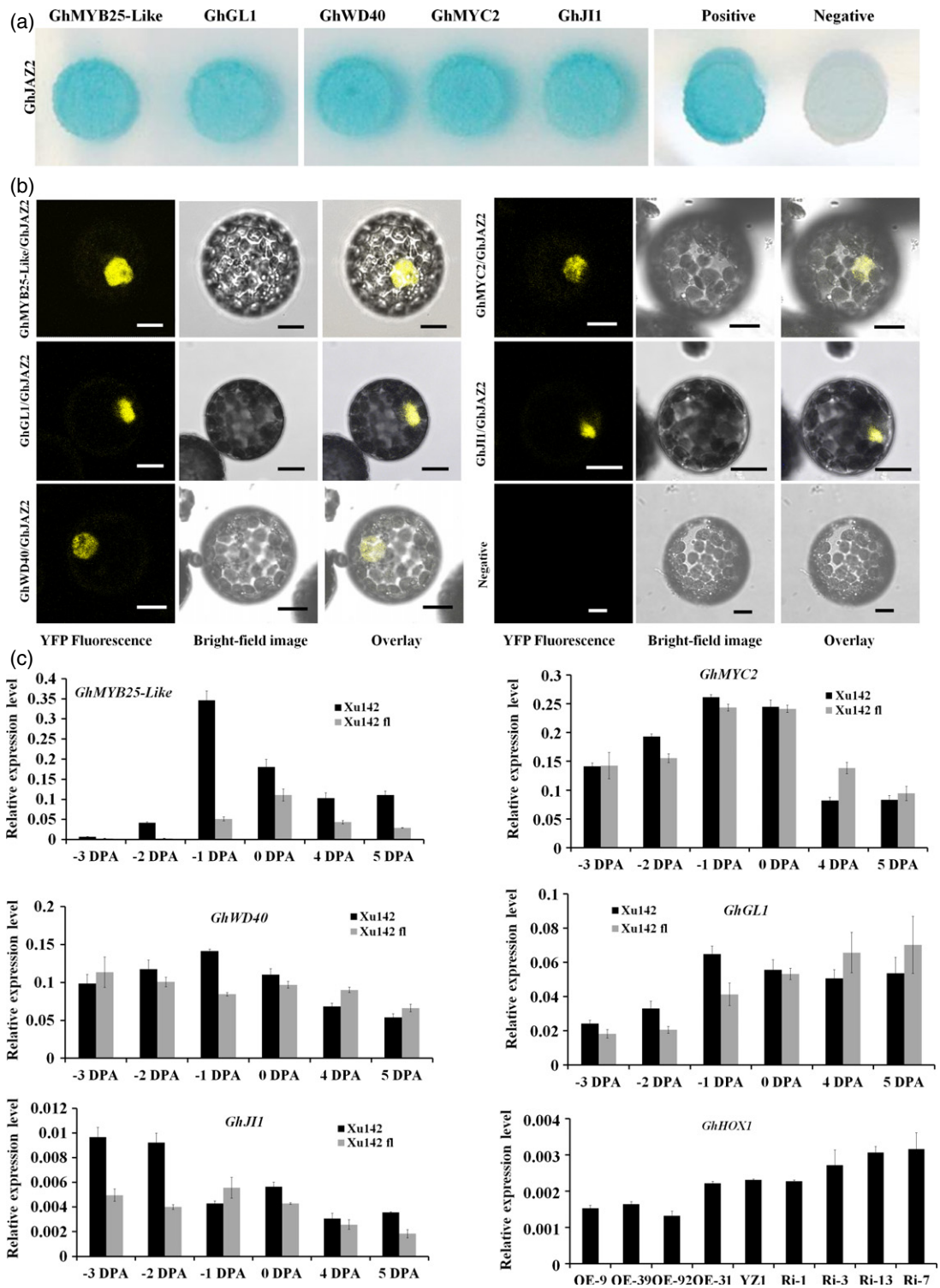

Figure 4. Interactions between GhJAZ2 and GhMYB25-like, GhGL1, GhMYC2, GhWD40, GhJI1 and their expression profiles.
(a) Yeast two-hybrid (Y2H) assays confirmed the interactions between GhJAZ2 and GhMYB25-like, GhGL1, GhMYC2, GhWD40 or GhJI1 by growth on SD medium with the -Trp/-Leu/X-α-Gal/AbA.
(b) The bimolecular fluorescence complementation (BiFC) assay confirmed the interactions between GhJAZ2 (fused with the N-terminal fragment of YFP) and GhMYB25-like, GhGL1, GhMYC2, GhWD40 or GhJI1 (fused with the C-terminal fragment of YFP). The construct pairs were co-expressed in protoplasts of *Nicotiana benthamiana*. Scale bar: 10 μm.
(c) The GhMYB25-like, GhGL1, GhMYC2, GhWD40 and GhJI1 expression levels were detected in Xu142 and Xu142 *fl* by quantitative real-time RT-PCR. The *GhHOX1* expression levels were detected in 0 days post-anthesis (DPA) ovules of the transgenic lines.

