GhAIL6, a Member of the AP2 Family Gene, Promotes Somatic Embryogenesis in Cotton.

Congcong Ma  
Zhengzhou University

Yilin Li  
Zhengzhou University

Xiaorui Zhang  
Zhengzhou University

Dan Ma  
Chinese Academy of Agricultural Sciences Cotton Research Institute

Ruibin Sun  
Chinese Academy of Agricultural Sciences Cotton Research Institute

Chuanliang Liu (liuchuanliang@caas.cn)  
Chinese Academy of Agricultural Sciences Cotton Research Institute

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Abstract

Background  Somatic embryogenesis (SE) is the process by which plant somatic cells are cultured in vitro without fertilization to regenerate embryos and develop into intact plants, the difficulty of cotton regeneration has severely limited functional gene research and transgenic breeding. The AP2 family is a relatively large family of transcription factor genes that regulate the process of growth and development, but the role of Aintegumenta-Like6 (AIL6) in cotton SE has not been reported.

Methods  The 35S::AIL6:GR vector was constructed and transformed into cotton JH713 by Agrobacterium-mediated method, after 3 years of self-breeding, stable genetic T3 generation positive plants were obtained, identified by Southern, and three lines were selected for the following regeneration experiments.

Results  The results showed that overexpression of GhAIL6 significantly inhibited the proliferation of callus during the first 30 days, and promoted the embryogenic callus production at about 45 days.

Conclusion  Our results indicated that GhAIL6 was a key regulator of cotton SE, overexpression of GhAIL6 helped to improve the regeneration efficiency of cotton SE

Introduction

Cotton is an important cash crop, providing important cotton fiber raw materials for the textile industry, at the same time, the seeds can be used to extract nutrient-rich edible cottonseed oil. Researchers have carried out extensive research on improving cotton yield, fiber quality, resistance to stress, disease and insect pests, both functional gene research and transgenic breeding of cotton cannot be separated from a good genetic transformation system, at present, the main method is to use agrobacterium-mediated method to transfer foreign genes, and then through tissue culture to complete the positive tissue screening and plant regeneration[1, 2]. However, its somatic embryo regeneration is restricted by genotype, which severely restricts functional gene research and transgenic breeding of cotton. Although the cotton genetic transformation system with Coker312, Zhong24, JH713, JH321 and JIN668 as receptors has been established at home and abroad[3], however, most cotton varieties with good agronomic traits are still difficult to regenerate by tissue culture. Therefore, an efficient cotton genetic transformation system was established by transgenic technology in this experiment.

AIL6 gene is a member of the plant AP2 transcription factor gene family, which is a key regulator of totipotency of plant cells, it plays a core and important role in embryogenesis, differentiation of stem cells, maintenance of meristem status and organ development[4, 5]. In many plants, AIL6 overexpression or heterologous expression can induce somatic embryo regeneration[6]. We explore the role of AIL6 gene in the regeneration of cotton somatic embryos, which will help improve the efficiency of cotton genetic transformation and greatly promote the research of cotton functional genes and genetic engineering breeding. There are eight members in the AP2 gene family in Arabidopsis, namely AINTEGUMENTA(ANT) [7, 8], AIL1[9, 10], PLETHORA1(PLT1), PLT2, AIL6[11], AIL2/PLT4/BBM,
The family genes are expressed in root tip, shoot tip and flower meristem, and they establish and maintain tissue meristem characteristics in a functionally redundant way and regulate organ initial development and growth, research has shown that $AIL2/BBM$, $AIL5/PLT5$, $PLT1$ and $PLT2$ genes are the main regulatory factors in embryonic initiation and early development, and also one of the most critical totipotence regulatory factors in plant cells, these genes play a synergistic regulatory role in the whole process of embryogenesis, including the acquisition and maintenance of embryogenesis at the early stage, and the maturation of embryos at the later stage, it is at the center of integration of hormone signals and plant development. $AIL$ transcription factors interact with auxin and some transcription factor genes such as $LEC1$, $ABI3$, $FUS3$ and $LEC2$, forming a complex gene regulatory network, recent studies suggest that $AIL2/BBM$ transcription factors induce somatic embryogenesis by activating the LAFL regulatory network, which may be upstream of many other embryogenesis and cell pluripotency related regulatory genes; it was found that the loss of function of these genes in $LEC1$, $LEC2$, $FUS3$ and $AGL15$ significantly reduced or even disappeared the ability of $AIL$ overexpression to induce somatic embryo regeneration. This family genes, have become one of the most powerful tools to promote somatic embryogenesis and plant regeneration, and have been applied in the genetic transformation of moss, maize, sorghum, coffee, coconut, camphor tree and other plants, these genes improve the efficiency of agrobacterium-mediated transgenic regeneration, have high application value. In poplars, FRT/FLP recombination system induced by heat shock is used to establish a highly efficient genetic transformation system that overexpressed $AtAIL2/BBM$ gene in Arabidopsis thaliana at the early stage of somatic embryo regeneration and then removed exogenous $AtAIL2/BBM$ gene to obtain normal transformed plants. For pepper with difficult regeneration, fusion of $BnAIL2/BBM$ and GR ligand, the instantaneous activation method effectively solves the problem of somatic embryo regeneration. DuPont Pioneer Company use a specific promoter to drive the $AIL2/BBM$ and $WUS$ genes to co-transform maize immature embryos, and cut the corn conversion cycle by half.

