

# *GhGASA10-1* promotes the cell and fiber elongation through the phytohormones IAA-induced

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## Research Article

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# Abstract

**Background:** Cotton is an important cash crop. The fiber length has always been a hot spot, but multi-factor control of fiber quality makes it complex to understand its genetic basis. Previous reports suggested that *OsGASR9* promotes germination, width, and thickness by GAs in rice, while the overexpression of *AtGASA10* lead to a reduction in silique length, which is likely to reduce cell wall expansion. Therefore, this study aimed to explore function of *GhGASA10* in cotton fibers development.

**Results:** To explore the molecular mechanisms underlying fiber elongation regulation concerning *GhGASA10-1*, we revealed an evolutionary basis, gene structure, and expression. Our results emphasized the conservative nature of *GASA* family with its origin in lower fern plants *S. moellendorffii*. *GhGASA10-1* was localized in the cell membrane, which may synthesize and transport secreted protein to the cell wall. Besides, *GhGASA10-1* promoted seedling germination and root extension in transgenic Arabidopsis, indicating that GhGASA10-1 promotes cell elongation. Interestingly, *GhGASA10-1* was upregulated by IAA at fiber elongation stages.

**Conclusion:** We propose that *GhGASA10-1* promotes fiber elongation by regulating the synthesis of cellulose induced by IAA, to lay the foundation for future research on the regulation network of *GASA10-1* in cotton fiber development.

## Background

Upland cotton, with higher yield properties, attributed to great proportion of cotton production worldwide to fulfill the everincreasing demands of taxtile industry. Although upland cotton is attributed to high production, the bottleneck in further improving fiber quality remains the fundamental concern [1]. Therefore, improving the fiber quality of cotton, specifically Upland cotton, is one of the current crucial research dimension in cotton breeding programs. Fiber length is important indicator in assessing the fiber quality [2]. Therefore, exploitation of fiber developmental mechanisms in different cotton species is fundamental to improve cotton cultivars. The mechanisms of fiber development constitute of four distinct yet overlapping phases viz., fiber initiation, fiber elongation, synthesis of cell secondary wall, and fiber maturation [3, 4]. Cotton fibers, generalyy described as single-celled trichomes, are excellent source material to study the single cell elongation [5, 6]. Many studies showed that phytohormones, i.e., IAA, GA3, BR etc., promoted cell and fiber elongation in cotton fiber development [7–9]. Previous research extensively emphasized that the *Gibberellic Acid Stimulated Transcript/Arabidopsis (GAST/GASA)* family genes were regulated by phytohormones to promote cell elongation and cell division in many plants [10].

*Gibberellic Acid Stimulated Transcript (GAST)*-like genes have been involved in complicated biological processes modifying further plant growth and development [11]. In most of the plants, *GASAs* domains are greatly conserved, resulting in conserved proteins [10, 12]. These proteins are generally divided into three well conserved subgroups, however, *OsGASTs* was divided into four subgroups in rice [13]. The encoded protein of *GA-stimulated transcripts (GAST)* family members, usually contains three sections:

the N-terminal signal peptide sequence, the hydrophilic, and C-terminal regions as GASA domain. GASA domains are generally comprised of 60 amino-acids, including 12 cysteine, key residues for the functional domain [14]. Exact subcellular localization of GASTs proteins is the sticking point to determine the protein function. Many *in vivo* studies demonstrated that GASTs proteins were discovered in the cell wall and/or apoplast. However, a few GASTs proteins were localized in plasma membrane, cytoplasm and nucleus. The above mentioned reports implies the divergent functional trends of GASTs, which are likely corresponding to functional association with cell elongation and division [15]. The presence conserved motifs is the main reason for *GASTs* to be conserved in vascular plants. Further, the phylogenetic studies of *GASTs* family genes from plant kingdom showed that *GASTs* might be initially evolved in *S. moellendorffii* [11]. *GAST1*, which is one of *GAST* family genes, initially detected in tomato [16] with subsequent discoveries in different species, such maize (*Zea mays* L.) [10], *Arabidopsis* [11], *Glycine max* [17], Grapevine (*Vitis vinifera* L.) [12].

Previously published reports emphasized the regulatory role of *GAST* family genes in cell elongation and cell division, but also involved in responses towards abiotic and biotic stresses [18–20]. *AtGASA* genes family members have been reported with their regulatory role in hormone synthesis such as ABA, GA, BR, IAA, JA, SA in *Arabidopsis* [15], while the *OsGASR9* regulated grain length, width and thickness in rice [21]. Interestingly, two vertical homologies from *Arabidopsis* (*AtGASA10*) and rice (*OsGAST9*) showed conserved (Functional and structural) positive regulatory role in germination, between dicot and monocot plants. Phytohormones may regulate both *AtGASA10* and *OsGASR9* through the signal element in their promoter regions and correspond to the signal element in likely feedback/response cycles in GA/ABA-mediated regulation [11]. GAs are among the universal plant phytohormones that play crucial role in the growth and development processes including germination, stem elongation, photosynthesis, flowering, and seed development, while *GASA*-like genes possess a key role in GAs signaling pathway [12, 22, 23] in different plants viz. gerbera [24], and maize [10]. Some *GASA* genes are also regulated by other hormones, as *RS17* was regulated by IAA [25], and *OsGSR1/2* was regulated by GA3 [26].

Reported statistics suggested that different *GASA*-like genes may exhibit differential expression patterns, mostly built on the spatiotemporal pattern of gene expression for regulating their speculated functions [27]. In *A. thaliana*, *AtGASA5* is highly expressed in the shoot tip and the inflorescence meristems of the reproductive stage [27]. Interestingly, *AtGASA6* is the key node in GA, ABA, and glucose signal interaction network, which is involved in the regulation of GA, ABA, and glucose in seed germination mesocotyl elongation [28]. The expression analysis in *Moso bamboo* revealed that most *PhGASTs* might be related to *M. bamboo* flower development and shoot growth. Importantly, *PhGASR1* was presumed to act a key role in rapid shoot growth involving in the ABA pathway [29]. However, the expression of *AtGASA10* was not significantly influenced by exogenous GA treatment of suspension cells in *Arabidopsis* [30].

However, the factors that the mechanism of exogenous plant phytohormones IAA and GA3 promoted in cotton fiber cells remain unknown. Given the important roles of *GASA* family proteins by exogenous plant phytohormones IAA and GA3 promoted in plant development and cell elongations, the analysis of *GASA* family is greatly valuable. Our study systematically aimed to uncover transcriptomic landscape of *GASA*

genes in fiber development of *G. arboretum*, *G. barbadense*, *G. hirsutum*, specifically *AtGASA10* and its vertical homologous genes, by utilizing RNA-Seq and qRT-PCR expression profiles. Furthermore, we exploited specific feature of GASA gene family including gene structures, conserved motifs, tissues specific expression and subcellular localization, expression patterns, overexpression of *Arabidopsis* as well as ovule culture in vitro. Using IAA, GA3 and their transport inhibitors, we demonstrated that *GhGASA10* plays a vital role promoted cell elongations in the overexpression of *Arabidopsis* and cotton fibers.

