

Identification and promoter analysis of a GA-stimulated transcript 1 gene from *Jatropha curcas*

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Research Article

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Abstract

Members of Gibberellic acid-stimulated Arabidopsis (GASA) gene family play roles in plant growth and development, particularly in flower induction and seed development. However, there is still relatively limited knowledge of GASA genes in *Jatropha curcas*. Herein, we identified a GASA family gene from *Jatropha curcas*, namely *JcGAST1*, which encodes a protein containing a conserved GASA domain. Sequence alignment showed that JcGAST1 protein shares 76% sequence identity and 80% sequence similarity with SIGAST1. *JcGAST1* had higher expression and protein levels in the male flowers than in the female flowers. Overexpression of *JcGAST1* in tobacco promotes plant growth but inhibits pistil development. *JcGAST1* expression was upregulated by GA and downregulated by MeJA. Promoter analysis indicated that the pyrimidine box and CGTCA motif were the GA- and MeJA-responsive elements of the *JcGAST1* promoter. Using a Y1H screen, six transcription factors were found to interact with the pyrimidine box, and three transcription factors were found to interact with the CGTCA motif. Overall, the results of this study improve our understanding of the *JcGAST1* gene and provide useful information for further studies.

Key Message

Overexpression of *JcGAST1* promotes plant growth but inhibits pistil development. The pyrimidine box and CGTCA motif of *JcGAST1* promoter was responsible for GA and MeJA response.

Introduction

Biodiesel is a green, renewable alternative to fossil fuel. *Jatropha curcas* L. (hereafter referred to as *J. curcas*), a member of the Euphorbiaceae family, which is native to tropical regions in the Western Hemisphere and has been considered an important biofuel plant (Maghuly and Laimer. 2013). The seeds of *J. curcas* contain a high content of oil (30 ~ 40%), and this seed oil contains high levels of unsaturated fatty acids, which are suitable for producing biodiesel (Gübitz et al. 1999; Openshaw. 2000; Thomas et al. 2008). However, due to low seed yield, this plant has limited economic benefit for exploitation and further expansion of the *Jatropha*-based biodiesel industry. *J. curcas* is a monoecious plant, and separate female and male flowers are present in the same inflorescence (Chen et al. 2017). The low ratio of female to male flowers (1/10 ~ 1/30) is an important factor that causes low seed productivity in *J. curcas* (Raju and Ezradanam. 2002). Despite the availability of transgenic breeding for *J. curcas*, the limited knowledge about the flowering mechanism in *J. curcas* has led to some gap toward the goal of producing high-yielding *J. curcas* cultivars. Thus, dissection of the molecular mechanisms of flowering and floral sex differentiation in *J. curcas* is beneficial for developing high-yielding *J. curcas* cultivars. Phytohormones are essential for plant developmental regulation and have been confirmed to be involved in floral development (Izawa. 2021). Among these phytohormones, gibberellin (GA) has been considered a florigen (King and Evans. 2003). GA regulates floral development by suppressing the function of DELLA

proteins, which can physically interact with and regulate the activity of many transcription factors related to flowering (Bao et al. 2020). In *J. curcas*, GA is thought to play essential and complicated roles in floral organ development. GA treatment could increase the number of both male and female flowers of *J. curcas* and promote the production of bisexual flowers (Pi et al. 2013; Pan et al. 2014).