**Materials And Methods**

**Plant materials, explant pre-culture**

Cotton materials: $JH713$, $Zhong12$, $Zhong24$, $TM-1$, $GhAIL6$ transgenic plants, these materials were all from the Cotton Research Institute of the Chinese Academy of Agricultural Sciences.

Seeds of wild type $JH713$ were used in the genetic transformation process, and seeds of $35S::GhAIL6:GR$ positive plants were used in the cotton SE experiment. The sterilized seeds were cultured in sterile seedling medium (10×MS bulk element mother liquor; $100\text{ml}/L$; Sucrose; $15\text{g}/L$; Agar; $6\text{g}/L$) for 7 days. Hypocotyls of the 7-day growth hypocotyl cut into 5-7mm sections as explants were placed on solid co-culture medium (M519, $4.4\text{g}/L$; $1\text{g}/L$ IAA, $0.1\text{ml}/L$; $1\text{g}/L$ 2,4-D, $0.1\text{ml}/L$; $1\text{g}/L$ KT, $0.1\text{ml}/L$; Glucose, $30\text{g}/L$; Plant gel, $2.5\text{g}/L$) [45], the explants pre-cultured at $23^\circ\text{C}$ under dim light of 1500 lux for 1-2 days, then, the culture was under natural light.
Agrobacterium, vectors and evolutionary analysis

The full-length *GhAIL6* was obtained from cDNA amplification of *JH713* cotton ECs, the primers used are: *GhAIL6-F* and *GhAIL6-R* (Table 1). The coding sequence (CDS) was inserted into the vector pCAMBIA2300-GR to construct the vector 35S::GhAIL6:GR by Gateway Technology (Invitrogen, Carlsbad, USA) for overexpression, the primers used are: *GhAIL6-GR-F* and *GhAIL6-GR-R* (Table 1). Escherichia coli Fast-T1, used for thermal transformation. The 35S::GhAIL6:GR vectors were transformed into *JH713* plants via *Agrobacterium* (LBA4404) [34]. *JH713* cotton ECs was from Functional Genes Research Group, the Cotton Research Institute of the Chinese Academy of Agricultural Sciences. The amino acid sequence alignments and phylogenetic relationship were analyzed with the Clustal X and MEGA6 software, respectively. Based on the full-length amino acid sequence of the AP2 family protein identified in upland cotton, and the corresponding gene family members in *Arabidopsis* as a reference, a phylogenetic tree was constructed using the maximum likelihood method.