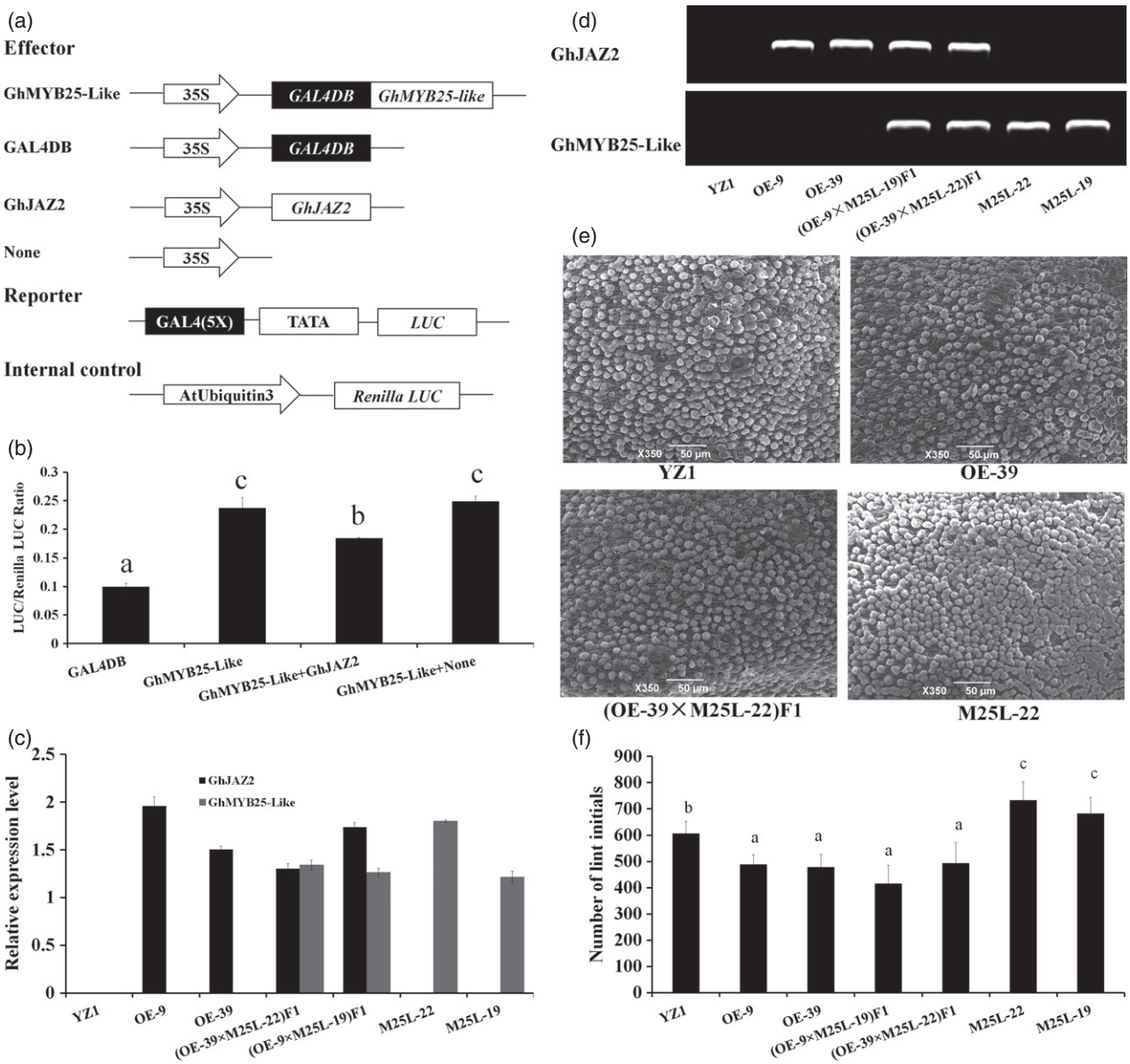

Figure 5. GhJAZ2 inhibits the transcriptional function of GhMYB25-like.
(a) Schematic diagram of the vectors used in transient expression assays in (b).
(b) Transient expression assays indicate that GhJAZ2 proteins can repress the transcriptional function of GhMYB25-like. The LUC/Renilla LUC ratios were measured in *Nicotiana benthamiana* leaf protoplasts after transient transfection with effectors, reporter and internal control. Four independent experiments were performed.
(c) Quantitative RT-PCR analysis of *GhJAZ2* and *GhMYB25-like* expression in leaves from transgenic lines, wild-type and the F_1 crosses between *GhJAZ2* overexpressing lines (OE-9, OE-39) and *GhMYB25-like* overexpressing lines (M25L-19, M25L-22).
(d) The positive detection of the F_1 plants, transgenic lines and wild-type.
(e) Scanning electron microscopy (SEM) images of 0 days post-anthesis (DPA) ovules of F_1 plants, transgenic lines and wild-type. Scale bars: 50 μm.
(f) Numbers of lint initials were calculated from over 40 ovules of 0 DPA. Error bars represent the standard deviations (SD). Significant differences (b, f) between the wild-type lines, transgenic lines and the F_1 plants were calculated by one-way analysis of variance (ANOVA) test ($P < 0.05$).

et al., 2007). Several JAZ proteins (as opposed to a specific JAZ) worked together to interact with the bHLH and MYB members of the WD-repeat/bHLH/MYB complexes to control trichome development, as revealed by Y2H assays (Qi *et al.*, 2011). To examine the details of the interaction between the GhJAZ family members and GhMYB25-like, GhGL1, GhMYC2, GhWD40 and GhJI1, we searched the whole genome and isolated all JAZ sequences in the *G. hirsutum* TM-1 [(AD)1 genome] sequence database (Zhang *et al.*, 2015). Thirty cotton *JAZ* members were identified and the sequences were downloaded (BioProject ID: PRJNA248163). A phylogenetic tree was constructed with

30 JAZ proteins in cotton and 12 JAZ proteins in Arabidopsis. GhJAZ2 (Gh_D06G0810), and two other JAZ proteins (Gh_D08G2564/Gh_A08G2199 and Gh_D05G0352/Gh_A05G0260) had high sequence similarity with AtJAZ2 (Figure S4a).

