

GhAGL15s, preferentially expressed during somatic embryogenesis, promote embryogenic callus formation in cotton (*Gossypium hirsutum* L.)

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Abstract Somatic embryogenesis is a useful tool for gene transfer and propagation of plants. *AGAMOUS-LIKE15* (*AGL15*) promotes somatic embryogenesis in many plant species. In this study, three homologous *AGL15* genes were isolated from *Gossypium hirsutum* L., namely *GhAGL15-1*, *GhAGL15-3*, and *GhAGL15-4*. Their putative proteins contained a highly conserved MADS-box DNA-binding domain and a less conserved K domain. Phylogenetic analysis suggested that the three *GhAGL15s* clustered most closely with *AGL15* proteins in other plants. Subcellular location analyses revealed that three *GhAGL15s* were localized in the nucleus. Furthermore, their expression levels increased following embryogenic callus induction, but sharply decreased during the embryoid stage. *GhAGL15-1* and *GhAGL15-3* were significantly induced by 2,4-D and kinetin, whereas *GhAGL15-4* was only responsive to 2,4-D treatment. Over-expression of the three *GhAGL15s* in cotton callus improved callus quality and significantly increased the embryogenic callus formation rate, while *GhAGL15-4* had the highest positive effect

on the embryogenic callus formation rate (an increase from 38.1 to 65.2 %). These results suggest that over-expression of *GhAGL15s* enhances embryogenic potential of transgenic calli. Therefore, spatiotemporal manipulation of *GhAGL15s* expression may prove valuable in improving cotton transformation efficiency.

Keywords *Gossypium hirsutum* · *AGL15* · Somatic embryogenesis · Expression pattern · Embryogenic callus formation

Introduction

Cotton is an economic crop cultivated for thousands of years. It is the most important textile crop, and is a valuable resource of oil and proteins, second only to soybean (Sunilkumar et al. 2006). Its cultivation and production have a profound effect on the world's economic and political affairs. Conventional breeding has led to substantial progress in improving cotton yield, quality, and disease resistance. However, further progress in conventional cotton breeding is severely constrained by limited cotton germplasm diversity, with many desirable traits not available in currently available germplasms. This has led to the use of gene transfer to introduce genes from other sources for cotton improvement. For example, transgenic varieties containing bacterial genes encoding herbicide resistance and BT endotoxins have been developed and released (Nobre et al. 2001; Perlak et al. 1990). *Agrobacterium*-mediated genetic transformation is the most powerful method for producing transgenic cotton. *Agrobacterium*-mediated cotton transformation depends on the efficient regeneration of transformed cells. However, only a few cotton cultivars are capable of regeneration, with most being recalcitrant to the process

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(Leelavathi et al. 2004). The genotype-dependent response is the principal cause that restricts high-frequency regeneration of cotton via SE (Kumria et al. 2003; Mishra et al. 2003; Ganesan and Jayabalan 2004). The identification of regulatory genes associated with cotton SE will further our understanding of the cotton SE process, and aid in the development of SE potential improvement strategies in cotton.

SE is a developmental process in which somatic cells cultured under appropriate induction conditions *in vitro* develop into embryos capable of regenerating into complete plants. In dicots, the process goes through globular, heart, torpedo, and cotyledon stages; these are analogous to zygotic embryo development (Zimmerman 1993; Schmidt et al. 1997). For example, in carrot callus cells, the SE process closely mimics zygotic embryo development in both spatial and temporal aspects (Ko and Kamada 2002). This developmental potential has subsequently been found in a variety of both dicot and monocot plant species (Quiroz-Figueroa et al. 2006; Mathieu et al. 2006). SE is a valuable tool for regenerating transgenic cells into plants. However, SE potential varies with species and even with genotypes. Additionally, the process is affected by the source and physiological status of the somatic cells. This has led to identification of tissue sources ranging from highly receptive to highly recalcitrant to embryogenesis induction. A plethora of differential gene expressions are associated with embryogenesis switching, including *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (SERK1)* (Schmidt et al. 1997), *LEAFY COTYLEDON (LEC)* (Curaba et al. 2004; Gaj et al. 2005), *FUSCA3 (FUS3)* (Gazzarrini et al. 2004; Curaba et al. 2004), *BBM* (Boutilier et al. 2002), *WUSCHEL (WUS)* (Zuo et al. 2002), and *AGAMOUS-LIKE15 (AGL15)* (Harding et al. 2003). *AGL15* encodes a MADS-domain regulatory protein, and belongs to a family of transcriptional regulatory factors known to play vital roles in diverse plant developmental events including the control of flowering time, homoerotic regulation of floral organogenesis, fruit development, and seed pigmentation (Parenicova et al. 2003). *AGL15* was initially identified as an embryo-expressed gene using differential display of mRNA, and by the characterization of MADS-box genes in *Arabidopsis* (Heck et al. 1995; Rounsley et al. 1995). The gene is preferentially expressed during embryogenesis, with protein accumulation occurring at its highest level in developing embryos. It is also expressed, at lower levels, following completion of germination (Heck et al. 1995; Rounsley et al. 1995; Perry et al. 1996; Fernandez et al. 2000). Constitutively expressed *AGL15* enhances production of secondary embryos from cultured zygotic embryos, and promotes somatic embryo formation (Harding et al. 2003). Likewise, in soybean (*Glycine max*), *GmAGL15* is preferentially expressed in developing embryos, and ectopic expression enhances somatic embryo development (Thakare et al. 2008). This implies that *AGL15* is an ideal

candidate for promoting crop somatic embryo formation. However, information regarding *AGL15* and its roles in cotton SE is lacking.