## Results

### Identification, phylogenetic analysis of the GASA gene family in the plant kingdom

According to the results of BLASTP and Query Sequence Searching of TBtools, the GASA protein sequences from different plants were collected and revised the correctness. To study the evolutionary relationships of GASA genes, the protein sequences, including *Arabidopsis*, *G. darwinii*, *G. mustelinum*, *G. tomentosum*, *G. raimondii*, *G. arboretum*, *G. barbadense*, *G. hirsutum* were exploited using phylogenetic tree (Fig. 1A). GASA proteins have a quite conservative in phylogenetic relationships between *Arabidopsis* and *Gossypium*, and further classified into three subfamilies viz. *GASA1/2/3/9/11/14*, *GASA4/5/6/12/13*, and *GASA7/8/8L/10*. The vertical homologous gene corresponding to each *AtGASA* gene could be found in different *Gossypium* species, and most GASA genes did not have doubling occurred in diploid cotton compared to *Arabidopsis* such as *GASA1/2/3/4/5/6/9/14/12*, and occurred doubled such as *GASA7/8/8L/10/11/13*. These results showed that the *GASA7/8/8L/10/11/13* might act as a key role for cottons. Allotetraploid cotton should have twice the number of diploid cotton genes, but the number of GASA genes were less than twice. This result showed that during the evolution process some GASA genes were lost, which is inline with the previously published statistics demonstrating higher gene losses in allotetraploid cotton as compared to diploid cotton [31]. Subsequently, GASA genes were explored in 20 species, extending from lower plants to higher plants, to make certain the origin and evolutionary relationship of these genes (Fig. 1B). Based on the genes number analysis, GASA genes were present in lower fern plants *Selaginella moellendorffii*, but not in the lower algae plants *Micromonas pusilla*, *Ostreococcus tauri*, and *Volvox carteri* and moss *Physcomitrella patens*, indicating that the GASA genes might have originated in fern. From the origin of ferns to angiosperms, the number of GASA genes has hardly changed in diploid plants. This result indicated that the number of GASA family gene are conserved in most plants.

### Structural characterizations of GhGASA genes and motif and analyses

To exploit the evolutionary relationships *GhGASA* family genes along with their structure and function, an unrooted phylogenetic tree was constructed utilizing GhGASA protein sequences. In general, *GhGASA* genes possessed one to three exons. Furthermore, genes structure and phylogenetic relationship displayed highly correlation. In total, 20 conserved motifs were identified in *GhGASA* protein sequence (Fig. S1). The number of conserved motifs in each GhGASA varied from 3 to 11. Most GASA proteins

contain the conserved Motif 1–3, showing that the three Motif of *GASA* proteins may have important role for the functional conservative.

To study the evolutionary relationships and functional divergence of the prominent *GASA* gene-family members, we extracted and examined the upstream 2.0 kb promoter regions. Many cis-acting regulatory elements, including 13 elements related to plant growth (including, Photo-responsive, cell cycle, and seed-specific reulation) and stress responses (including, hormone-response, wound-response, and defense response towards stresses), were analyzed (Fig. 2B).

We mainly focused on cis-acting regulatory elements to verify gene functions concerning cotton fiber development. *GhGASA* genes promoter active elements and cotton transcriptomic data revealed that fiber development might not be linked to the cell cycle regulation, and seed-specific regulation, it might be linked to hormone-responsive elements such as IAA, GAs (Fig. 2A). It might be suggested that IAA and GAs act important roles in fiber development and cell elongation.

### **RNA-seq expression profile of *GASA* genes in three major cotton species**

Expression profile and tissue specificity were explored using transcriptomic data of *G. arboreum*, *G. barbadense*, *G. hirsutum*. Firstly, we constructed phylogenetic tree of *GASA* family genes of three major cotton species using the relative expression profiles with TBtools. The majority of *GASA* family-genes from the same subfamily had similar expression patterns in six varieties in three major cotton species (Fig. 3, Fig. S2, and Fig. S3).

In *G. hirsutum* (Fig. 3), only four genes are highly expressed at fiber development stages. Gh.A09G018000 and Gh.D04G053600 depicted relatively high expression at the early stages of fiber development. However, the Gh.A04G144000 and Gh.D04G1827 were highly expressed throughout whole fiber development stages, especially the critical period of fiber elongation for 5–15 DPA.

In *G. arboreum* (Fig. S2), the study found that the Ga14G0224.1 significantly higher expression level in all the tissues. While Ga07G1350.1 was only high expressed in leaves, and Ga04G0326.1 was only highly expressed in different fiber development stages, especially during the critical period of fiber elongation.

In *G. barbadense* (Fig. S3), only three genes were highly expressed at crucial fiber development stages; especially two genes viz. Gbar.D04G017490 and Gbar.A04G012790 showed significant expression levels at the critical period for fiber elongation (10-DPA).

Interestingly, the five genes Ga04G0326.1, Gbar.D04G017490, Gbar.A04G012790, Gh.A04G144000, and Gh.D04G182700 were all vertical homologous of *AtGASA10* in three cotton species. Regardless of the evolutionary relationship, gene structure, and expression changes were consistent among the six varieties of *G. arboreum*, *G. barbadense*, *G. hirsutum*. These results emphasized that higher expression and tissue specificity of these genes viz. Ga04G0326.1, Gbar.D04G017490, Gbar.A04G012790, Gh.A04G144000, and Gh.D04G182700 might play a direct critical role in fiber development and fiber cell elongation.

In this study, we identified five *GASAs* genes sited at A04/D04, they were explicitly expressed at critical fiber development stages in three major cotton species, which emphasized that the *GASA10* might have a vital role in fiber development, specifically fiber cell elongation. To understand the genetic basis, characteristics, and functions of *GhGASA10-1* (Gh.D04G182700), we further performed functional verification.

### **Subcellular localization of *GhGASA10-1***

According to the online tool analysis, TMHMM2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) predicted that GhGASA10-1 has the 26 N-term signal sequence with transmembrane and the other sequence outside the membrane. The CELLO version 2 [32] and Euk-mPLoc 2.0 [33] predicted that the subcellular localization of *GhGASA10-1* is extracellular. YLoc [34] and BaCelLo [35] predicted that localization of *GhGASA10-1* is a secreted pathway.

To verify this prediction, the full-length CDS of *GhGASA10-1* was ligated with 35S-1300-GFP vector. The constructed vector was infiltrated into *N. benthamiana* mature leaves and visualized by confocal microscopy. The fluorescence of 35S-GFP was detected in nucleus and the cytomembrance (Fig. 4). In contrast, the GhGASA10-1::GFP fusion protein was localized in cell membrane appearing green, and cell membrane presented red fluorescence stained by cell membrane marker Dil. Subsequently, the cell membrane was merged into yellow by both GhGASA10-1::GFP fusion protein and cell membrane marker Dil. The above results demonstrated that *GhGASA10-1* was localized in the cell membrane, which may synthesize secreted protein transport to the cell wall involved in cell wall synthesis and promote cotton fibers cell wall development through the secreted pathway.

### **Overexpression and tissues specificity analysis of *GhGASA10-1* in *Arabidopsis***

To further confirm the gene function, *GhGASA10-1* was overexpressed in *Arabidopsis*. Among 10 lines *GhGASA10-1*-overexpressing transgenic *Arabidopsis* of T3 generation, 3 lines were selected for further analysis. Tissue specificity expression analysis (Fig. 5A) showed that *GhGASA10-1* is explicitly expressed in the roots at the seedling stage. However, *GhGASA10-1* is significantly down-regulated in the roots and specifically expressed in the flower buds at the flowering stage.