A heat map based on the FPKM values of different tissues for the *JAZ* members was constructed (Figure S4b). By aligning the sequences, we found that *GhJAZ2* was *Gh_D06G0810* (FPKM 14, 0 DPA) from the D_T sub-genome. The other five *JAZ* genes that were preferentially expressed at the fiber initiation stage were also found (*Gh_A07G0156*, *Gh_D07G0152*, *Gh_A10G2244*, *Gh_D10G0531* and *Gh_D05G0379*) with higher FPKM values. *Gh_A07G0156* and *Gh_D07G0152* were homoeologous genes from the A_T and D_T sub-genomes with FPKM values of 57 and 81 in the 0 DPA ovules, respectively. Similarly, *Gh_A10G2244* (FPKM 21) and *Gh_D10G0531* (FPKM 23) were also homoeologous genes. The *Gh_D05G0379* FPKM value was 98 in the 0 DPA ovules (Table S2).

The expression patterns of the *GhJAZ* proteins were validated in different tissues from TM-1 using qRT-PCR (Figure S5) and showed trends similar to the digital expression profiling from the public database (Figure S4). We found another JAZ protein gene (*Gh_D01G1406*) that had an obvious expression peak in the −1 DPA ovule (FPKM 4 in the 0 DPA ovule in the digital expression profile; Figure S5; Table S2). According to the above result, two JAZ proteins had high sequence similarity with GhJAZ2 and four *JAZ* members had similar expression patterns to *GhJAZ2*. Therefore, we selected six *JAZ* members (*Gh_D07G0152*, *Gh_D10G0531*, *Gh_D05G0379*, *Gh_D01G1406*, *Gh_D08G2564* and *Gh_D05G0352*) for Y2H analysis. We cloned the full-length sequences of *Gh_D07G0152*, *Gh_D10G0531*, *Gh_D05G0379*, *Gh_D01G1406*, *Gh_D08G2564* and *Gh_D05G0352*, and fused them to the Y2H system vector PGBKT7; these constructs were used for yeast mating to detect the interaction between GhJAZ and each of the five transcription factors (GhMYB25-like, GhGL1, GhMYC2, GhWD40 and GhJI1). The results showed that only Gh_D01G1406 had a positive interaction with GhMYB25-like, GhGL1, GhMYC2, GhWD40 and GhJI1 (Figure 6a), which suggested that *Gh_D01G1406* (whose expression level was lower than *GhJAZ2*) might have some functional redundancy with *GhJAZ2*. The *Gh_D01G1406* expression level was also measured in the 0 DPA ovule in the transgenic lines; the result showed that its expression was increased in two RNAi lines (Ri-3 and Ri-13), but did not change in the OE lines (Figure 6b). This result implies that there may be other cotton JAZ genes with functional redundancy during cotton lint and fuzz initiation.