In this study, three cotton *AGL15*-like MADS-box genes, *GhAGL15-1*, *GhAGL15-3*, and *GhAGL15-4*, were isolated, and their proteins were found to be localized in nucleus. Expression analysis revealed that their transcription levels increased following embryogenic callus induction, but decreased sharply during the embryoid stage. *GhAGL15-1* and *GhAGL15-3* were significantly induced by 2,4-D and KT treatments, while *GhAGL15-4* responded only to 2,4-D treatment. Over-expression of the three *GhAGL15s* in cotton callus, especially *GhAGL15-4*, improved callus quality and increased competency of embryogenic callus formation. Our results indicated that *GhAGL15s* enhanced embryogenic potential of transgenic calli. Therefore, spatiotemporal manipulation of *GhAGL15s* expression has the potential to improve cotton transformation efficiency.

Materials and methods

Plant materials and growth conditions

Callus of CCRI24 was initiated in callus induction medium as previously described (Zhang et al. 2011). After 40 days, calli following a non-embryogenic callus induction program were collected and maintained at -80°C for gene expression analysis. The remaining calli were subcultured on an embryogenic callus induction medium (Zhang et al. 2011). Sample batches were collected at relevant stages, i.e., 7, 14, 21, 22, 23, 24, 25, 26, 27, 42, and 49 days after culture on the embryogenic callus induction medium, and embryogenic callus was periodically collected, and stored at -80°C for subsequent gene expression analyses. The embryogenic callus began to appear on the 21st day. Concurrently, non-embryogenic tissues or organs, including roots at the aseptic seedling stage, stems at the aseptic seedling stage, and leaves, were harvested. These were used to extract total RNA for detection of expression levels using real-time PCR (RT-PCR). For the detection of the three *GhAGL15s* response to auxin and cytokinins, 40-day-old calli were transferred to liquid MS medium with $10\ \mu\text{M}$ 2,4-D or $10\ \mu\text{M}$ KT, with mock treatment used as a control.

RNA extraction and cDNA synthesis

Total RNA was extracted from each sample using an RNA extraction kit (Tianze Gene Co., China) according to the manufacturer's instructions. The concentration and quality of the RNA samples were determined by absorbance at 260 nm using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA), and evaluated on

an agarose gel. First-strand cDNA was synthesized using a PrimeScript RT reagent kit with an eraser to eliminate potential DNA (Takara, China) in accordance with the manufacturer's protocol.

Cloning of *GhAGL15s*

Blast analysis was performed using *AtAGL15* protein as a query in our cotton transcript database (Yao et al. 2011; Zhang et al. 2013) and the genome of *Gossypium raimondii* (Wang et al. 2012). Three candidates possessing highly homologous amino acid sequences were obtained. Primers were then designed using Premier Primer 5 software for amplification of the target genes. PCR was performed using PrimeSTAR polymerase (Takara, China). The reaction mix consisted of 0.2 mM dNTP, 0.3 μ M forward primers, 0.3 μ M reverse primers, 4 μ l PrimerSTAR buffer, 0.8 μ l cDNA, 1.25U PrimeSTAR Polymerase, and 12.2 μ l double distilled water. Gradient PCR was performed under the following conditions: 2 min predenaturation at 94 °C, followed by a total of 37 amplification cycles as follows: 12 cycles (98 °C for 10 s, 56 °C for 15 s, 72 °C for 1 min), 10 cycles (98 °C for 10 s, 59 °C for 15 s, 72 °C for 1 min), and 15 cycles (98 °C for 10 s, 62 °C for 15 s, 72 °C for 1 min), and a final incubation at 72 °C for 3 min. PCR products were separated on 1.0 % agarose gels, and unique bands cloned into the pMD18-T vector (Takara, China) for sequencing. Gene-specific primers for *GhAGL15-1*, *GhAGL15-3*, and *GhAGL15-4* were designed from sequence annotations in the cotton transcripts (Table S1).

Sequence analysis

Homology comparisons were conducted using the BLAST program (<http://www.ncbi.nlm.nih.gov/>). Multiple sequence alignment was performed using DNAMAN software (version 5.2.2.). A phylogenetic tree was constructed using molecular evolutionary genetics analysis (MEGA) 5.10 software. Conserved domains were analyzed using the RPSBLAST program in the NCBI website (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>).

Subcellular localization analysis

The coding sequences of *GhAGL15-1*, *GhAGL15-3*, and *GhAGL15-4* were amplified by PCR, and subsequently cloned into pCAMBIA2301-GFP vectors. Next, expression constructs encoding a *GhAGL15*-GFP fusion protein were transferred into onion epidermal cells via *Agrobacterium tumefaciens*-mediated transformation (Xu et al. 2014), and a construct encoding GFP alone served as a control. Localization of GFP was monitored using an Olympus BX53F fluorescence microscope (Olympus, Japan).

Real-time PCR analysis

Tissue samples were prepared as described above. Primers for target genes were designed using the Primer Premier 5 program. Endogenous histone-3 (Accession: AF024716.1) was used as an internal standard for RT-PCR analysis. Gene-specific primers used for *GhHIS-3*, *GhAGL15-1*, *GhAGL15-3*, and *GhAGL15-4* are given in Table S1. Thermal cycling was performed under the following conditions: initial denaturation at 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. RT-PCR was carried out on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, USA).