Genetic transformation and cotton SE experiments

The genetic transformation of *GhAIL6* mainly includes 11 steps. Culture of sterile seedlings: flushed with concentrated sulfuric acid followed by disinfection with 3% H2O2, the sterilized seeds were cultured in sterile seedling medium for 7 days; co-culture: hypocotyls of the 7-day growth hypocotyl cut into 5-7mm sections as explants were placed on solid co-culture medium, agrobacterium infection the explants, the explants pre-cultured at 23°C under dim light of 1500 lux for 1-2 days; callus induction: hypocotyls were transferred to induction medium B29 (M519, 4.4g/L; 1g/L IAA, 0.1ml/L; 1g/L 2,4-D, 0.1ml/L; 1g/L KT, 0.1ml/L; Glucose, 30g/L; Plant gel, 2.5g/L, K+, 50µg/mL Cef+, 250µg/mL PH=6.3), callus were induced by dark culture for 20-30 days, followed by light culture for 20-30 days; callus subculture: the induced callus was transferred to the subculture medium DB29 (M519, 4.4g/L; 1g/L IAA, 0.01ml/L; 1g/L 2,4-D, 0.01ml/L; 1g/L KT, 0.01ml/L; Glucose, 30g/L; Plant gel, 2.5g/L, K+, 50µg/mL PH=6.3) for subculture, subculture every 20 days, and the hormone gradually decreased; embry-like induction: the embryoids were transferred into embryoid induction medium (MSC, 6.46g/L; sucrose, 30g/L; Plant gel, 2.5g/L, PH=6.3); the embryoids were transferred to the emergence medium (MSC, 6.46g/L; sucrose, 30g/L; Plant gel, 2.5g/L, PH=6.3); grafting and transplanting.

In contrast to genetic transformation, cotton SE experiments do not require the process of *Agrobacterium* infection. In cotton SE experiment, the experiment was divided into three groups, one group of explants was transgenic positive plants with medium supplemented with dexamethasone (DEX) (experimental group); one group of explants was transgenic positive plants with medium without DEX (CK group); the other group of explants was *JH713* with medium without DEX. The final concentration of dexamethasone was 30 µM [35]. the three groups were counted separately. The experiment had three biological replicates, GR1, CK1, and WT controlled each other, GR3, CK3, WT controlled each other, and GR7, CK7, WT controlled each other.

Callus proliferation rate (CPR) and embryonic differentiation rate (EDR) calculation
Callus Proliferation Rate (CPR): after the sterile seedlings were cultured for 6 days, we cutted the hypocotyls into 6-8 mm and placed them on the induction medium for induction culture. We weighed the explants in each petri dish on 20days and 30days. The callus proliferation rate (CPR) was calculated as follows: the weight of the explants in each petri dish after 20 days or 30 days of culture minus the initial weight before the induction culture, and then divided by the initial weight of the explants before the culture. The specific steps were carried out in accordance with the articles published by predecessors[36].

Embryogenic callus differentiation rate (EDR): continue the induction process, and count the number of explants with embryogenic callus at 45 d, 55 d, 65 d and 85 d of induction culture. The EDR is calculated as follows: the number of embryonic callus or embryonic explants divided by the total number of explants.

RNA extraction, cDNA synthesis and Quantitative Real-time PCR

RNA extraction: The RNA of each tissue (root, stem, leaf, calycle, non-embryogenic callus (NEC), embryogenic callus (EC)) of cotton was extracted by Easy Pure plant RNA kit (Trans Gen, Beijing, China). Checked the integrity of RNA by agarose electrophoresis. Detected the concentration and purity of RNA through Nano Drop 2000.

cDNA synthesis: Reversed transcription of RNA to synthesize the first-strand cDNA by Tran Script® All-in-One First-Strand cDNA Synthesis Super Mix for qPCR kit. The cotton cDNA obtained by reverse transcription was used as a template. Determined the concentration and purity of cDNA by Nano drop 2000 nucleic acid analyzer.

Quantitative Real-time PCR (q-PCR): q-PCR was performed using SYBR Green fluorescent dye method on Roch LIGHtcycler480Ⅲsystem using Ultra SYBR Mix Ture (With ROX) kit (purchased from CWBIO). The results were analyzed using CFX Manager TM1.6 software and Ghhis3 gene as internal reference by relative quantitative method. Snap Gene was used to design the primer sequence of the gene, and the Sangon Biotech company was commissioned to synthesize the primer. all primers used in the vectors construction are listed in Table 1.