DISCUSSION

Cotton fiber development is mediated by a balance of hormones, and each hormone has its corresponding protein targets to control development. Appropriate concentrations of IAA and GA_3 produced the longest fiber in the ovule culture system (Momtaz, 1998), and the balance between IAA and ABA determines cotton fiber elongation and secondary cell wall deposition (Guinn and Brummett, 1988). Moreover, *G. hirsutum* had medium level of ethylene compared with *G. arboreum* and *Gossypium raimondii*, ethylene may be an important modulator during cotton fiber development during the evolutionary process (Li *et al.*, 2015). GA and JA antagonistically and synergistically regulate plant growth in Arabidopsis. JAZ proteins and DELLA, a repressor in GA signaling pathway, bind to the WD-repeat/bHLH/MYB complex to modulate trichomes initiation (Qi *et al.*, 2014), and exogenous JA increases both trichome density and the numbers of Arabidopsis leaves (Traw and Bergelson, 2003). In cotton, JA also plays an important role in cotton fiber development. High concentrations of JA inhibit cotton fiber initiation (Tan *et al.*, 2012) and lower concentrations promote fiber elongation (Hao *et al.*, 2012). Our results show that JA promotes fiber initiation at the 0.001 μM concentration, suggesting that an optimal JA content is required for fiber initiation.

The JAZ protein family contains key repressors of the JA pathway. COI1 is a subunit of the SCF^{COI1} E3 ligase complex that targets AtJAZ1 for degradation through the 26S proteasome system in the presence of JA. In Arabidopsis, the jasmonate-response mutant *coi1*, which is characterized by a mutated subunit of the SCF^{coi1} E3 ligase complex, has fewer trichomes on the leaves (Feys *et al.*, 1994). AtJAZ1 protein cannot be degraded in the mutant, resulting in a high level of AtJAZ1 accumulation (Thines *et al.*, 2007). AtJAZ1 interacts with the WD-repeat/bHLH/MYB transcription factors and represses their transcriptional activity, resulting in reduced trichome initiation (Qi *et al.*, 2014). In our study, we overexpressed the cotton gene *GhJAZ2* in Arabidopsis and found that *GhJAZ2* also reduces trichome initiation. In the cotton lines stably overexpressing *GhJAZ2*, both lint and fuzz fiber initiation were also inhibited.

Jasmonic acid content was found to be lower in the fiberless or fuzzless mutants compared with the wild-type cultivars, and we found that *GhJAZ2* expression levels were lower in the wild-type cultivars compared with the fiberless or fuzzless mutants (Figure S3d). Also, opposing trends were found in the JA contents in 0 DPA ovules of the mutants (XinWX, Xu142 *fl*, GZnn, GZNn and n2) and WT varieties (Xu142 and TM-1; Figure 1b). We therefore speculate that GhJAZ2 might regulate fiber initiation and length, potentially explaining the differences between the mutant and cultivated varieties. Based on our transcription analysis, a lower level of *GhJAZ2* expression and higher JA content should promote trichome or fiber initiation.