Plasmid construction

The 35S:*GhAGL15-1*, 35S:*GhAGL15-3*, and 35S:*GhAGL15-4* over-expression vectors used in this study were constructed on the backbone of a pBI121 vector. The reporter gene encoding β -glucuronidase (GUS) was removed from pBI121 by digesting with the restriction enzymes *BamH* I and *Sac* I. Meanwhile, *BamH* I and *Sac* I restriction sites were introduced into the upstream and downstream coding regions of the target genes. Following purification, the digested pBI121 vector and target gene fragment were ligated using T4 DNA ligase. PCR analysis and restriction enzyme digestion were performed to validate the recombinant vectors.

Agrobacterium tumefaciens-mediated cotton transformation

The recombinant plasmid was transferred into *Agrobacterium* strain LBA4404. Hypocotyls of aseptic cotton seedlings (*G. hirsutum* 'CCRI24') were cut into 0.5–0.8 cm segments and used for transformation as described previously (Shang et al. 2009). The empty vector of pBI121 was used as a control in the transformation experiments. The differentiation rate was scored by measuring the percentage of transgenic calli that formed embryogenic callus.

Results

Cloning and sequence analysis of three *GhAGL15* genes

To clone the *AGL15* genes in allotetraploid cotton, our cotton transcript database (Yao et al. 2011; Zhang et al. 2013) and the draft genome of *Gossypium raimondii*, which is the putative D-genome parent of *Gossypium hirsutum* (Wang et al. 2012), were blasted with *AtAGL15* (Accession number: NP_196883.1). The sequences with high similarity to *AtAGL15* were cloned and sequenced, and

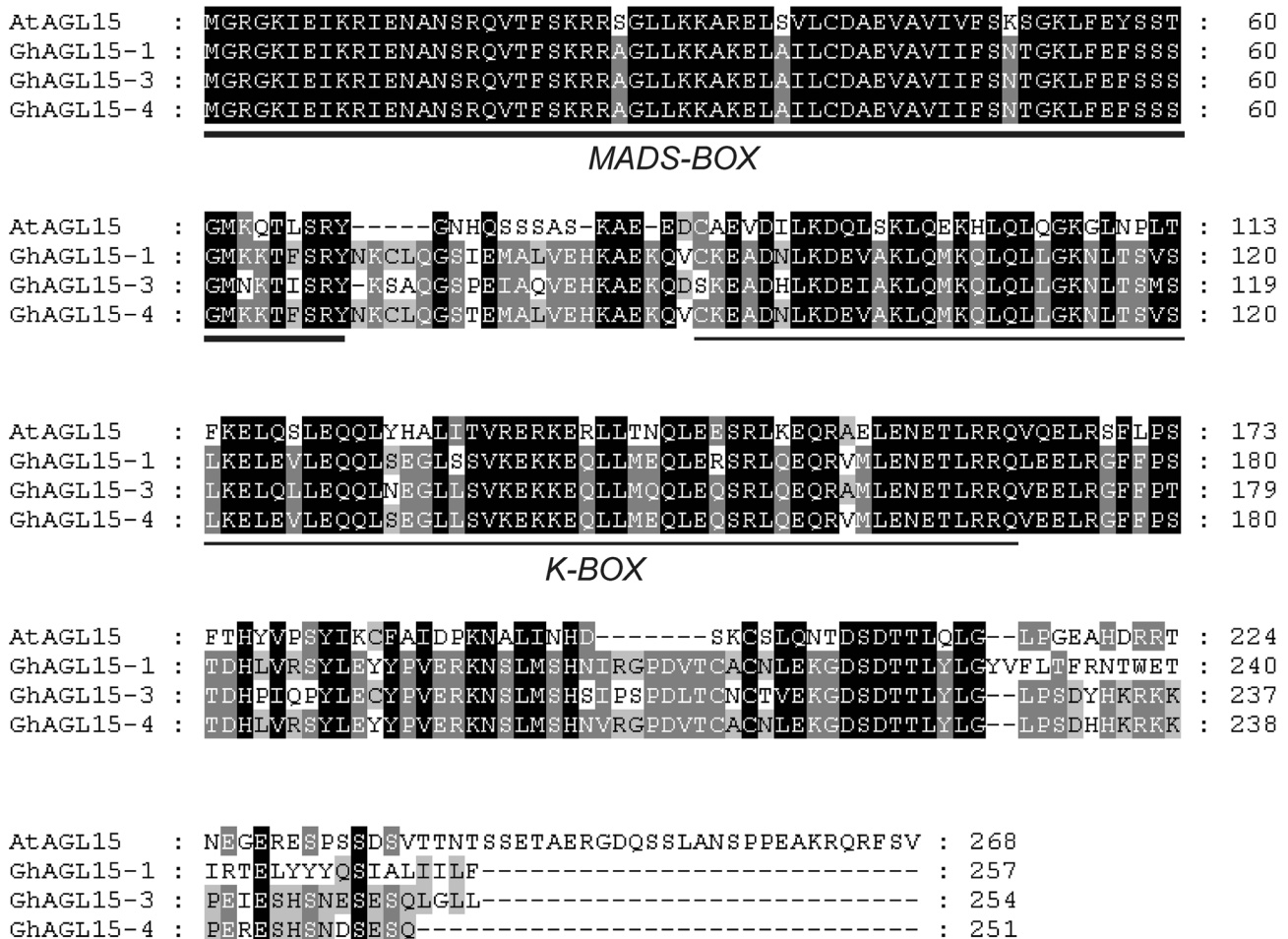


Fig. 1 Multiple sequence alignment of GhAGL15-1, GhAGL15-3, GhAGL15-4, and AtAGL15 proteins from cotton and *Arabidopsis*. The MADS-box (1–70) and K-box domains (90–160) are underlined with a thick line and a thin line, respectively

three *AtAGL15* ortholog genes were named *GhAGL15-1*, *GhAGL15-3*, and *GhAGL15-4*, respectively. The deduced amino acid sequences of these genes were compared with the *AtAGL15* amino acid sequence, and GhAGL15-1, GhAGL15-3, and GhAGL15-4 shared 58, 49, and 59 % identity, respectively. According to domain analysis results obtained using the NCBI RPSBLAST program, the three deduced amino acid sequences contained a highly conserved MADS-box DNA-binding domain, a relatively less conserved K domain (a peculiarity in plant MADS-domain proteins; Theissen et al. 1996), a poorly conserved domain I (a short linker between the MADS- and K-domains), and a variable C-terminal domain (Fig. 1).