When grown on 1/2 MS medium, wild-type and 3 transgenic lines exhibited a noticeable phenotypic difference in *Arabidopsis* seedling germination stages. The seedling germination after 14 days of standard cultivation (Fig. 5B), *GhGASA10-1*-overexpressed seedlings germinated vigorously than wild type seedlings. Statistics on seeds germination rate showed that transgenic seeds' germination rate was significantly higher than the wild type, especially on the third day (Fig. 5C). The length of the taproot after 14 days of vertical cultivation (Fig. 5D), *GhGASA10-1*-overexpressed seedlings formed more main roots than wild type seedlings. The seed from wild type and transgenic plants viz., OE1, OE2, OE3 were germinated on MS-agar medium to further verify the transgenity. The results comprehend the trasgenity, as shown in Fig. 5D, the root growth was observed with significantly higher expression in the transgenic lines than in the wild-type plants. Biological statistics (Fig. 5E) showed that the length of the root of

transgenic lines was twice than wild-type of the length of *Arabidopsis* seedling stages. These results showed that *GhGASA10-1* promotes seedling germination and root extension in *Arabidopsis*.

As mentioned above *GhGASA10-1* was screened as a putative candidate gene for fiber cell elongation. However, the tissue specificity of this gene in *Arabidopsis* suggested marked changes in root elongation. To validate our hypothesis that *GhGASA10-1* play a crucial role in cell elongation, we further examined the roots of *Arabidopsis* and compared both the transgenic and wild type. Interestingly, we observed that overexpression of *GhGASA10-1* extensively promotes root length with the elongation of root cells instead of an increase in the number of cells (Fig. 5F/G). These results strengthen our hypothesis that *GhGASA10-1* may be important in fiber cell elongation.

### ***GhGASA10-1* expression level associates with cellulose synthesis**

As the over-expression of *GhGASA10-1* in *Arabidopsis* promote cell elongation resulting in root elongations, we speculated that *GhGASA10-1* might promote downstream transcription factors, leading to high expression of cellulose synthase genes, further promoting cell elongation. Comparing the expression of cellulose synthase genes (*AtCesAs*) (Fig. 6) in wild-type and overexpressing *Arabidopsis*, five members of the *AtCesAs* family were found to be upregulated. Among them, *AtCesA5b/9* were upregulated twice, while *AtCesA4/7* were upregulated three times, and *AtCesA10* was upregulated more than ten times. Taken together, this data provides strong evidence that over-expression of *GhGASA10-1* strongly induced cellulose synthesis associated genes and promote cell elongation.

### ***GhGASA10-1* induced by IAA but not GA<sub>3</sub>**

It has been shown that *GASA* family genes being involved in the regulation of phytohormones in different plants and act as binding promoter element in upland cotton. *IAA* or *GA3/IAA* might regulate *GhGASA10-1* or *GA3* might regulate *GhGASA10-1*, so we take hormone-treated ovule in vitro culture to verify *GhGASA10-1* expression level. Surprisingly, qRT-PCR (Fig. 7) results showed that *GhGASA10-1* was upregulated during crucial fiber elongation stages. Moreover, *GhGASA10-1* was regulated by IAA, but not GA3 (Fig.S4) during cotton fiber development when treated with different concentrations of hormone IAA, GA3 and their inhibitors in cotton ovule.

## **Discussions**

The whole-genome sequences (WGS) of different cotton species have been accomplished in recent years, which has boosted the research on genetic breeding and functional genes discovery of cotton [36, 37]. Moreover, cotton fibers are single-celled trichomes, and are excellent model materials for studying single-celled elongations [38]. Some studies showed that phytohormones IAA, GA3, BR etc., promoted cell elongation in cotton fiber development [39], and reported that the *GASA* family genes regulated by different phytohormones to promote or inhibit cell elongation and cell division as well as other function in many plants [12]. Therefore, it is particularly important to exploit and understand the functional

mechanism of some crucial genes of the *GASA* family regulating fiber cell elongations for different fiber development stages in cotton.

In this study, we identified *GASA* genes in seven representative cotton species, including three wild allotetraploid cotton *G. mustelinum*, *G. darwinii*, *G. tomentosum* with 48, 45, 47 *GASA* genes respectively, two cultivated allotetraploid cotton *G. hirsutum*, *G. barbadense* with 45, 41 *GASA* genes respectively, and its two diploid ancestors, *G. arboreum* and *G. raimondii* with 24, 25 *GASA* genes respectively. This differential distribution of *GASA* family genes emphasized the loss of genes in different allotetraploid cotton species, consistent with the higher rate of gene loss in different allotetraploid cotton than in diploid species [15, 40]. The *GASA* proteins are quite conservative in higher plants, mainly divided into three subgroups in *Arabidopsis* and *Gossypium*, which is consistent with *GASA* family of *Zea mays* [10], *Oryza sativa* [11, 21], *Vitis vinifera* [12], and *Glycine max* [17]. *GASA* genes are present in lower fern plants *S. moellendorffii* [11]. In the life tree, the number of *GASA* genes have hardly changed from ferns to angiosperms, firstly reported in our study. The above results showed that *GASA* family genes are relatively conservative in gene structure and quantity [10, 12].

Promoter region elements and expression profile of *GhGASA* family genes in cotton were analyzed, and fiber specific expression of *GASA10* member in different cotton species. The results emphasized the involvement of phytohormones viz. GA3 and IAA in promoting cell wall and fiber elongation. Most of the *GASA* genes regulated by GA, ABA, SA [11] are involved in the hormonal signaling pathway in different plants, specifically influencing many plant functions such as bud dormancy, bud germination, root length, stem elongation, seed size, and yield [12, 36, 41, 42].

According to the expression profile of *GASA* genes in three major cotton species, different *GASA* family genes showed tissue-specific expression such as *GhGASA1/2* was highly expressed in leaves of both cultivars, whereas *GhGASA10* showed high expression in the fruit and seed of both cultivars. *GASA* genes have tissue-specific expression; that is also consistent with the previous report suggesting flower-tissue-specific expression of *CcGASA4*, *OsGASR1/9* genes [21, 23, 42]. Numerous studies have found that the function of *GASA* genes not only promotes cell elongation and other tissue development, but also resists various abiotic stresses i.e., salinity, drought, cold, fungal, and paclobutrazol (PBZ) [29, 43, 44].

*GhGASA10-1* was localized in the cell membrane, which may synthesize secreted protein transform into the cell wall involved in cell wall compound for fiber cell elongations. *Citrus clementina* *CcGASA4* and Rice *OsGASR9* localized to the plasma membrane and nucleus [23], while *Pyrus pyrifolia* *PpyGAST1* localized to the cytoplasm and *AtGASA5* protein localized in the cell wall/extracellular matrix [45]. This study results showed that *GhGASA10-1* might be consistent with the function of *AtGASA10*.