Our research shows that GhJAZ2 interacts with the R2R3-MYB transcription factor GhMYB25-like. GhMYB25-

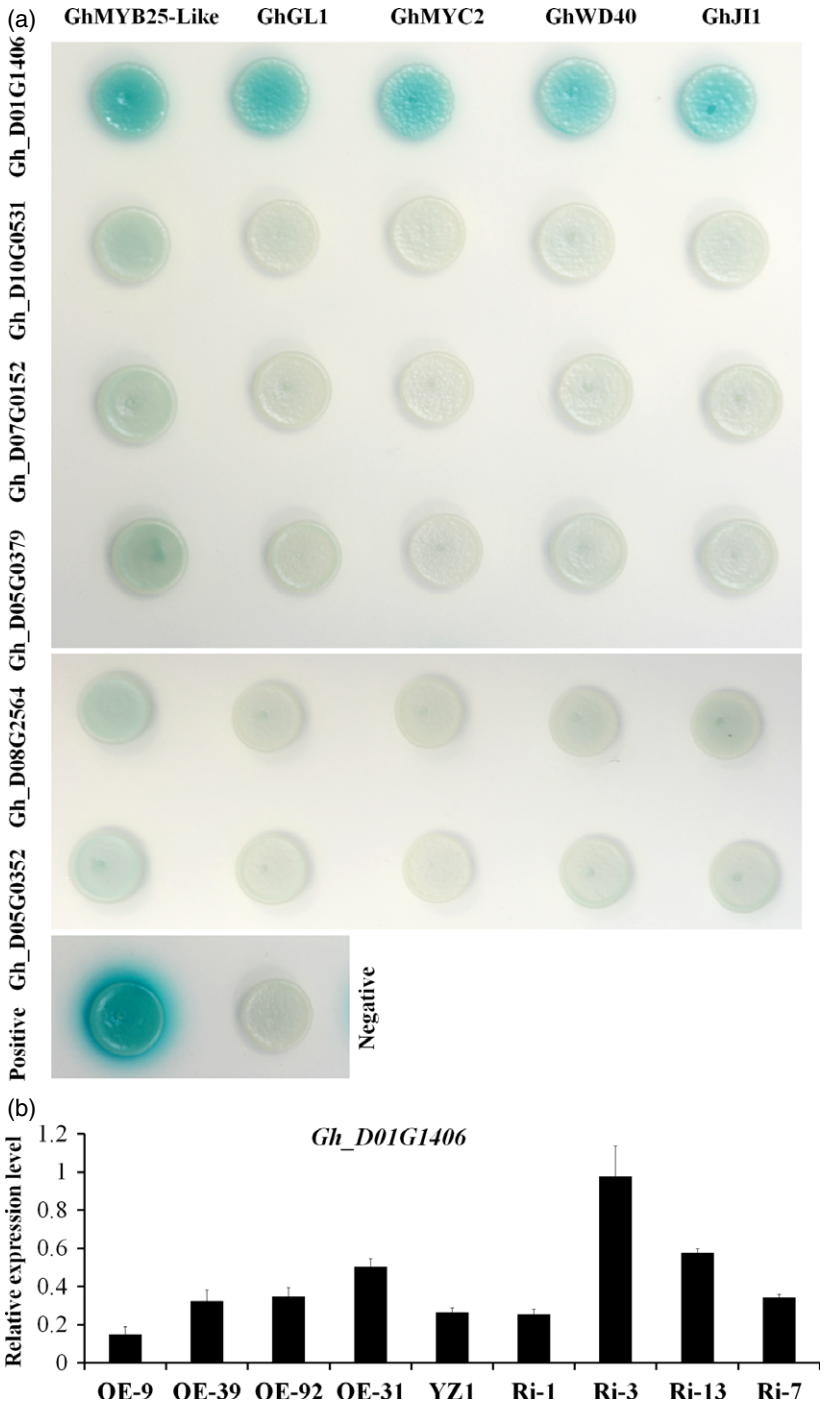

Figure 6. Only one member (Gh_D01G1406) of the JAZ family can interact with GhMYB25-like, GhGL1, GhMYC2, GhWD40 or GhJI1, shown by growth on SD medium with the -Trp/-Leu/X-α-Gal/AbA. The expression level of *Gh_D01G1406* was also upregulated in the RNAi transgenic lines.
(a) Gh_D01G1406 showed a positive interaction with GhMYB25-like, GhGL1, GhMYC2, GhWD40 and GhJI1. The interactions of Gh_D07G0152, Gh_D10G0531, Gh_D05G0379, Gh_D08G2564 and Gh_D05G0352 showed negative results.
(b) Quantitative RT-PCR analysis of *Gh_D01G1406* expression in 0 days post-anthesis (DPA) ovules from the transgenic lines and controls.