GASA proteins is a cysteine-rich low molecular weight peptide that is extensively widespread in the plant kingdom (Nahirñak et al. 2012). The GASA gene family is unique in plants, and most members are regulated by GA (Zhang and Wang. 2017). Tomato (*Solanum lycopersicum* L.) GA-stimulated gene *GAST1* (GA-stimulated transcript 1) was the first GASA gene reported in 1992 (Shi et al. 1992). Subsequently, a large number of GASA genes and proteins were identified in *Arabidopsis* (*Arabidopsis thaliana*), *Petunia* (*Petunia hybrida*), rice (*Oryza sativa*), soybean (*Glycine max*), poplar (*Populus trichocarpa*) and tobacco (*Nicotiana tabacum*) (Herzog et al. 1995; Ben-Nissan and Weiss. 1996; Furukawa et al. 2006; Ahmad et al. 2019; Han et al. 2021; Li et al. 2022). Most GASA proteins contain a GASA domain consisting of 60 amino acids, typically including 12 cysteine residues (Sun et al. 2013). A previous study reported that lack of a GASA domain or mutation of conserved cysteine residues in the GASA domain will result in loss-of-function of the GASA protein (Rubinovich and Weiss. 2010). Growing evidence has revealed that GASA genes play an important role in the regulation of plant growth and development, including seed germination, stem elongation, root growth, floral induction and flower organ development (Zhang and Wang. 2017). In the same GASA gene family, different members may play an opposite biological function. In *Arabidopsis*, *AtGASA4* was reported to contribute to flowering, and suppression of both *AtGASA4* and *AtGASA6* causes late flowering (Qu et al. 2016). However, *AtGASA5* inhibits flowering by upregulating the expression of the flowering repressor *FLC* (LOWERING LOCUS C). GASA proteins also act as phytohormonal signaling transducers and integrators (Zhang et al. 2009). It has been reported that *AtGASA6* plays a role as an integrator of GA, ABA (abscisic acid), and Glc (glucose) signaling to regulate seed germination by linking *AtRGL2* and *AtEXPA1* functions (Zhong et al. 2015). In rice, the GASA protein *OsGSR1* interacts with brassinolide (BR) synthase *DIM/DWF1* directly to promote the biosynthesis of BR (Wang et al. 2009). Additionally, the GASA gene family is also involved in plant responses to biotic and abiotic stresses. Overexpression of the beech *FsGASA4* gene in *Arabidopsis* improves tolerance to salt, reduce oxygen species (ROS), and heat stress (Alonso-Ramírez et al. 2009). Sun et al. reported that the *Arabidopsis atgasa14* mutant is sensitive to ABA and reduced salinity tolerance, and overexpression of *AtGASA14* could reduce ROS to resist ABA and salt stress (Sun et al. 2013). Similar to *AtGASA14*, overexpression of *AtGASA4* also inhibits ROS accumulation (Rubinovich et al. 2010).

Although some GASA genes have been studied in *Arabidopsis* and other plant species, there is still relatively limited knowledge of GASA genes in *J. curcas*. Herein, we identified a homolog of tomato GA-stimulated transcript 1 from *J. curcas*, termed *JcGAST1*. The mRNA and protein levels of *JcGAST1* were higher in male flowers than in female flowers. Overexpression of *JcGAST1* in tobacco not only promotes the growth of plants but also inhibits the development of the stigma. The promoter of *JcGAST1* contains GA- and MeJA-responsive elements, and the activity of the *JcGAST1* promoter was induced by GA but

inhibited by MeJA. Taken together, our study sheds light on the potential role of JcGAST1 in floral development of *J. curcas*.

Materials And Methods

Plant materials and growth conditions

Flower bud samples at different developmental stages were collected from thirty *Jatropha curcas* trees in Zhenfeng, Guizhou Province, China (36°14'50.2"N, 87°51'47.8"E). The flower bud samples were dipped immediately in RNAlocker (Tiandz, Inc., Beijing China) on ice for RNA isolation. *Jatropha curcas* or tobacco (*Nicotiana benthamiana*) seeds were sown in a soil mixture (peat moss, perlite, and vermiculite, 8:1:1) and grown in a greenhouse at 25°C under a 16-h light/8-h dark cycle at 50% relative humidity.

Bioinformatic Analysis

The coding sequence (CDS), peptide and 2,249 bp upstream of the translational start site (ATG) promoter region sequence information of *JcGAST1* is available in the NCBI database (<https://www.ncbi.nlm.nih.gov/>). The functional domains of JcGAST1 were predicted using SMART online software (<http://smart.embl-heidelberg.de/>). A phylogenetic tree was constructed using the neighbor-joining (NJ) method with MEGA 6.0 software. The PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) was used to identify potential cis-elements of the *JcGAST1* promoter.

Rna Isolation And Qpcr

Total RNA was isolated from *Jatropha curcas* flower buds or leaves using E.Z.N.A Total RNA Kit (OMEGA) according to the manufacturer's protocol. cDNA was synthesized with an AMV RNA PCR Kit 3.0 (Takara) in 10 µL of reaction mixture containing 1 µg of total RNA according to the manufacturer's instructions. Quantitative PCR was performed with the ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Inc. USA) using 2 × SYBR green PCR mix (QIAGEN, Shanghai, China). To analyze the expression of *JcGAST1* in flower development, the flower bud samples were divided into eight development phases as previously described (Xu et al., 2019). To quantify the transcriptional levels of *JcGAST1* in response to phytohormones treatment, three-month-old *Jatropha curcas* seedlings were treated with 100 µM MeJA, 100 µM GA₃, 100 µM IAA or 100 µM SA, respectively, and leaves that collected at 3 h and 24 h after phytohormones treatment were used for total RNA extraction. The primers used for qPCR are listed in Supporting Information Table S1. *JcTUB* was used as an internal positive control.