Phylogenetic analysis

To confirm subfamily identities and understand possible functionality of the three MADS-box genes, a phylogenetic tree was constructed using the MEGA 5.10 program with 16

protein sequences. These consisted of the three GhAGL15s obtained in this study, and the other known AGL15s: BnAGL15-type 1 (*Brassica napus*), BnAGL15-type 2, DIAGL15 (*Dimocarpus longan*), TaAGL15 (*Triticum aestivum*), AcAGL15 (*Aquilegia coerulea*), AtAGL15 (*Arabidopsis thaliana*), and seven other *Arabidopsis* AGL proteins. The AGL15s clustered into one subgroup (Fig. 2). The three GhAGL15s showed significant similarity with each other and clustered together. These results further confirmed that the three GhAGL15s belonged to the AGL15 subfamily.

Subcellular localization analysis of GhAGL15 proteins

The main function of transcriptional factors requires nuclear localization. To investigate the subcellular localization of three GhAGL15 proteins, their C terminals were fused to green fluorescent protein (GFP), and driven by the constitutive CaMV 35S promoter. They were then transferred into onion epidermal cells via *Agrobacterium*

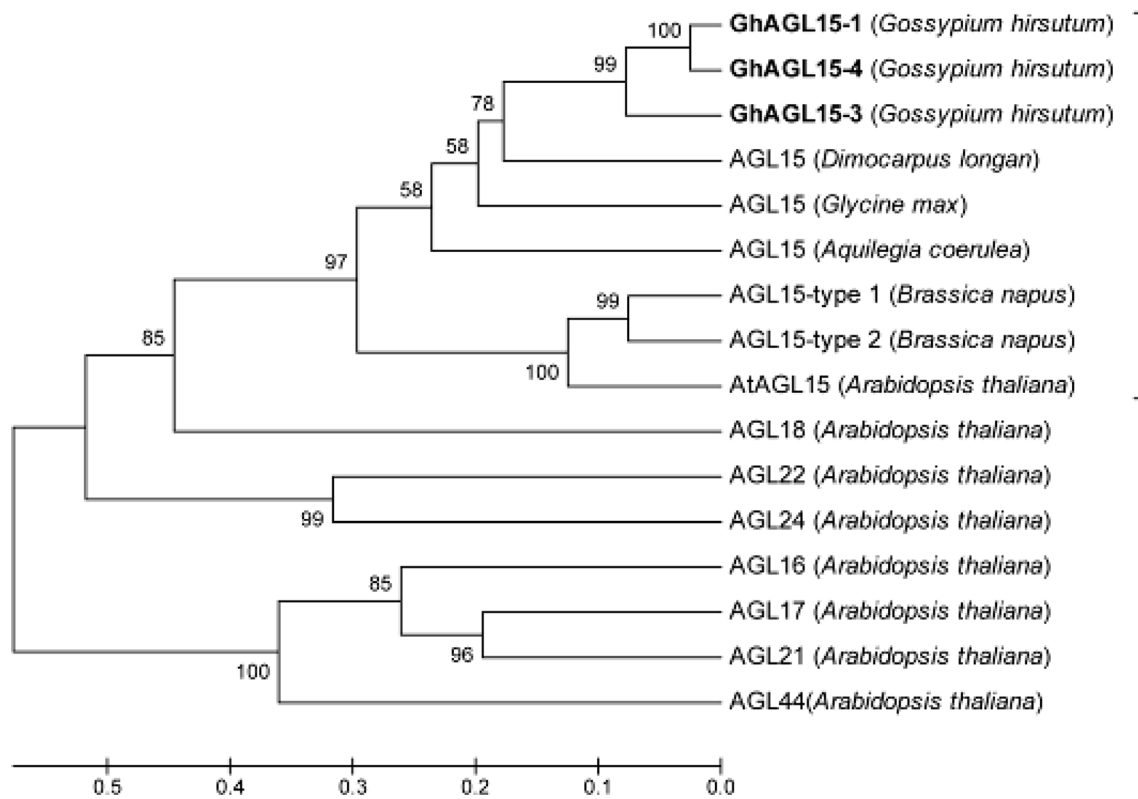


Fig. 2 Phylogenetic tree generated from three GhAGL15s, DIAGL15, GmAGL15, AcAGL15, two BnAGL15s, AtAGL15, and seven *Arabidopsis* AGL proteins. The tree was generated using the neighbor-joining method by MEGA 5.10 software. Bootstrap values,

as labels of the corresponding nodes of the tree, were obtained based on 1,000 replications. AGL15 subfamilies are labeled with brackets at the right-hand side

tumefaciens-mediated transformation. Fluorescent imaging of the GhAGL15-GFP fusion protein by microscopy revealed that the three GhAGL15-GFPs were exclusively localized to the nucleus, while the fluorescence localization signals of the control GFP-only vector were ubiquitous in the cell (Fig. 3). These results demonstrated that the three GhAGL15s were nuclear-localized proteins, which are consistent with AtAGL15 (Heck et al. 1995; Rounsley et al. 1995).