like, GhMYB25, GhMYB109 and GhMYB2 belong to the R2R3-MYB family. The *GhMYB25-like* expression level was reduced in the fiberless mutant Xu142 *fl* compared with Xu142 during early fiber development. Suppressing *GhMYB25-like* expression levels resulted in totally fiberless seeds (Walford *et al.*, 2011). *GhMYB25*-silenced transgenic cotton show shorter fibers and less fiber initiation. Conversely, overexpression of *GhMYB25* significantly increases fiber initiation in 0 DPA ovules and leaf trichomes (Machado *et al.*, 2009). Cotton fiber length is shorter when expression of *GhMYB109* is reduced (Pu *et al.*, 2008). *GhMYB2* overexpression also results in thicker leaf trichomes (Huang *et al.*, 2013). Two WD-repeat genes from cotton (*GhTTG1* and *GhTTG3*) are able to rescue trichome formation in the Arabidopsis *ttg1-1* mutant (Humphries *et al.*, 2005).

All of these transcription factors therefore have been shown to be involved in fiber initiation and early elongation in cotton or in Arabidopsis trichome initiation. *GhMYB25-like* is a key transcription factor in cotton fiber initiation, as suppressing *GhMYB25-like* completely blocks fiber production (Walford *et al.*, 2011). Our results show that GhJAZ2 represses the transcriptional activation of GhMYB25-like in *N. benthamiana* protoplasts and the F_1 crosses between *GhJAZ2* OE lines and *GhMYB25-like* OE lines had the same phenotype as *GhJAZ2* OE lines, which indicates that GhJAZ2 regulates fiber initiation through interacting with GhMYB25-like and affecting its transcriptional activation capability. GhJAZ2 also interacted with GhGL1, GhMYC2, GhWD40 and GhJI1, but no report shows how the four genes may be related to fiber development. *GhMYB25-like* expression was greatly decreased in Xu142 *fl* at all stages (−3 DPA to 5 DPA), but the expression levels of *GhGL1*, *GhMYC2*, *GhWD40* and *GhJI1* were only reduced at some stages and the tendency towards reduced expression was not obvious compared with *GhMYB25-like* (Figure 4c). Thus, JAZ may not regulate cotton fiber initiation through interacting with these particular four genes.

The gene *GaHOX1* that is strongly expressed in cotton fiber cells is a functional homolog of Arabidopsis *GL2*. *GL2* is downstream of the R2R3-MYB/bHLH/WD40 transcription complex, and *GaHOX1* can rescue the glabrous mutant of *gl2-2* in Arabidopsis (Guan *et al.*, 2008). Therefore, *GaHOX1* may be downstream of GhMYB25-like to directly regulate cotton fiber initiation. Our results show that *GhHOX1* transcription level in *GhJAZ2*-overexpressing transgenic lines was reduced, possibly due to a suppressed GhMYB25-like activity caused by GhJAZ2, which results in the suppression of fiber initiation.

In conclusion, GhJAZ2 represents a key repressor in the JA pathway, and inhibits lint and fuzz fiber initiation by repressing the transcriptional activation of GhMYB25-like.

EXPERIMENTAL PROCEDURES

Plant materials

The cotton plants *G. hirsutum* cv. TM-1, YZ1, and Xu142, the fuzzless mutants n2, GZNn and GZnn, and the lintless-fuzzless mutants Xu142 *fl* and XinWX used in this study were planted in the experimental field at Huazhong Agricultural University in Wuhan, China, under standard farming conditions. Transgenic cotton lines were grown in the greenhouse (28–35°C by day and 20–25°C by night) under a 16/8 h light/dark cycle according to relevant national approvals for biotechnology research (China, http://pg.natesc.gov.cn/sites/pg/). Ovules and fibers at different developmental stages were separated from the flower buds or bolls of the plants in the greenhouse. Roots, hypocotyls and leaves were collected from 15-day-old seedlings. All materials were collected and immersed in liquid nitrogen and then frozen at −70°C for later use. The flower buds 1.3–1.6 cm in length were identified as the −2 DPA bolls; the −1 DPA bolls were large enough to be distinguished with the naked eye. *Nicotiana benthamiana* was grown in 16/8 h light/dark conditions under white fluorescent light at 20°C and used for BiFC analysis. To analyse trichome initiation in the *GhJAZ2* overexpression lines, *Arabidopsis thaliana* (Columbia) was grown at 20°C under 16/8 h light/dark conditions.