Plasmid Construction And Plant Transformation

The full-length coding sequence (CDS) of *JcGAST1* was cloned from *Jatropha curcas* cDNA and inserted into the pCambia1305 vector to generate the *35S::JcGAST1* construct. The construct was transformed into *Agrobacterium tumefaciens* GV3101 through heat shock transformation. To generate stable tobacco transgenic plants, plants were transformed by *Agrobacterium*-mediated transformation through the leaf disc transformation regeneration method. The *JcGAST1* promoter fragments of different lengths were inserted upstream of the *GUS* gene coding region of the pCambia1304 vector to replace the cauliflower mosaic virus (CaMV) 35S promoter, obtaining a series of *proJcGAST1-GUS* constructs named FL (-2249/-1, 2249 bp), P1 (-1799/-1, 1799 bp), P2 (-1215/-1, 1215 bp), P3 (-713/-1, 713 bp) and P4 (-359/-1, 359 bp). The promoter constructs were transformed into tobacco leaves using the transient transformation method for the GUS activity assay.

Protein Extraction And Immunoblotting

Total proteins of *J. curcas* flower buds were extracted using a Plant Total Protein Extraction Kit (Sangon Biotech, China) according to the manufacturer's instructions. For immunoblotting, the proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to a 0.22 µm PVDF membrane. The membrane was incubated with an anti-JcGAST1 antibody (DetaiBio, China). The blot was developed using an enhanced chemiluminescent kit (Biosharp) and detected by a Tanon-5500 system.

Gus Histochemical And Fluorometric Assay

For histochemical GUS staining, tobacco leaf discs expressing various *Promoter-GUS* were immersed in GUS staining solution and incubated at 37°C overnight. The chlorophyll was removed by immersion of the leaf disc samples in 70% ethanol. Images were taken using a stereomicroscope (Leica M80). A fluorometric assay of GUS activity was performed as previously described (Jefferson et al., 1987) by using 4-methylumbelliferyl- β -glucuronide (4-MUG) as a substrate. The tobacco leaves transformed with various *promoter-GUS* vectors were treated with 100 µM MeJA, 100 µM GA₃, 100 µM IAA or 100 µM SA and then collected for protein extraction. The protein concentration was quantified using the Bradford protein assay. Fluorescence was measured by using a Thermo Varioskan™ LUX Microplate Reader.

Yeast One-hybrid Assay

The coding sequences of *JcRF2a*, *JcIAA9*, *JcAHL9*, *ERF106*, *JcbZIP11*, *JcPAT1*, *JcSAP8* and *JcRAG1* were cloned into the pGADT7 vector. The primer pairs used for gene cloning are listed in Supplemental Table S1. The cis-elements of the *JcGAST1* promoter, like 3×CCTTTT and 3×CGTCA, were inserted into the pHIS2 vector, respectively. All of the constructs were transformed into the yeast Y187 strain using the lithium acetate method. The transformed cells were harvested and evenly coated on minimal synthetic

dextrose medium lacking leucine, tryptophan and histidine (SD/-LWH) containing 3-amino-1,2,4-triazole (3-AT) and grown at 30°C for three days.

Dual-luciferase Assay

A dual-LUC assay was performed as previously described (Zhang et al. 2015). Briefly, the cis-elements of the *JcGAST1* promoter, like 3×CCTTTT and 3×CGTCA, were inserted into the pGreenII 0800-LUC plasmid to generate the reporter vectors, respectively. The coding sequences of *JcRF2a*, *JcIAA9*, *JcAHL9*, *ERF106*, *JcbZIP11*, *JcPAT1*, *JcSAP8* and *JcRAG1* were cloned into the pCAMBIA1305 plasmid driven by the CaMV 35S promoter as the effector vectors, respectively, and *35S::YFP* served as a negative control. The resultant constructs were subsequently transiently expressed in tobacco leaves. The relative LUC/REN activity was examined using a Dual-Luciferase Assay System Kit (Promega).

Accession Numbers

Sequence data of this article can be found in the NCBI database (<https://www.ncbi.nlm.nih.gov>) under the following accession numbers: *JcGAST1* (XM_012234671.3), *JcTUB* (XM_012218402.3), *JcRF2a* (XM_012229037.3), *JcERF106* (XM_012235114.3), *JcIAA9* (XM_012212698.3), *JcAHL9* (XM_012228026.3), *JcbZIP11* (XM_012211837.3), *JcPAT1* (XM_012232033.3), *JcSAP8* (XM_012234577.3), *JcRAG1* (XM_012212711.2) and *Nt18S* (HQ384692.1).