Southern blotting

Take 15 µg of good quality transgenic cotton leaves genomic DNA, digest it with HindIII (NEB, USA) at 37 ºC for 48 h, and separate it by electrophoresis on a 0.8% agarose gel, and transfer it to Hybond by the alkaline transfer method. On N+ nylon membrane, and immobilize DNA. The method of probe labeling adopts digoxin labeling (Roche). For specific experimental operation methods, please refer to this article[36].

Results
**GhAIL6 is specifically expressed in cotton somatic embryogenesis**

The open reading frame of *GhAIL6* is 1,473bp long, encoding 491 amino acids, is a protein of AP2 subfamily. Based on the full-length amino acid sequence of the AP2 family protein identified in upland cotton, and the corresponding gene family members in *Arabidopsis* as a reference, a phylogenetic tree was constructed using the maximum likelihood method. According to the ML tree, the AP2 gene family was divided into 4 different groups, all groups contain *Arabidopsis* and cotton, this result showed that AP2 family genes were relatively conserved in the evolution process between *Arabidopsis* and cotton, *GhAIL6* (Gohir.A10G088200) and *AtAIL6* (*AT5G10510*), *AtAIL7* (*AT5G65510*) were homologous (Fig. 1.c), previous studies have reported that *AtAIL6* and *AtAIL7* played a role in *Arabidopsis* SE and *GhAIL6* may have the same function [37].

The results of q-PCR and transcriptome sequencing showed that the expression level of *GhAIL6* in root, stem, leaf, calyx, and non-embryogenic callus was very low, and the expression level in embryogenic callus was the highest (Fig. 1.a). And the expression of *GhAIL6* was significantly up-regulated during the transformation from non-embryogenic callus to embryogenic callus. The expression level of *GhAIL6* is directly proportional to the regeneration efficiency of cotton. AE1 represents *Zhong12* embryogenic callus after 45 days post-induction, AE2 represents *Zhong12* embryogenic callus after 55 days post-induction, AE3 represents *Zhong12* embryogenic callus after 65 days post-induction; AN1 represents *Zhong12* non embryogenic callus after 10 days post-induction, AN2 represents *Zhong12* non embryogenic callus after 20 days post-induction, AN3 represents *Zhong12* non embryogenic callus after 30 days post-induction; BE1 represents *Zhong24* embryogenic callus after 45 days post-induction, BE2 represents *Zhong24* embryogenic callus after 55 days post-induction, BE3 represents *Zhong24* embryogenic callus after 65 days post-induction; BN1 represents *Zhong24* non embryogenic callus after 10 days post-induction, BN2 represents *Zhong24* non embryogenic callus after 20 days post-induction, BN3 represents *Zhong24* non embryogenic callus after 30 days post-induction. To understand the function of *GhAIL6* in cotton (Fig. 1.b). The vector 35S::*GhAIL6*:GR (glucocorticoid receptor) were constructed and transformed into cotton *JH713*. Several single T-DNA insertion lines were identified by Southern blot analysis and selected for further experiment (Fig. 1.d).

**GhAIL6 positively regulates cell fate specification during cotton SE**

We selected three transgenic lines (GR1, GR3, GR7) for further study. The experiment is divided into three groups, the experimental group (explants are transgenic positive materials, the medium is supplemented with DEX), the control group (explants are transgenic positive materials, the medium is not supplemented with DEX), and the WT group (explants are cotton *JH713*, the medium does not add DEX). GR1, CK1 and WT were mutually controlled, GR3, CK3 and WT were mutually controlled, GR7, CK7 and WT were mutually controlled. The GR induction expression system is constructed based on the response
Characteristics of the structure domain of the GR, this structure domain contains 93 amino acids, in the absence of steroid hormones, GR interacts with the heat shock protein HSP90, and the binding of the target gene to GR has no activation activity and is anchored in the cytoplasm, so it cannot enter the nucleus to play a role, but in the presence of the steroid hormone DEX, the interaction between GR and HSP90 is interrupted, and HSP90 is released, so that GR can bind to the target gene and enter the nucleus, in the nucleus, the target gene is activated and expressed[38, 39].