Gene cloning, vector construction and transformation

The open reading frame (ORF), protein sequence and the conserved domain of GhJAZ2 were analysed in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). *GhJAZ* family members were identified from *G. hirsutum* (*Gh*) TM-1((AD)1), whose genome sequences are available (Zhang *et al.*, 2015). The *JAZ* sequences were downloaded from genomic sequencing information (BioProject ID: PRJNA248163). The phylogenetic analysis was performed with Clustal X version 1.83 and MEGA 5. Gene-specific primers (Table S1) were designed to amplify *GhJAZ2* using YZ1 leaf cDNA as the template. The sequence of *GhMYB25-like* comes from a previous report (Walford *et al.*, 2011). Subsequently, *GhJAZ2* and *GhMYB25-like* were inserted into pK2GW7.0 with CaMV 35S promoter (Ghent University, http://www.plantgenetics.rug.ac.be/gateway/) to construct the overexpression vector. The sequence from the 337th to the 734th position after the *GhJAZ2* ATG was amplified by the primers with attB1 and attB2 adaptors (Table S1) to construct the RNAi vector with pHellsgate4 with CaMV 35S promoter (Helliwell *et al.*, 2002). The expression constructs were transferred into *A. tumefaciens* strain LBA4404 by electroporation. The strain LBA4404 containing the constructs was used to transform cotton (YZ1) plants via *A. tumefaciens*-mediated transformation (Jin *et al.*, 2006).

Ovule culture

The −2 DPA and 0 DPA ovules were collected from the flower buds at 08:00–09:00 hours. The culture method was performed according to the previous report (Momtaz, 1998); −2 DPA ovules were cultured in liquid BT medium supplemented with 0, 0.0005, 0.001, 0.005, 0.01 or 0.05 μM JA. After 2 days of culture, the ovules were collected to observe fiber initiation by SEM. The 0 DPA ovules were cultured in liquid BT medium with 0 and 0.05 μM JA for 10 days. Three biological replicates were performed for each treatment (three biological replicates indicate that we cultured and analysed the ovules three times). Each time and each treatment with different hormones concentrations was performed for three bottles. All of the cultured ovules were collected for SEM and photographed.

DNA extraction, Southern blotting and expression analysis

Genomic DNA was extracted from young leaves of transgenic and wild-type cotton using the plant genomic DNA kit DP305 (Tiangen Biotech, Beijing). Approximately 20 μg of genomic DNA digested with *Hind*III was used for Southern blotting. The digested DNA was transferred to a Hybond-N^+ nylon membrane as previously reported (Tu *et al.*, 2007). *NPT*II was used as the probe to detect copy numbers. λ DNA digested with *Hind*III was used as the marker DNA.

To confirm the expression level of *GhJAZ2* in the transgenic and wild-type cotton plants, RNA was isolated from all samples according to the previously reported method (Tu *et al.*, 2007). Approximately 3 μg of total RNA was reverse-transcribed using the SuperScript III reverse transcriptase (Invitrogen, Cat. no. 18080-093, Waltham, MA, USA) in a 20-μL reaction mixture to obtain cDNA. The qRT-PCR was performed using the ABI Prism

7000 system (Applied Biosystems, Waltham, MA, USA). *GhUBQ7* (DQ116441) was used as the internal control to normalize the expression levels. The primers used in the real-time PCR are shown in Table S1.

Protein extraction and Western blotting

Nuclear protein was extracted from young leaves of transgenic and wild-type cotton using the nuclear protein extraction method (Sigma, Lot # 035M4060V). Polyclonal antibody of GhJAZ2 was generated by the Abmart company (http://www.bioabmart.bioon.com.cn/) using a peptide sequence (C-PKDDALKSSMNKL). Western blotting experiments were performed as previously reported (Hu *et al.*, 2011).

The observation of fiber initiation by SEM

To observe the initiation of fiber cells, bolls were collected at 0 DPA from similar positions in the transgenic and wild-type plants grown in the greenhouse; all ovules were taken from the same positions of the cotton bolls (Sun *et al.*, 2005) and fixed in 2.5% (v/v) glutaraldehyde at 4°C. Ovules cultured *in vitro* for 3 days were also fixed in 2.5% (v/v) glutaraldehyde at 4°C. After dehydration with an ethanol series, the samples were transferred into isoamyl acetate and dried to the critical point. Fiber initiation was observed and photographed with a JSM-6390/LV SEM (Jeol, Tokyo, Japan).