Expression analysis of *GhAGL15-1*, *GhAGL15-3*, and *GhAGL15-4*

To learn more about the expression patterns of the three *GhAGL15* genes, their expression profiles were examined by RT-PCR analysis during somatic embryogenesis and in different cotton organs, namely roots, stems, and leaves of aseptic seedlings. All three genes exhibited extremely low expression levels in the leaves and stems of aseptic seedlings (Fig. 4a). *GhAGL15-3* was constitutively expressed during SE; when cultured on embryogenic callus induction medium, the level of expression increased during the SE process, peaked at day 42, and followed by a reduction

in expression level (Fig. 4a). The trend of the *GhAGL15-4* transcripts during SE was analogous to that of *GhAGL15-3*, reaching highest detectable levels at days 27–42 (Fig. 4a). *GhAGL15-1* was highly expressed in callus of different stages, and reached its highest level in embryogenic callus (Fig. 4a). The lowest expression levels of *GhAGL15-1* were detected in the embryoid stages, including globular-stage embryos, heart-shaped embryos, torpedo-shaped embryos, and cotyledon-stage embryos; this was the same for *GhAGL15-3* and *GhAGL15-4* (Fig. 4a). These results implied that *GhAGL15s* play a vital role during cotton embryogenic callus formation.

Auxin and cytokinins play vital roles in cotton somatic embryogenesis (Xu et al. 2013). To assess *GhAGL15s* response to the two hormones, non-embryogenic callus was treated with the synthetic cytokinin KT, and the synthetic auxin 2,4-D. KT treatment significantly increased the transcripts of *GhAGL15-1* and *GhAGL15-3*, whereas *GhAGL15-4* showed no significant response. The three *GhAGL15s* all responded significantly to the 2,4-D treatment; the expression level of *GhAGL15-3* peaked at 12 h, while *GhAGL15-1* and *GhAGL15-4* were continually induced (Fig. 4b).

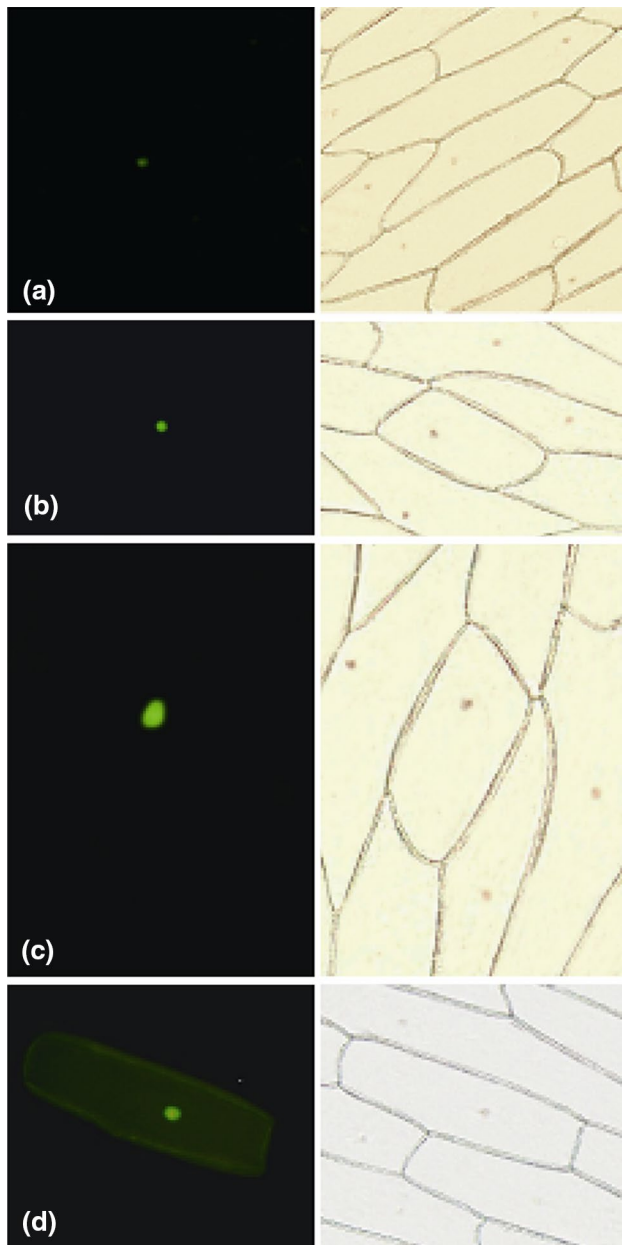


Fig. 3 Subcellular localization of GhAGL15-GFP fusion proteins. **a** GhAGL15-1-GFP fusion protein, **b** GhAGL15-3-GFP fusion protein, **c** GhAGL15-4-GFP fusion protein, and **d** free GFP control. Fluorescence images (left); visible light images (right)

Ectopic expression of three *GhAGL15s* promote embryogenic callus formation

To explore the influence of the three *GhAGL15* genes on the development of cotton callus, *Agrobacterium*-mediated transformation was performed using three *35S:AGL15s* or empty vector controls (Fig. 5a). Following coculture with *Agrobacterium tumefaciens*, the hypocotyl segments were cultured in callus induction medium containing kanamycin.