Calluses were induced from 3 transgenic lines on MSB medium in vitro. Seven days post-induction, calluses could be observed at the ends of the CK group explants and the WT group, while only a little expansion with adventitious roots was observed at the ends of the experimental group explants. During the development of SE, the growth was evident for the experimental group on 20 and 30 days post-induction. However, the explant of the CK group showed little difference. The callus proliferation rate (CPR) was then measured among those lines during the dedifferentiation stage. The results showed that overexpression of GhAIL6 inhibited callus proliferation at both ends of the hypocotyls (Fig. 2.a, b, c).

After 45 days of induction culture, overexpression of GhAIL6 promoted embryonic cell differentiation, the EDR of GR1, GR3 and GR7 at 45 days were 12.5%, 12.3% and 14.5%, respectively, while the embryogenic callus of CK1, CK3, CK7 and WT began to appear at 55 days (Fig. 3. a, b).

ABI3 and FUS3 are considered to be marker genes for embryonic cell formation[23], therefore, detecting the expression of embryonic cell formation marker genes during the induction of somatic embryogenesis can identify the process of embryonic cell differentiation. The expression of GhABI3 and GhFUS3 genes was not detected in the hypocotyls at 7 d of induction, and there was higher expression of these three embryonic development-related genes in the embryonic guarantor tissues overexpressing GhAIL6 relative to the control and WT explants. It can be hypothesized that the hypocotyls overexpressing GhAIL6 had a large number of embryonic cell production after 45 d of induction, while the control and WT explants did not see a large number of embryonic cell production after 55 d of induction. Thus, overexpression of GhAIL6 accelerated the formation of embryonic cells during somatic embryogenesis.
Discussion

The results showed that the gene inhibited callus proliferation significantly in the first 30 days of SE, and promoted embryogenic callus production at about 45 days, the underlying mechanism were unclear careful. *In vitro* regeneration of plant organs requires cell dedifferentiation to form callus, then differentiation to form root and stem meristem, and finally root and bud[40], early dedifferentiation and later embryogenic callus proliferation were two different stages of regeneration, and *GhAIL6* may participate in different regulatory mechanisms in these two processes. Some recent studies have also discussed this problem[36], when studying the process of dedifferentiation and re-differentiation of tea tissue, some researchers found the miRNA up-regulated in dedifferentiation phase transition was down regulated in the process of regenerated roots and buds, but less up-regulated; On the contrary, the down-regulated miRNAs in dedifferentiation phase transition are more up-regulated in the process of regenerated roots and buds, and the down-regulated miRNAs account for a small proportion[41]. How *GhAIL6* participates in the two-stage regulation mechanism, we need further follow-up research.