Results

Characterization of JcGAST1

Based on the gene expression profile of developing flowers in *J. curcas*, a homolog of the tomato GA-stimulated transcript 1 (*GAST1*) gene was isolated from differentially expressed genes, termed *JcGAST1*. The cDNA of the *JcGAST1* gene is composed of a 348 bp ORF that encodes a protein of 115 amino acids. Sequence comparisons showed that JcGAST1 shares 76% identity and 80% similarity with SIGAST1. Similar to SIGAST1, JcGAST1 also contains a GASA domain at the C-terminus (Fig. 1A). The GASA domain could be found in all GASA proteins, and its impairment would result in loss of function (Rubinovich and Weiss. 2010). In addition, phylogenetic analysis demonstrated significant evolutionary relevance between JcGAST1 and its homologs in 10 plant species (Fig. 1B). To investigate the function of JcGAST1 during floral development in *J. curcas*, we first performed quantitative PCR (qPCR) to monitor the expression of *JcGAST1* at nine different stages for sex differentiation and floral development. As shown in Fig. 2A, the transcripts of *JcGAST1* could be detected in all of the development phases, and the JCFIII stage had the highest level, followed by the JCFII stage. Compared to the JCUN stage, *JcGAST1* expression was higher at the four developmental stages of the male flower but lower at the other four developmental stages of the female flower. Subsequently, we examined the accumulation of JcGAST1 protein at the nine stages of *J. curcas* flowering. Immunoblot analysis showed that the

amount of JcGAST1 protein was highest at the JCFII stage. Furthermore, the protein levels of JcGAST1 in male flowers were higher than those in female flowers (Fig. 2B). These results indicated that JcGAST1 may play an opposite role during male and female flower development in *J. curcas*.

Overexpression of JcGAST1 inhibits the development of stigma in transgenic tobacco

To further investigate the function of JcGAST1 during flower development and sex differentiation, the *JcGAST1* gene was overexpressed in tobacco plants (cultivar K326). RT–PCR analysis showed that *JcGAST1* transcripts were undetectable in wild-type (WT) tobacco plants but could be detected in the three transgenic lines (*35S::JcGAST1* #1, #2 and #3), which were selected for further study (Fig. S1). As shown in Fig. 3A, the *35S::JcGAST1* plants showed increased growth compared with the WT plants. To determine the extent of increased growth of *35S::JcGAST1* plants, the plant height, leaf length and leaf width were measured. We found that there was a significant elevation in the plant height, leaf length and leaf width of *35S::JcGAST1* plants compared with those of WT plants (Fig. 3C–E), suggesting that overexpression of the *JcGAST1* gene could promote the growth of plants. Significantly, the stigma development of these selected *35S::JcGAST1* plants was obviously inhibited (Fig. 3B). Compared to 0.97 ± 0.11 of that of the WT plants, the filament/stigma length ratio of *35S::JcGAST1* plants was enhanced to 1.13 ± 0.06 , 1.12 ± 0.09 and 1.47 ± 0.15 , respectively (Fig. 3F). These results indicated that JcGAST1 may be involved in the regulation of flower development.

Sequence and deletion analysis of the JcGAST1 promoter

To predict the potential regulatory mechanism mediated by JcGAST1 during flower development, the cis-acting elements of the promoter were analyzed. Except for the basic elements CAAT box and TATA box, the *JcGAST1* promoter also contains the light response elements (G box and Box 4), the meristem expression element (CAT box) and the stress responsive element (TC-rich repeats). Some hormone response elements were also found in the *JcGAST1* promoter, including the TCA element (salicylic acid responsiveness), the GARE motif (gibberellin responsiveness), the TGA element (auxin responsiveness), the CGTCA motif (MeJA responsiveness), and the ABRE motif (abscisic acid responsiveness) (Fig. S2 and Table S2). Moreover, pollen-specific elements (GTGANTG10 and POLLEN1LELAT52) and embryo-specific elements (DPBFCORED CDC3) were present in the *JcGAST1* promoter. The coordination of phytohormone signaling plays an important role in the regulation of flower development (Ma et al. 2018). Considering that the *JcGAST1* promoter contains hormone response elements, the effects of phytohormones on *JcGAST1* expression were investigated. qPCR showed that the expression of *JcGAST1* could be induced by IAA, GA and SA treatment but inhibited by MeJA treatment (Fig. S3).