The identified putative transformants (with kanamycin resistance) were further confirmed by PCR analysis, using primers designed between the CaMV 35S promoter and the *GhAGL15s* (Fig. 5a, c). The transgenic calli were then sub-cultured into new callus induction medium. After 100 days, the transgenic calli were light yellow, looser, and less moist compared to the control, which was tan, and comparatively tight and moist (Fig. 5b). To investigate whether constitutive expression of *GhAGL15s* affected the differentiation rate (as measured by the embryogenic callus percentage of the transgenic callus), transgenic calli were transferred to embryogenic callus induction medium. After culture for 30 days, the differentiation rate of the transgenic callus was scored. Constitutive expression of *GhAGL15s* significantly increased differentiation rates of the transgenic callus ($P < 0.05$ or 0.01 ; Fig. 5d). In particular, the differentiation rate of *35S:AGL15-4* transgenic callus significantly increased to 65.2 % ($P < 0.01$), compared with 38.1 % for the empty vector (Fig. 5d). These results indicated that over-expression of the three *GhAGL15s* promoted embryogenic callus formation, and that *GhAGL15-4* had more pronounced effects.

Discussion

SE represents an atypical developmental process for somatic cells, leading to the formation of somatic embryos (Zimmerman 1993; Kumar et al. 2005; Firoozabady et al. 2006). While a variety of factors, such as the source of explants and exogenous hormone regime, are known to affect this process (Norma and Goodin 1987), the genetic regulation of the somatic embryogenesis process remains unclear. *SERK1* plays an imperative part in SE, and is often considered as a potential marker for embryogenic competence (Schmidt et al. 1997; Hecht et al. 2001; Nolan et al. 2003). *AGL15* was identified as a component of the *SERK1* protein complex (Karlova et al. 2006), and both *SERK1* and *AGL15* are expressed in response to auxin treatment (Nolan et al. 2003; Perry et al. 1996; Zhu and Perry 2005). *AGL15* is associated with embryo development and increases somatic embryo production (Wang et al. 2004; Tokuji and Kuriyama 2003; Chen and Chang 2003). However, the molecular nature of *AGL15* orthologs in cotton is unknown.

In the present study, three *AGL15*-like MADS-box genes *GhAGL15-1*, *GhAGL15-3*, and *GhAGL15-4* were firstly isolated from cotton using a homologous cloning method. Sequence analysis suggested that the three deduced amino acid sequences contained a highly conserved MADS-box DNA-binding domain (Fig. 1), indicating that they belonged to the MADS-box family. Multiple sequence alignment and phylogenetic analyses revealed