<table>
<thead>
<tr>
<th>Primer number</th>
<th>Primer sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>GhAIL6</em>-F</td>
<td>ATGGCATCAGCGAGTAACTGGC</td>
<td>The CDS sequence of <em>GhAIL6</em> was amplified, and the size was about 1473 bp, Kpn1 / Bamh1 enzyme digestion pBinGFP4 ligation</td>
</tr>
<tr>
<td><em>GhAIL6</em>-R</td>
<td>TTAATAGGATCGGTGAGGCC</td>
<td></td>
</tr>
<tr>
<td><em>GhAIL6</em>-GR-F</td>
<td>CCACGTGACCGGAGTGACGC</td>
<td>35S::GhAIL6:GR vector was constructed, and the amplification size was about 600 bp</td>
</tr>
<tr>
<td><em>GhAIL6</em>-GR-R</td>
<td>CCAGTCAACTGAGTTGACCC</td>
<td></td>
</tr>
<tr>
<td>q-PCR-F</td>
<td>ATGGCATCAGCGAGTAACTGG</td>
<td>q-PCR</td>
</tr>
<tr>
<td>q-PCR-R</td>
<td>TGAATAATGAGGAGAAGCAGCA</td>
<td></td>
</tr>
<tr>
<td><em>GhABI3</em>-F</td>
<td>CCCACACCCATTTCACAGACAGT</td>
<td>q-PCR</td>
</tr>
<tr>
<td><em>GhABI3</em>-R</td>
<td>TGTCTGCAGCGGCTGACTT</td>
<td></td>
</tr>
<tr>
<td><em>GhLEC2</em>-F</td>
<td>GCCTGAAATTCCCATACAAACCA</td>
<td>q-PCR</td>
</tr>
<tr>
<td><em>GhLEC2</em>-R</td>
<td>GCATCTACTTGGTGTTGAAGCCC</td>
<td></td>
</tr>
<tr>
<td><em>GhFUS3</em>-F</td>
<td>AACCAACATCAAACACCTCTCC</td>
<td>q-PCR</td>
</tr>
<tr>
<td><em>GhFUS3</em>-R</td>
<td>GCCTTCAAGCTCCACAGTC</td>
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</table>
Conclusion

In this study, we identified a gene GhAIL6 in somatic embryogenesis in upland cotton. The results of the study show that: in upland cotton, overexpression of GhAIL6 inhibits the dedifferentiation process in the early stage and promotes the growth of embryogenic callus in the later stage. Our research provided some new ideas for improving the regeneration efficiency of upland cotton, and more work is needed for in-depth research in the future.

Declarations

Compliance with Ethical Standards:

Funding This work was supported by the National Science and Technology Major Special Projects China(2016YFD0101006).

Conflict of interest The authors declare that they have no conflict of interest for this study.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

References

8. Mizukami Y, Fischer RL. Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. PLANT BIOLOGY 6


35. Li K (2014) Isolation, characterization and expression analysis of the BABY BOOM (BBM) gene from Larix kaempferi×L. olgensis during adventitious rooting. 8


**Figures**

![Figure 1](image-url)

Figure 1

<p>(a) The expression of *GhAIL6* in different organizations. (b) The expression of *GhAIL6* of in the *Zhong12* non-embryogenic callus, *Zhong12* embryogenic callus, *Zhong24* non-embryogenic callus, and *Zhong24* embryogenic callus. The gene expression level was calculated by comparing the sequenced reads with the upland cotton genome using</p>
Tophat software (Version 2.0.8), and the expression level was expressed by the number of sequencing fragments (FPKM) contained in each thousand transcriptional sequenced bases per million sequenced bases. (c) A phylogenetic tree of <em>Arabidopsis</em> and cotton AP2 gene family, based on the full-length amino acid sequence of the <em>AP2</em> protein, constructed using the maximum likelihood method. (d) Southern blot copy number detection of genetically modified cotton, GR1, GR3 and GR7 represent three different transgenic cotton lines respectively, and M is the marker.

**Figure 2**

Overexpression of <em>GhAIL6</em> inhibits dedifferentiation of somatic embryos in upland cotton during early regeneration. (a) The phenotype of the three groups cultured on the medium for 20 days (scale bars=1cm). (b): The phenotype of the three groups cultured on the medium for 30 days (scale bars=1cm). (c): The callus proliferation rate (CPR) of the three groups at 20 days and 30 days (scale bar = 1 cm). The error line represents the standard deviation of three biological replicates.

**Figure 3**

Overexpression of <em>GhAIL6</em> promotes embryogenic callus production of upland cotton. (a): The phenotypes of the three groups 45 days after induction on the culture medium (scale bars=1cm). (b): The embryonic differentiation rate (EDR) of the three groups 45, 55, 65 and 85 days after induction on the culture medium (scale bar = 1 cm). The error line represents the standard deviation of three biological replicates.

**Figure 4**

q -PCR was used to detect the expression levels of GhFUS3, GhABI3 and GhLEC2 in experimental group, control group and wild-type at 20 d, 30 d and in embryogenic callus. The error line represents the standard deviation of three biological replicates.