The overexpression of *GhGASA10-1* in *Arabidopsis* was analyzed, which promoted seedling germination. Interestingly, the overexpression of *GhGASA10-1* remarkably promoted the main root extension, and the cellular level of *Arabidopsis* roots length was found that *GhGASA10-1* promotes *Arabidopsis* roots cell elongations, which further indirectly confirmed that *GhGASA10-1* promote cell and fiber elongation of cotton. *AtGASA10* is involved in changing the hydroxyl length facilitating cell wall growth by regulating

cell elongation. The above results showed that the functions of *GhGASA10-1* differ from *AtGASA10* function, and might have different regulation mechanisms, which is crucial for further characterization of *GhGASA10-1* gene for its involvement in fiber cell elongation [30].

Most previous studies have shown that changes in plant organs may be due to the regulation of *CesAs* genes. different *CesAs* genes perform different functions of primary and secondary cell wall synthesis [46]. Our results suggested that *GhGASA10-1* in *Arabidopsis* promotes root elongations, which lead to the hypothesis that *GhGASA10-1* may promote downstream transcription factors, leading to high expression of cellulose synthase and further promoting cell elongation.

Our study found that the expression of *GhGASA10-1* was not promoted by the exogenous phytohormones GA3, which is consistent with the expression of *AtGASA10* being not regulated by GA3 [30]. *OsGASR9* is involved in response to GA in rice [21]. Interestingly, *GhGASA10-1* was upregulated by the exogenous phytohormones IAA; the result showed that IAA might play a crucial role in fiber cell elongation and development, which is further researched for cotton fiber quality main as fiber elongations.

## Conclusions

In this study, the evolutionary relationships of *GASA* gene family were identified in different *Gossypium* species, which were classified with 3 distinct subclasses and quite conservative. *GASA* genes might have originated in lower fern plants *Selaginella moellendorffii*. From the origin of ferns to angiosperms, the number of *GASA* genes has hardly changed. The *GhGASA10-1* were localized in the cell membrane, which may synthesize secreted protein transform into the cell wall involved in cell wall compound for fiber cell elongations. The overexpression of *GhGASA10-1* promote seedling germination in advance and promote *Arabidopsis* roots cell elongations in *Arabidopsis*, further indirectly confirmed that *GhGASA10-1* might promote cell and fiber elongation of cotton. *GhGASA10-1* promote *AtCesA4/5b/7/9/10* in *Arabidopsis*, especially *AtCesA10* being more remarkable, which revealed that *GhGASA10-1* not only promotes primary wall synthesis but also primarily promotes secondary wall synthesis. *GhGASA10-1* was upregulated by IAA, IAA may play a crucial role in fiber cell elongation and development. These results reveal the structural characteristics and expression patterns of *GhGASA* gene family and functional verification of *GhGASA10-1* in fiber elongation and provide crucial information for further regulation mechanism/network of fiber elongation.

## Materials And Methods

### Database search and sequence retrieval

Cotton Functional Genomics Database (CottonFGD) (<https://cottonfgd.org/>) platform was employed to obtain the genomic datasets and protein sequences of different cotton species i.e., *G. arboreum* L, *G. raimondii* Ulbr, *G. hirsutum* L, *G. barbadense* L. The protein sequences of three wild cotton such *G. darwinii*, *G. mustelinum*, *G. tomentosum* were downloaded from NCBI(<https://www.ncbi.nlm.nih.gov/>)

[47]. The protein sequences of *Arabidopsis thaliana* were downloaded from the *Arabidopsis* Information Resource (TAIR) (<https://www.arabidopsis.org/>). The other species protein sequences come from NCBI (<https://www.ncbi.nlm.nih.gov/>). Based on the sequence similarity of the protein domains, GASA protein sequences in different plant species were extracted using TBtools [48].

TBtools, with default parameters [48], was employed to search for various GASA protein sequences where the GASA domain (PF02704), obtained from pfam database (<http://pfam.xfam.org/>) was used as a query sequence. Repeated proteins were redundant, thus deleted and only GASA protein sequences with e-value > 30 were kept and double checked through NCBI-CDD (NCBI conserved domain database, <https://www.ncbi.nlm.nih.gov/cdd>) for further analysis.

### **Phylogenetics, Gene Structure, And Motif Analysis**

Multiple sequence alignments of obtained GASA protein sequences, including *Arabidopsis* and *Gossypium* (as mentioned above), was done using Muscle wrapper in TBtools. Subsequently, IQ-TREE in TBtools was utilized to generate phylogenetic tree with 1000 bootstraps [48]. A bar graph was made by the number of GASA family protein sequences of different cotton species.

Furthermore, The genes structure of *GASA* were analyzed using Gene Structure Shower of TBtools [48]. We also exploited motifs with conserved domains of GASA proteins using MEME (<http://meme-suite.org/tools/meme>) with default parameters. The *GASA* family genes characteristic was visualized and integrated into graphics using TBtools [48].

### **Analysis Of CIS-elements Related To Plant Hormone**

2.0 kb upstream sequences of *GASA* family genes in *G. hirsutum* and *GASA10* genes from other four cotton species *G. arboreum* L, *G. raimondii* Ulbr, *G. hirsutum* L, *G. barbadense*, were extracted by TBtools, and the cis-elements were determined utilizing the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

### **Plant Materials And Growth Conditions**

The three major cotton species of *G. hirsutum* cv. J02-508 and ZRI-015; *G. arboreum* cv. 971 and 972; *G. barbadense* cv. XINHAI133 and MAROAD were grown in Anyang cotton farm. Three different tissues and 0 DPA ovule(day post-anthesis), 3, 5 DPA ovule, and 10, 15, 20, & 25 DPA fibers in *G. hirsutum*, 0, 3, 5 DPA ovule as well as 8 DPA fibers in *G. arboreum*, 0 DPA (Days to post anthesis) ovules as well as 15, 25 DPA (Days to post anthesis) fibers in *G. barbadense* were sent to Biomarker Technologies company for completing transcriptome sequencing.

Col-0, ecotype of *Arabidopsis thaliana*, seeds were put in 4°C for vernalization and later grown on agar-solidified Murashige and Skoog (MS) medium, which were placed in an incubator with a 16h / 8h (light / dark) cycle at 22°C. The seedlings were transplanted in mixed soil (vermiculite: humus = 1:1). the *Agrobacterium tumefaciens* strain (GV3101) with constructed overexpression vector was transformed into *Arabidopsis* plants using the floral dip method [49].

## RNA Extraction And Quantitative PCR

The total RNA of cotton stem tips and leaves were isolated by the RNAprep Pure Plant kit (Tiangen, China). Approximately 1000 ng of RNA were reversely synthesized into cDNA by MonScript RTIII Super Mix with dsDNase (Two-Step) (Monad, China).

The real-time PCR detection system (RT-PCR) utilized ABI 7500 real-time PCR system and ChamQ Universal SYBR qPCR Master Mix (Vazyme, China). The Histone3 gene which express stably in upland cotton was used as an internal control. The relative expression levels of *NCED* genes were calculated by the  $2^{-\Delta\Delta C_T}$  method.

The cotton ubiquitin gene (*Gh\_A10G005800*) and *Arabidopsis*  $\beta$ -actin genes (*actin 2*, *actin 8*) were used as internal references [50, 51]. To further explore *OE-GhGASA10-1* in *Arabidopsis* whether through regulation of cell wall synthesis cellulose synthase genes to promote main root elongation, and related 12 *AtCesAs* primer pairs [46]. The primers used in the quantitative PCR analysis are shown in Table S1. All qRT-PCR experiments were executed for at least 3 biological replicates.