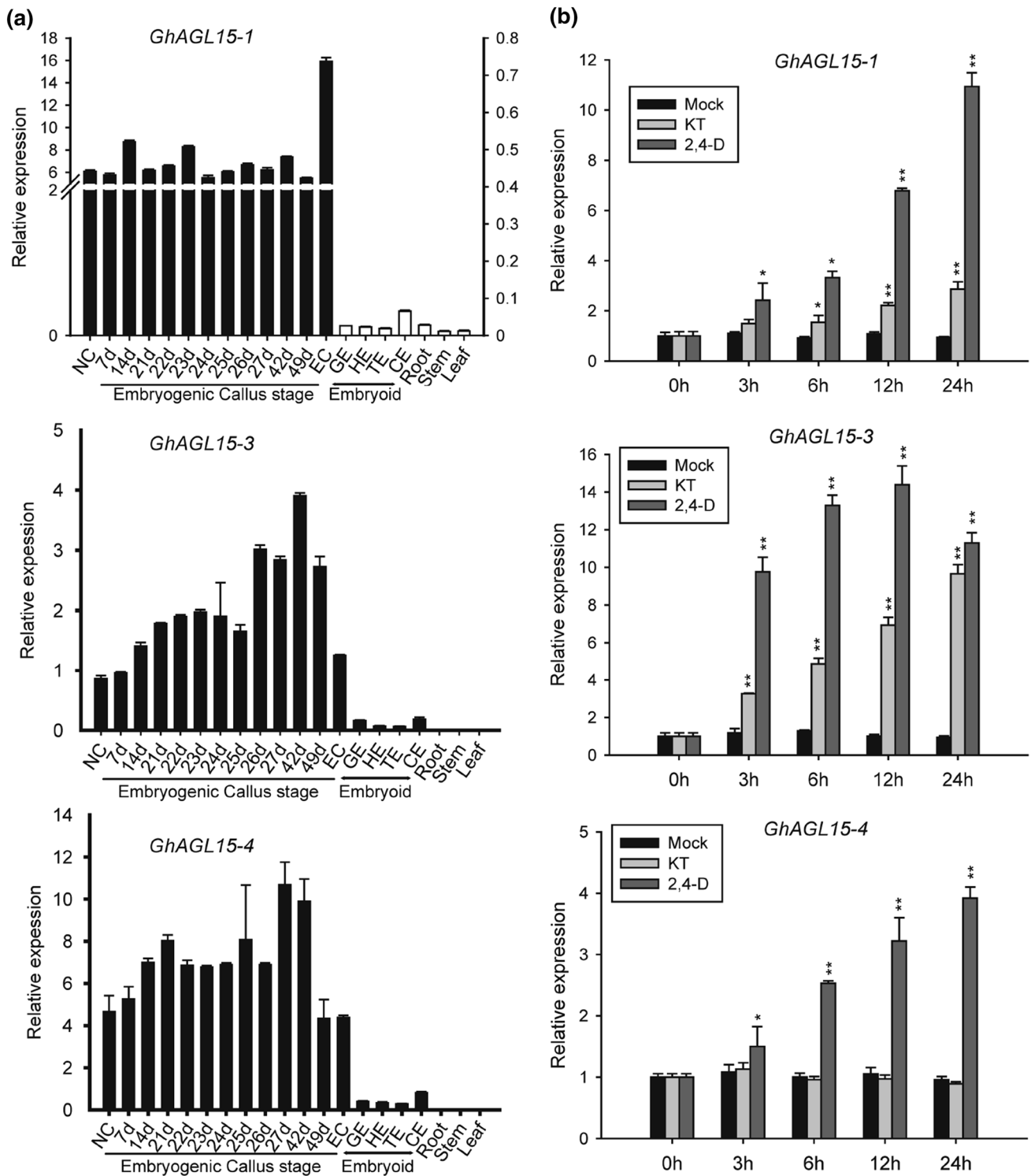


Fig. 4 Expression profile of *GhAGL15s*. **a** Relative expression levels of *GhAGL15-1*, *GhAGL15-3*, and *GhAGL15-4* in different tissues of *GhAGL15-1*, *GhAGL15-3*, and *GhAGL15-4* in different tissues. *NC* non-embryonic callus. Embryogenic callus stages represent 7-, 14-, 21-, 22-, 23-, 24-, 25-, 26-, 27-, 42-, and 49-day-old calli cultured on embryogenic callus induction medium as well as mature embryogenic callus (EC). *GE* embryoid shows globular-stage embryos, *HE* heart-shaped embryos, *TE* torpedo-shaped embryos, *CE* cotyledon-stage

embryos. *Error bars* represent SD from three independent experiments. **b** Expression patterns of *GhAGL15s* in response to KT and 2,4-D. Non-embryonic calli were treated with 10 μM 2,4-D or 10 μM KT for the given time. *Error bars* represent SD from three independent experiments. *Asterisks* indicate significant differences from the mock treatment by *t* test at **P* < 0.05 and ***P* < 0.01

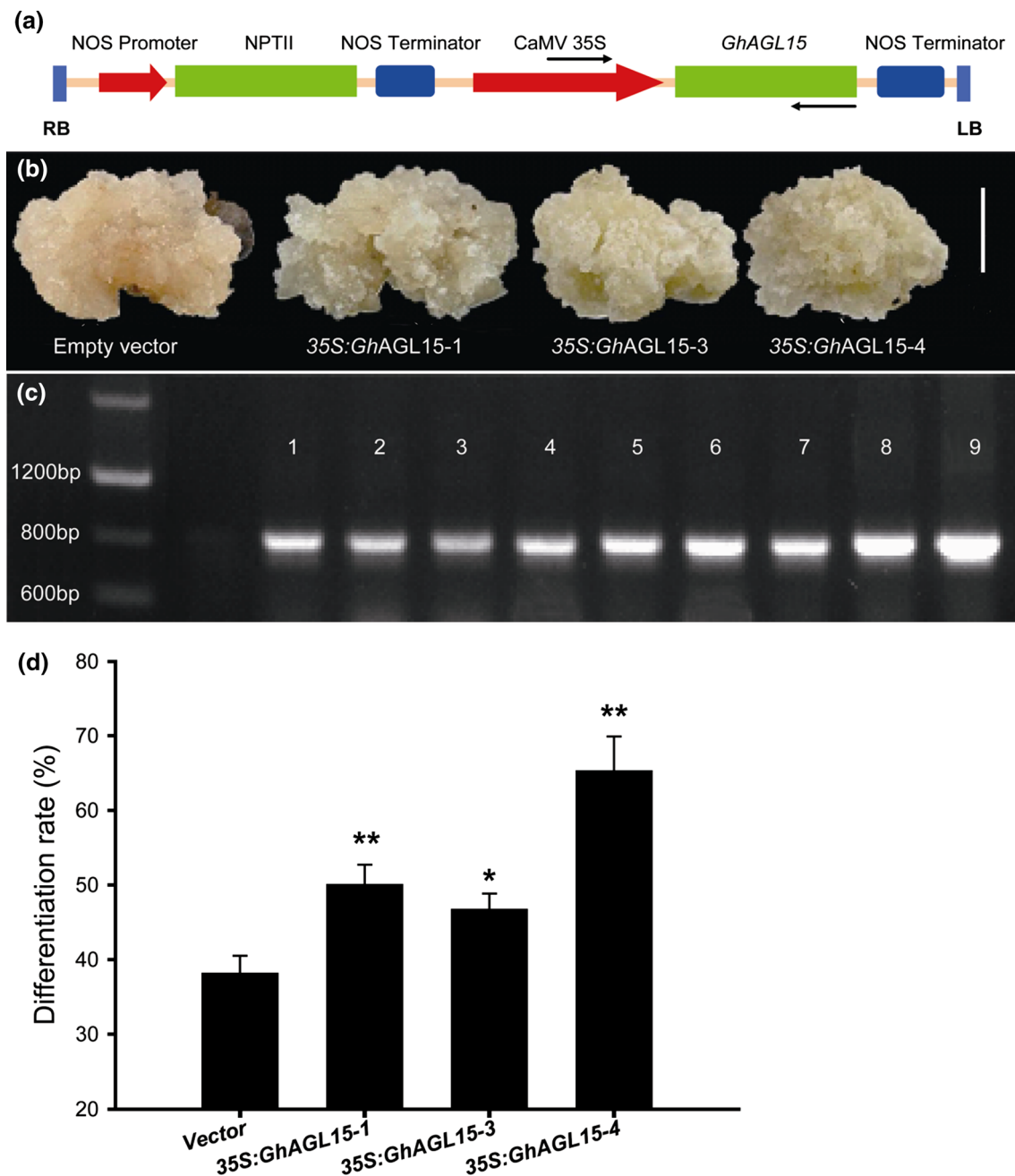


Fig. 5 Generation and characterization of transgenic callus. **a** Schematic representation of the T-DNA region in the binary vector. Black arrows indicate the position of the primers used for transgenic confirmation. **b** Growth status of transgenic callus cultured for 100 days. Scale bar 1 cm. **c** PCR verification of transgenic callus. 1–3 35S:GhAGL15-1; 4–6 35S:GhAGL15-3; 7–9 35S:GhAGL15-4. **d** Over-expression of *GhAGL15s* increased the transgenic callus dif-