To determine the regulatory effects of different regions of the *JcGAST1* promoter, a series of 5'-deleted fragments of the promoter were fused with the *GUS* gene (Fig. 4A). As Fig. 4B shows, except for P4 (-359/-1 region), the *GUS* activity of these deleted promoter constructs was detectable, and P3 had the highest *GUS* activity. Consistently, the *GUS* staining intensity was notably enhanced in P3 compared with FL, indicating that P3 (-713/-319 region) may contain the core region of the *JcGAST1* promoter. Using *GUS* staining and the 5'-deleted constructs of P3, we further identified the core region of the *JcGAST1*

promoter and found that the – 381/-1 region had the highest GUS activity. These results suggest that the 22-bp region (AATAACCAGTGAAATTTGTTGT) may be the core region of the *JcGAST1* promoter.

GA and MeJA response analysis of the *JcGAST1* promoter

Due to the hormone response elements were predicted in the *JcGAST1* promoter, the effects of GA, MeJA, IAA and SA on the activity of the *JcGAST1* promoter were investigated. As shown in Fig. 5A, there were no obvious differences in GUS activity in FL and P1 upon treatment with these phytohormones. However, the GUS activity was significantly increased in P2 after GA treatment and decreased with SA treatment. Additionally, significantly decreased GUS activity was also observed in P3 after IAA, SA and MeJA treatment. Considering that the expression of *JcGAST1* was induced by GA treatment but downregulated with MeJA treatment (Fig. S3), we further identified the GA- and MeJA-responsive cis-acting element of the *JcGAST1* promoter. We found that P2 contains a predicted GA responsive element (pyrimidine box, CCTTTT), and a MeJA responsive element (CGTCA motif) was predicated in P3. We substituted the CCTTTT motif in P2 with CCGTTT to generate the mP2-GUS construct. In the presence of GA₃, the GUS activity of P2 was approximately 2-fold higher than that of mP2 (Fig. 5B). Meanwhile, CGGCA was substituted for the CGTCA motif of P3 to generate the mP3-GUS construct. In the presence of MeJA, the GUS activity of mP3 was approximately 9-fold higher than that of P3 (Fig. 5C). These results suggest that the transcripts of *JcGAST1* regulated by GA and MeJA are associated with the pyrimidine box and CGTCA motif.

Identify the interactors of GA- and MeJA-responsive elements in *J. curcas*

To identify potential regulators acting upstream of GA- and MeJA-responsive elements of the *JcGAST1* promoter, the 3×CCTTTT and 3×CGTCA motifs were inserted into a pHis2 vector for yeast one-hybrid (Y1H) screening using a cDNA library of *J. curcas* flowers. As a result, some cDNA fragments for 3×CCTTTT screening or 3×CGTCA screening were obtained. Using the NCBI database, these cDNA fragments were matched with *J. curcas* genes. The potential transcription factors are listed in Table S4, including six proteins for 3×CCTTTT screening (JcRF2a, JcIAA9, JcPAT1, JcERF106, JcAHL19 and JcbZIP11) and three proteins for 3×CGTCA screening (JcIAA9, JcAHL19 and JcSAP8). Subsequently, the interaction between these proteins and GA- or MeJA-responsive elements was verified with a Y1H assay. The results showed that yeast cells containing the construct pairs pHis2-CCTTTT and pGADT7-JcRF2a/JcERF106/JcAHL19/JcbZIP11/JcIAA9/JcPAT1 or pHis2-CGTCA and pGADT7-JcAHL19/JcSAP8/JcIAA9 grew well on SD/-Trp/-His/-Leu media supplemented with 3-AT, suggesting the potential interaction between these proteins and the two motifs (Fig. S6A). To determine the effects of the interaction between GA- or MeJA-responsive elements and their interactors on gene transcription, a dual-luciferase assay was performed. The 3×CCTTTT and 3×CGTCA motifs were fused to the *LUC* gene as the reporter. The coding sequences of the interactors were inserted into the pCAMBIA1305 vector as the effector constructs, and the *YFP* gene was used as the control (Fig. 6B). The dual-LUC assay results showed that the LUC/REN value was increased by coexpression with the 35S::JcRF2a/JcAHL19/JcbZIP11 and 3×CCTTTT-LUC constructs or the 35S::JcAHL19/JcSAP8 and

3×CGTCA-LUC constructs. These results indicated that these element interactors could bind to the CCTTTT or CGTCA motif to enhance the transcription of downstream genes.