## Subcellular Localization Of Ghgasa10-1

The amplified exonic region of *ChGASA10-1*, using specific primers (Table S1) corresponding *Sma I* and *Kpn I* restriction enzyme sites, was fused to the 5' terminal of the GFP gene and consequently generated *GhGASA10-1*-GFP fusion construct comprising CaMV 35S promoter. The *GhGASA10-1*-GFP vector and positive control (empty vector) were transformed into the *Agrobacterium tumefaciens* strain (GV3101), then transformed into tobacco (*Nicotiana tabacum*) leaves [52]. Leaves of the seedlings were stained with cell membrane CM-Dil (10  $\mu$ M, Sigma-Aldrich) and visualized using a laser confocal microscope (Zeiss LSM710, Germany).

## In Vitro Cotton Ovule Culture And Hormone Treatment

Randomly selected cotton bolls (TM-1) were collected and sterilized in 0.1% (w/v) HgCl<sub>2</sub> and 75% (v/v) ethanol for 15 min and 5 min, respectively. Collected bolls were washed with sterilized distilled water after sterilization. Ovule samples were collected from air dried bolls under sterile conditions. Collected ovules were then cultured in BT medium as control treatment in dark environment at 28–30°C, as previously described by [53]. The ovules were also cultured for hormone treatment assay with different concentrations of GA<sub>3</sub> (1  $\mu$ M, 3  $\mu$ M, 5  $\mu$ M), GA inhibitor (PAC, 5  $\mu$ M), IAA (1 $\mu$ M, 5 $\mu$ M, 10 $\mu$ M), and IAA inhibitor (NPA, 5  $\mu$ M), respectively.

## Abbreviations

*GAST/GASA*: Gibberelic Acid Stimulated Transcript/*Arabidopsis*; DPA: Days post anthesis; CDS: Coding sequence; OE: Over-expression; qRT-PCR: Quantitative real-time PCR; WGS: whole-genome sequences;

CottonFGD: Cotton Functional Genomics Database; FPKM: Fragments per kilobase of exon per million fragments mapped.

## Declarations

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### Authors' contributions

B.C. and Y.S. carried out the experiments and drafted the manuscript. B.C., Z.T., G.F., X.P., Z.P. and H.L. analyzed the data. B.C., X.W. and N.Q. participated in figure preparation. B.C., S.H., X.D., S.S. and Y.M. participated in experimental design and manuscript. M.N. participated in its manuscript revision. S.H. and X.D. conceived the study. All authors read and approved the final manuscript.

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### Availability of data and materials

*G. arboreum* L, *G. raimondii* Ulbr, *G. hirsutum* L, *G. barbadense* L were obtained from the Cotton Functional Genomics Database (CottonFGD) (<https://cottonfgd.org/>). The protein sequences of three wild cotton such *G. darwinii*, *G. mustelinum*, *G. tomentosum* were downloaded from NCBI(<https://www.ncbi.nlm.nih.gov/>). The protein sequences of *Arabidopsis thaliana* were downloaded from the *Arabidopsis* Information Resource (TAIR) (<https://www.arabidopsis.org/>). The other species protein sequences come from NCBI (<https://www.ncbi.nlm.nih.gov/>).

### Ethics approval and consent to participate

The collection of plant materials used in our study complied with institutional and national guidelines. Field studies were conducted in accordance with local legislation.

### Consent for publication

Not applicable

### Competing interests

Conflict of interest the authors declare that they have no conflict of interest.