ferentiation rate (as measured by the embryogenic callus percentage of the transgenic callus). Calli were scored after 30 days of culture on embryogenic callus induction medium. Error bars represent SD from three independent transformations. Asterisks indicate significant differences from the mock treatment by *t* test at **P* < 0.05 and ***P* < 0.01

that GhAGL15s share very high similarity with AGL15s from other dicot species, and are evolutionally conserved (Fig. 2); this added support that they are members of the AGL15 subfamily. All three GhAGL15s were localized to the nucleus (Fig. 3), indicating that like AGL15s from other

organisms, GhAGL15s may function in transcriptional regulation. There are more than one *AGL15* members in *Brassica napus* (Heck et al. 1995), likewise three GhAGL15s exist in allotetraploid cotton (*G. hirsutum*) and show high similarity with each other (>80 %; Fig. 2). Polyploidy

results in extensive genetic redundancy, and the diversification of paralogous genes is often associated with different functionalization (Roulin et al. 2013). Therefore, the three individual *GhAGL15s* may have specific roles in regulating cotton growth and development.

GhAGL15-1, *GhAGL15-3*, and *GhAGL15-4* display very low expression in non-embryogenic organs (roots, stems, and leaves; Fig. 4). This is similar to observed for *AGL15s* of *Brassica napus*, whose transcripts were present at very low levels in roots, and undetectable in mature leaf samples (Heck et al. 1995). Previous research has shown that *AGL15s* are expressed highly in developing embryos. *BaAGL15* expression started to increase at the late globular stage, peaked at approximately the torpedo stage, and then fell gradually through the remainder of embryo maturation (Heck et al. 1995). *GmAGL15* was preferentially expressed in young developing embryos and in somatic embryo cultures (Thakare et al. 2008). Constitutive expression of *AGL15s* enhanced the production of secondary embryos from cultured zygotic embryos (Harding et al. 2003; Thakare et al. 2008). Nevertheless, the expression levels of the three *GhAGL15s* increased following embryogenic callus induction, and their expression, especially that of *GhAGL15-1*, decreased during the embryoid stage (Fig. 4a). Therefore, the expression patterns of the *GhAGL15s* are different from that reported for other *AGL15s*, implying that *GhAGL15s* may play a predominant role in cotton embryogenic callus formation.

The acquirement of somatic embryos and regenerated plants correlates to callus qualities such as color and texture. Previous studies suggested that a loose light yellow callus is more competent to induce embryogenesis formation in callus, and is beneficial to the development of the somatic embryo (Trolinder and Goodin 1987; Wu et al. 2004). When *GhAGL15s* were over-expressed in cotton callus, the transgenic calli were light yellow and much looser than that of the control (Fig. 5b). Furthermore, embryogenic callus formation rates were significantly increased, especially for *GhAGL15-4* (Fig. 5d). These results suggest that over-expression of *GhAGL15s* enhances embryogenic competence of the transgenic calli. The different expression profiles and promotional effects imply that *GhAGL15-1*, *GhAGL15-3*, and *GhAGL15-4* have a precise division in promoting somatic embryogenesis.

Similar to *AGL15*, *GhAGL15s* are induced by auxin, one of the essential components for somatic embryogenesis (Fig. 4b). *LEC2*, one essential gene for induction of somatic embryo, is responsive to auxin and may directly control the expression of *AGL15* (Braybrook et al. 2006). *AGL15* controls ethylene biosynthesis, and directly regulates the ethylene response factor *SERF1* and gibberellin catabolism genes *AtGA2ox6*. So it promotes somatic embryogenesis by influencing ethylene signal transduction

and reducing the gibberellin/abscisic acid ratio (Wang et al. 2004; Zheng et al. 2013). *SERK1* is also induced by auxin treatment and enhances somatic embryo development when ectopically expressed (Hecht et al. 2001). *AGL15* is one component of *SERK1* protein complex (Karlova et al. 2006). *GhAGL15-1* and *GhAGL15-3* responded to KT treatment (Fig. 4b). Therefore, *GhAGL15s* may be a modulator of these phytohormones and gene interactions in promoting embryogenesis competence. Future research is required to determine the division and corporation between *GhAGL15s* in regulating embryogenesis callus and the embryoid development.

Transgenic research has made considerable progress in cotton improvement (Sunilkumar et al. 2006; Shadmanov et al. 2013; Zhang et al. 2000; Wilkins 2000; Wilkins et al. 2000). Cotton genetic transformation largely depends on regeneration via somatic embryogenesis (Zhang et al. 2011). Embryogenic callus formation is the key step to accomplish regeneration in cotton plants, so *GhAGL15s*, which are highly expressed in embryogenic callus, can improve the embryonic competency of transgenic calli. Therefore, the control of *GhAGL15s* expression, using temporal or specific inducible promoters, may prove a valuable tool for improving transformation efficiency and recovery of transgenics.

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