Measurement of fiber length, lint percentage, lint index and seed index

To collect the lint index, seed index and lint percentage data, mature bolls from the same position of the plants were harvested from seven independent T3 transgenic lines and the wild-type control lines grown in the greenhouse in 2013 and 2014, respectively. For each independent transgenic line, at least 30 plants were grown; 10 randomized plants comprised one biological replicate. All mature fibers were collected from the middle part of the cotton plants at the same time for analysis. The fiber lengths of the transgenic lines and ovules that were cultured for 10 days were measured using the method of Tan *et al.* (2013). The lint percentage, lint index and seed index were calculated by recording the lint and seed weight of 100 seeds (with fibers attached). Each line was a biological replicate, and three replicates were performed for the measurement of the fiber length, lint index, seed index and lint percentage.

Extraction and quantification of JA content

The method used for JA extraction was as reported previously (Shindy and Smith, 1975). Samples (100–200 mg) were extracted with 80% cold methanol (v/v) and shaken overnight at 4°C. Each sample was extracted twice to ensure that JA was fully extracted. The aqueous phase was evaporated with N_2 and then dissolved in 0.2 mL of 10% methanol by shaking overnight at 4°C. The samples were stored at −70°C prior to measurement. JA was quantified using an Applied Biosystems 4000Q-TRAR high-performance liquid chromatography-mass spectrometry system (Japan). Each sample had three biological replicates.

Y2H assays

The −2 DPA to 4 DPA ovule/fiber cDNA library for Y2H screening was constructed with the Matchmaker Gold Yeast Two-Hybrid System (Clontech, Cat. no. 630489). The cotton *GhJAZ2* gene was fused with the GAL4 DNA-binding domain in pGBKT7 to ensure that there was no autoactivation and toxicity due to the X-α-Gal assay in yeast; and the GhJAZ2 fusion protein was used as bait to identify interacting proteins. To detect protein–protein interactions between GhJAZ2 and the identified proteins, the full-length *GhGL1*, *GhMYC2*, *GhMYB25-like*, *GhWD40* and *GhJI1* genes were cloned into pGADT7. pGBKT7 fused with *GhJAZ2* was used to transform Y2H, and pGADT7 fused with *GhGL1*, *GhMYC2*, *GhMYB25-like*, *GhWD40* and *GhJI1* was transferred into the Y187 yeast using the Transformation System (Clontech, Cat. no. 630489). Interactions between these transcription factors after mating were determined by growth on SD medium with the -Trp/-Leu/X-α-Gal/AbA assay as described by the manual (Clontech, Cat. no. 630489).

BiFC analysis and transcriptional activation function analysis

Nicotiana benthamiana was grown in the greenhouse for 1 month. Leaf protoplasts were isolated as previously described (Yoo *et al.*, 2007). The CDS of *GhJAZ2* was cloned into pVYNE(R), the full-length *GhMYB25-like*, *GhMYC2*, *GhGL1*, *GhWD40* and *GhJI1* sequences were cloned into pVYCE(R), and the constructs were transformed into *Escherichia coli* (Waadt *et al.*, 2008). The CDS of *GhJAZ2* was cloned into vector None (Ohta *et al.*, 2001), the full-length *GhMYB25-like* was cloned into GAL4DB (Ohta *et al.*, 2001). These plasmids were isolated from *E. coli* using QIAGEN® Plasmid Maxi Kits (Cat. no. 12262) and transferred to *N. benthamiana* protoplasts using the PEG-calcium method (Yoo *et al.*, 2007). The interactions were detected under fluorescence light using a Leica TCS SP2 confocal spectral Microsystems laser-scanning microscope (Leica, Heidelberg, Germany). Dual-Luciferase system was used to detect the LUC and Renilla LUC (Promega, Cat. no. E1910). The primers used for vector construction were listed in Table S1.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Expression level and Southern blotting analysis in transgenic cotton lines.

Figure S2. Response of seedling and ovules to JA *in vitro*.

Figure S3. The phenotype of Arabidopsis transgenic plants over-expressing *GhJAZ2* and the *GhJAZ2* expression level in cotton ovules.

Figure S4. Phylogenetic and expression profile analysis of the cotton JAZ family.

Figure S5. The expression levels of cotton JAZ family members.

Table S1. Primers used in the research.

Table S2. The FPKM values of JAZs family in different tissues in TM-1.

Table S3. The proteins interacted with GhJAZ2.

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