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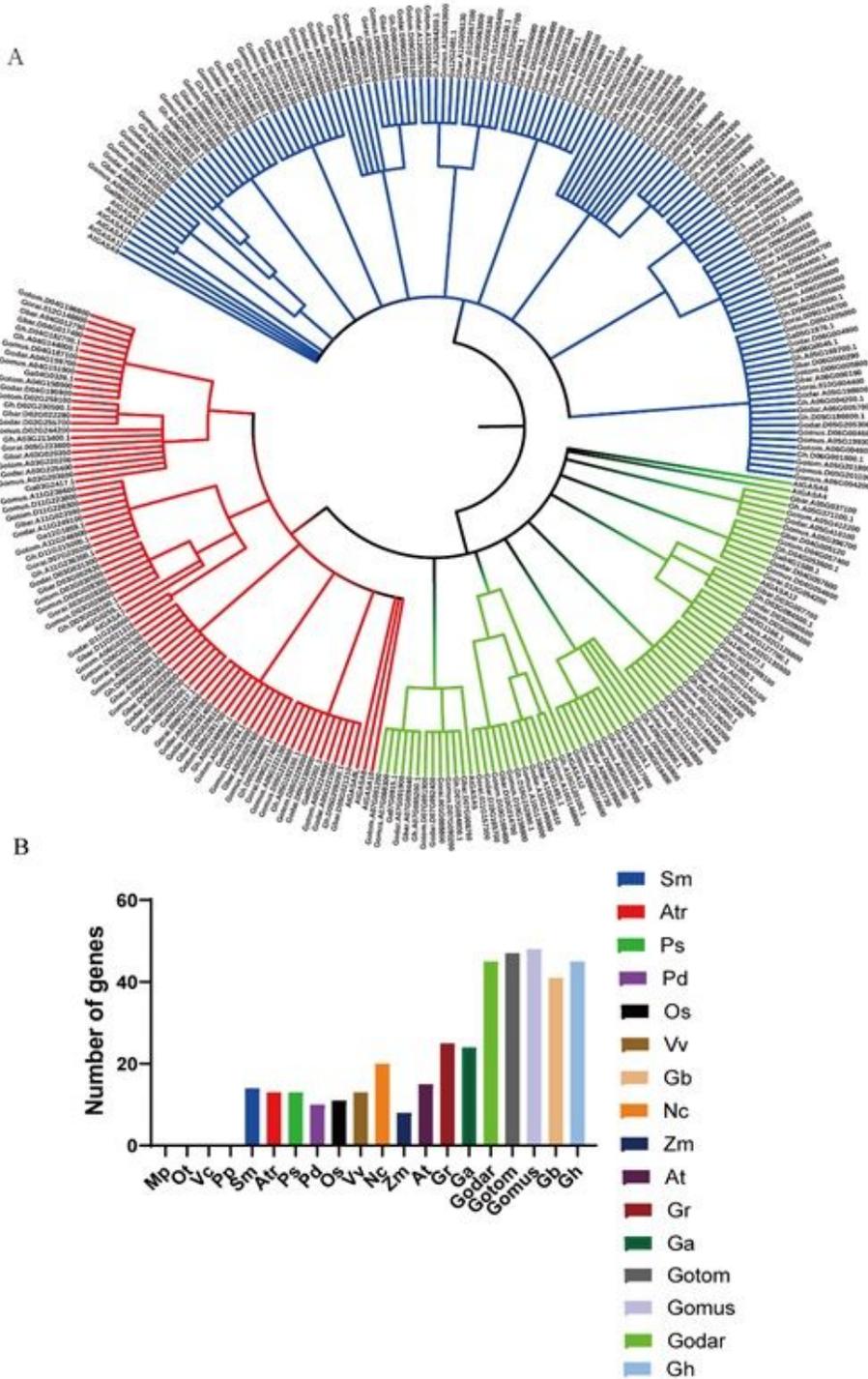
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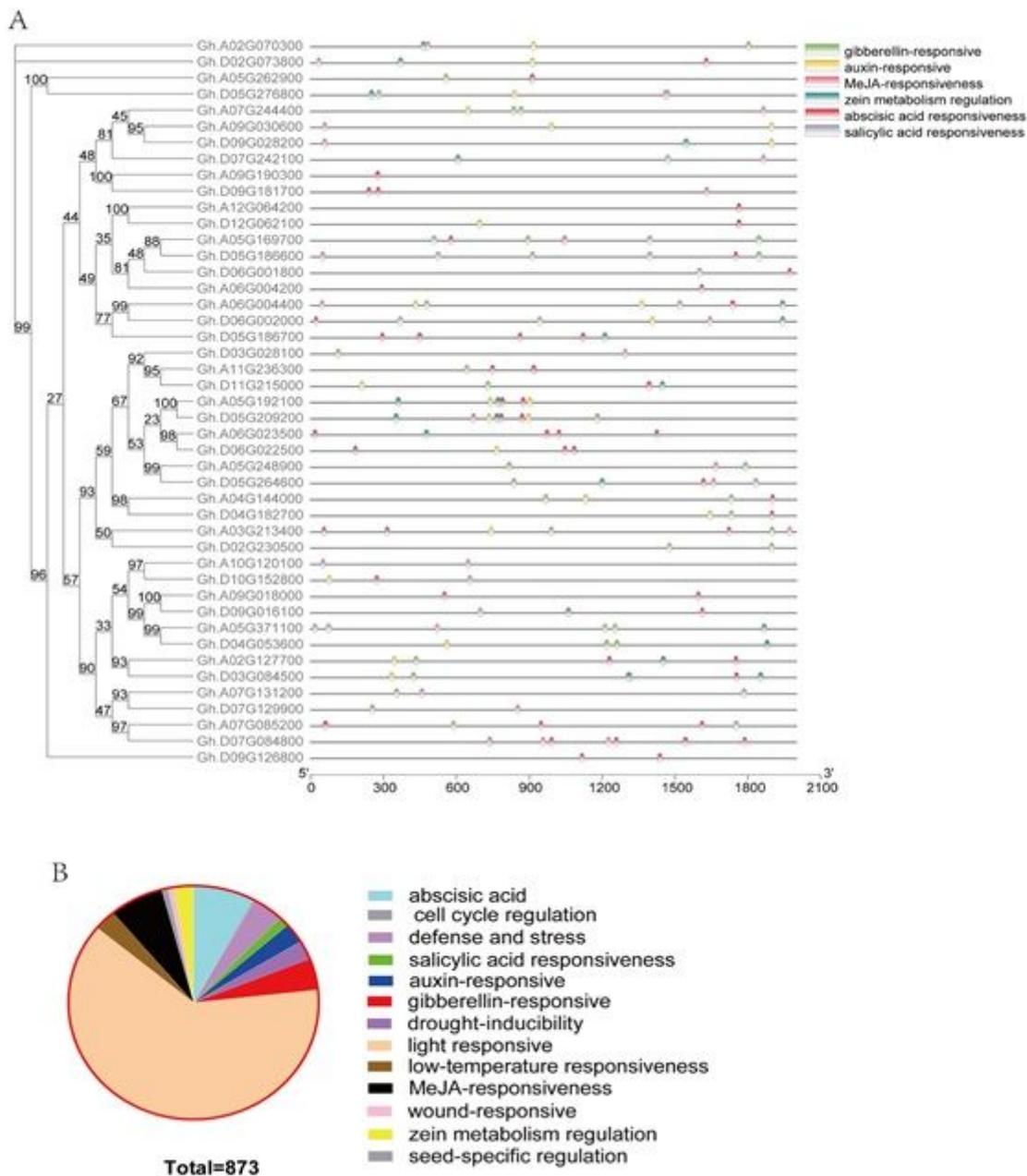
## Figures



**Figure 1**

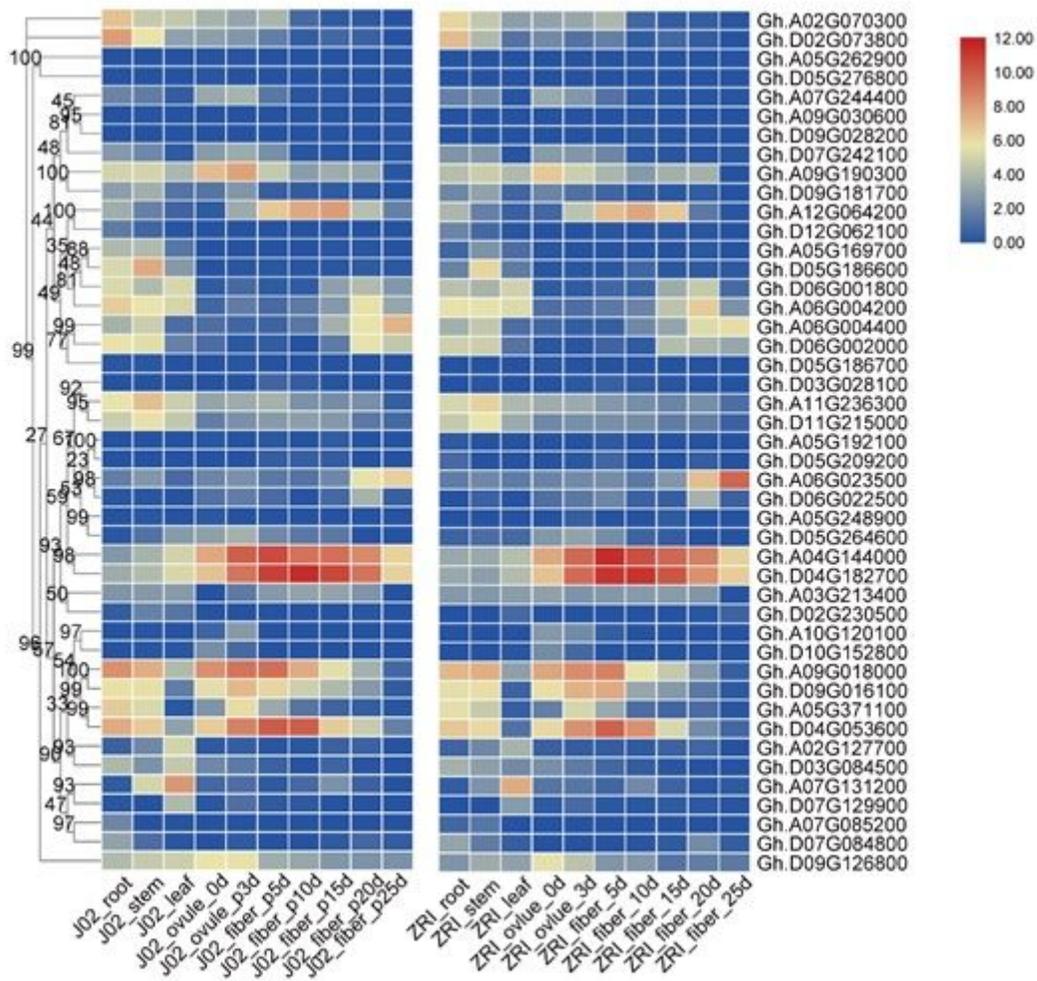
Phylogenetic and evolutionary analysis of the GASA gene family in different plant species. (A) An unrooted phylogenetic tree of GASA protein sequences from *Arabidopsis thaliana*, and seven *Gossypiums*. The phylogenetic tree was constructed using GASA protein sequences and the Neighbor-Joining (NJ) method in MEGA 6.0 software. (B) Comparisons of GASA protein number across a wide range of plant species. Mp, *Micromonas pusilla*; Ot, *Ostreococcus tauri*; Vc, *Volvox carteri*; Pp,

Physcomitrella patens; Sm, Selaginella moellendorffii; Atr, Amborella trichopoda; Ps, Picea sitchensis; Pd, Phoenix dactylifera; Os, Oryza sativa Japonica; Vv, Vitis vinifera; Nc, Nymphaea colorata; Zm, Zostera marina; At, A. thaliana; Gr, G. raimondii; Ga, G. arboretum; Gd, G. darwinii; Gt G. tomentosum; Gm, G. mustelinum; Gb, G. barbadense; Gh, G. hirsutum.



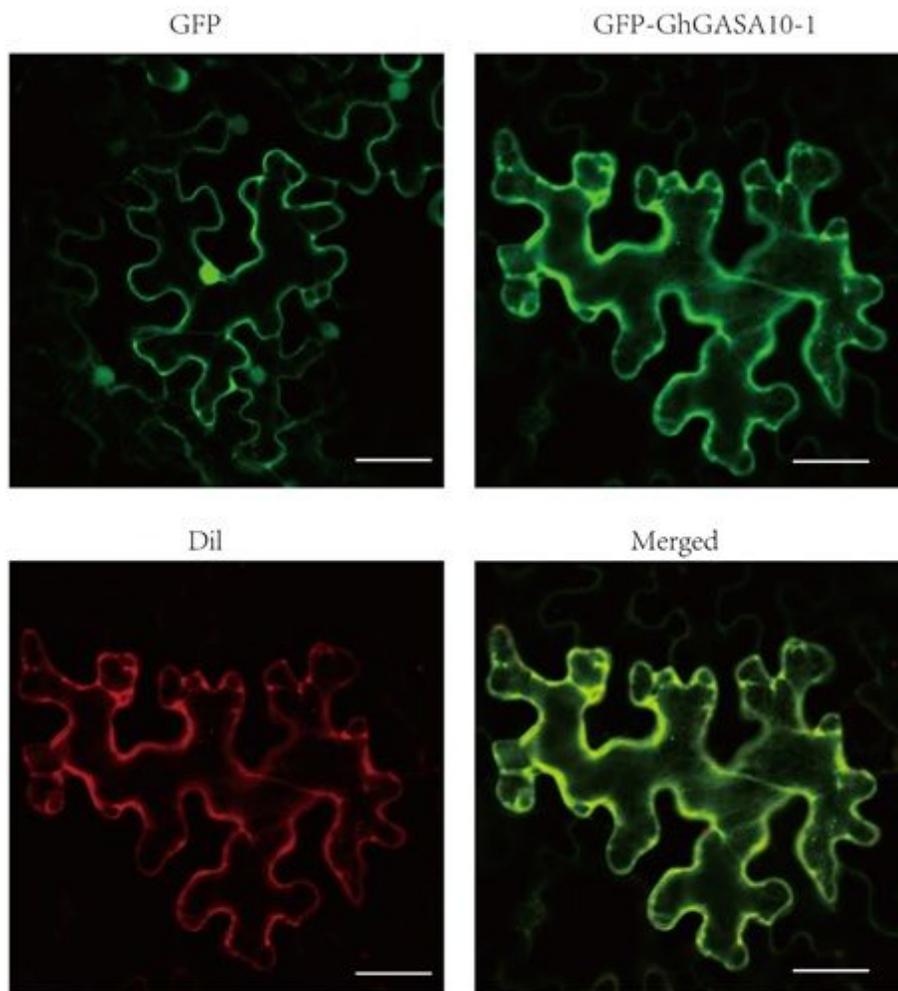
**Figure 2**

Promoter elements analysis of the GASA genes family in upland cotton. (A) The six phytohormones elements in the upstream 2.0 kb promoter regions; (B) The number of cis-acting regulatory elements of GASA genes family promoter sequences in upland cotton, and the sequences analysis were used by online software PlantCare.



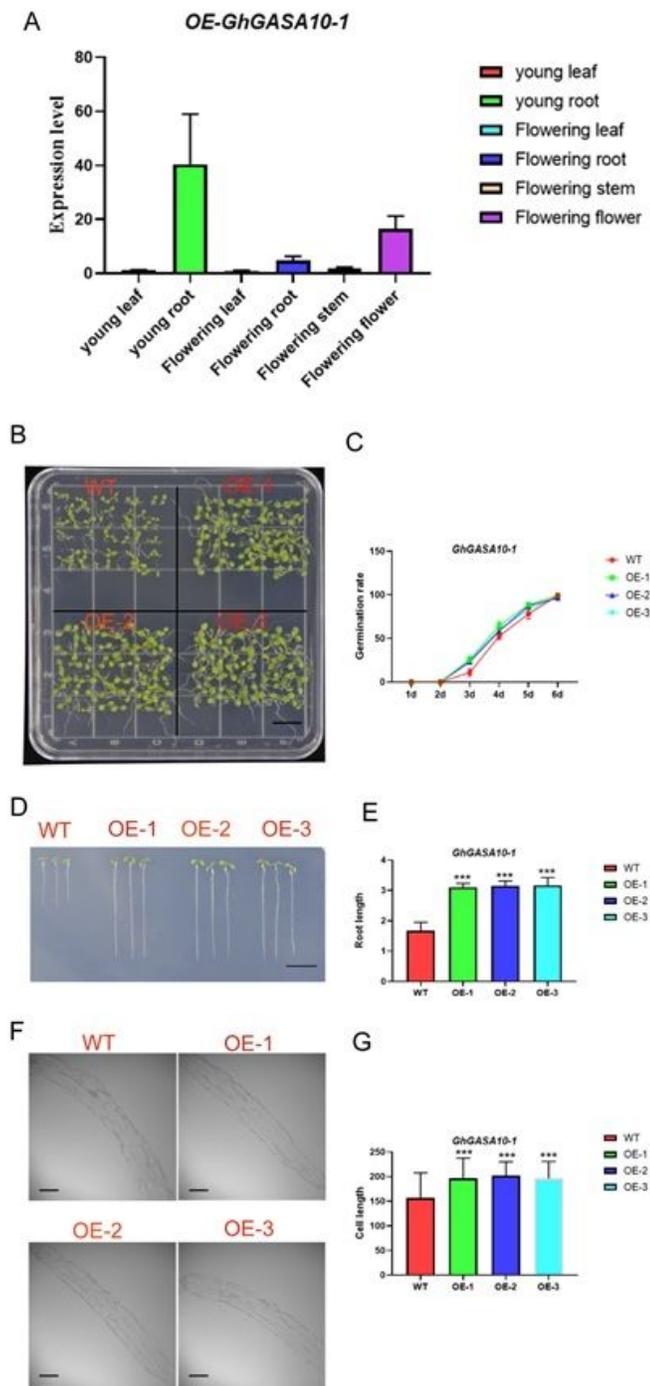
**Figure 3**

Heat map of GhGASA family genes based on RNA-seq data. Root, stem, leaf and different stages (0, 3, 5, 10, 15, 20, 25 DPA) of seed fiber growth in two upland cotton.



**Figure 4**

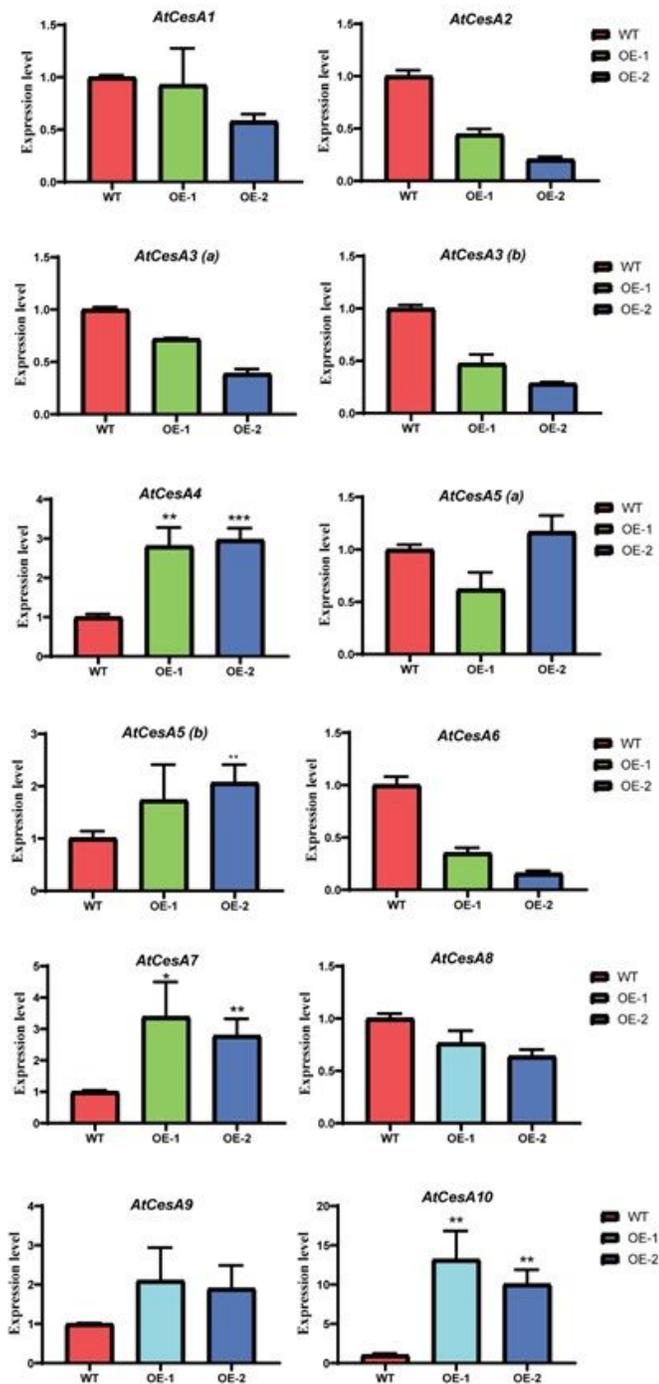
Subcellular localizations of GhGASA10-1 protein in the epidermal cells of tobacco leaf. GFP was green fluorescent protein and as a blank control; GFP-GhGASA10-1 protein was only located in cell membrane; Dil was the most commonly used cell membrane fluorescent probe; Merged was GFP-GhGASA10-1 and Dil collective effect. Bar = 20 μm



**Figure 5**

Various characteristics of overexpressed GhGASA10-1 in Arabidopsis. (A) Different expression characteristics of different organs. The data are shown in (A) as mean  $\pm$  SD,  $n = 3$ ,  $**P < 0.01$ ,  $***P < 0.001$ , Student's t-test. (B/C) Verification and statistics of the germination experiment of wild-type and transgenic Arabidopsis. The data are shown in (C) as mean  $\pm$  SD,  $n = 3$ ,  $**P < 0.01$ ,  $***P < 0.001$ , Student's t-test. (D/E) Comparing and statistics of root lengths wild-type and transgenic Arabidopsis. The data are

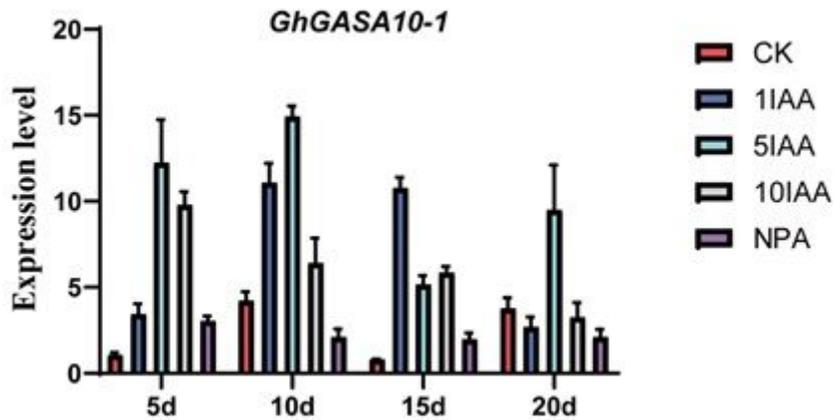
shown in (E) as mean  $\pm$  SD,  $n = 6$ ,  $**P < 0.01$ ,  $***P < 0.001$ , Student's t-test. (F/G) Comparing wild-type and transgenic root lengths at the cellular level. The data are shown in (G) as mean  $\pm$  SD,  $n = 60$ ,  $**P < 0.01$ ,  $***P < 0.001$ , Student's t-test. Bars in (B), (E) = 1 cm. Bars in (F) = 100  $\mu$ m.



**Figure 6**

Over-expression of GhGASA10-1 promote cellulose synthase genes in Arabidopsis. Over-expression of GhGASA10-1 promote cellulose synthase genes to regulate root elongations in Arabidopsis. The data are

shown as mean  $\pm$  SD,  $n = 3$ ,  $**P < 0.01$ ,  $***P < 0.001$ , Student's t-test. ACTIN was used as internal control.



**Figure 7**

IAA induce the expression of GhGASA10-1 promote cotton fiber elongations. Different concentrations of (1 $\mu$ mol, 5 $\mu$ mol, 10 $\mu$ mol) IAA and their inhibitor 10 $\mu$ mol NPA treatment ovules on the day of flowering, respectively. Relative expression of GhGASA10-1 in different periods (5 d, 10 d, 15 d, 20 d) of *G. hirsutum*. The data are shown as mean  $\pm$  SD,  $n = 60$ ,  $**P < 0.01$ ,  $***P < 0.001$ , Student's t-test. UBQ7 was used as internal control.

## Supplementary Files

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- [TableS1.xlsx](#)