Discussion

Members of the GASA gene family play an important role in the regulation of plant growth (Zhang and Wang, 2017). A large number of GASA genes have been identified in some plant species, but there have been no studies on the GASA gene in *J. curcas*. In this study, *JcGAST1* was cloned from *J. curcas*, which encodes a protein containing a highly conserved GASA domain (Fig. 1A). The regulative function of GASA genes in floral development has been described in previous studies, most of which were in bisexual flower plants (Kotilainen et al. 1999; Roxrud et al. 2007; Zhang et al. 2009). *J. curcas* is a monoecious plant, and sex differentiation of flowers occurs after the initiation of five petal primordia. Xu et al. reported that the development of *J. curcas* female flowers requires a hermaphroditic period: stamens first develop with carpels and then gradually degenerate as the three carpels to be fused (Xu et al. 2016). In contrast to the female flowers, the male flowers of *J. curcas* did not undergo a hermaphroditic period. Our transcriptome data indicate that the *JcGAST1* gene is differentially expressed in female and male flowers (data not shown). Both the transcript and protein levels of *JcGAST1* were higher in the male flowers than in the female flowers (Fig. 2). This means that *JcGAST1* may play different roles in female and male flowers. In *Arabidopsis*, *AtGASA14* is speculated to function in the promotion of cell elongation, and overexpression of *AtGASA14* promotes an increase in leaf size (Sun et al. 2013). Additionally, we observed that transgenic tobacco plants overexpressing the *JcGAST1* gene increased leaf size and plant height (Fig. 3A, C-E), suggesting that *JcGAST1* might be a contributor to vegetative growth in plants. Although *JcGAST1* showed a high level of expression in the male flowers (Fig. 2A), the development of stamens was not influenced in *35S::JcGAST1* transgenic tobacco plants. In contrast, significant inhibition was observed in the stigmas of transgenic tobacco plants (Fig. 3B, F). These phenotypic studies revealed a negative role for *JcGAST1* in the development of female organs, which may be the reason why *J. curcas* female flowers had a lower *JcGAST1* expression level. In gynoeceious plants, GA treatment represses pistil development in female flowers to produce neutral flowers but does not resume the development of stamens (Chen et al. 2013). Most GASA genes were upregulated by GA, such as tomato *SlGAST1*, *Arabidopsis AtGASA4*, *AtGASA6*, *AtGASA7*, *AtGASA8* and *AtGASA13*, and beech *FsGASA4*. (Shi et al. 1992; Zhang and Wang. 2008; Alonso-Ramírez et al. 2009; Rubinovich and Weiss. 2010). Similar to these genes, *JcGAST1* expression was also induced by GA. These results implied that the regulatory role of *JcGAST1* in plant reproductive growth was largely associated with the GA signaling pathway.

GASA family genes are thought to be integrators of phytohormone signals. In *Arabidopsis*, all GASA genes contain GA signaling-associated GARE and ABA signaling-related ABRE elements (Zhang and Wang. 2008). The promoter sequence analysis showed that the *JcGAST1* promoter not only contains GARE and ABRE elements but also contains TCA-element (SA responsive element), TGA-element (Auxin responsive element) and CGTCA-motif (MeJA responsive element). Our deletion analysis found that the promoter region containing the GARE element was nonessential for the GA response. However, substitution of a base in the pyrimidine box decreased the activity of the *JcGAST1* promoter upon GA

treatment, suggesting that the pyrimidine box may be the key element for the GA response. The pyrimidine box was first found in cereal, and mutations in the pyrimidine box caused a reduction in GA induction (Huang et al. 1990; Gubler and Jacobsen. 1992; Rogers and Rogers. 1992). Previous studies reported that the transcription factors of the DNA Binding with One Finger (DOF) class could interact with the pyrimidine box (Mena et al. 2002). DOF transcription factors have been confirmed to be involved in the regulation of pollen development in maize (Yanagisawa. 2000; Chen et al. 2012). Except for the pyrimidine box, we noticed that the *JcGAST1* promoter also contains the GTGANTG10 and POLLEN1LELAT52 motifs, which are both pollen-specific expression elements. These results prompted us to speculate that, perhaps, there is a DOF-GAST1 module that regulates pollen development in *J. curcas*. This possibility should be addressed in the future. JA and MeJA are naturally occurring plant hormones that function as key signaling molecules in response to biotic and abiotic stresses. In strawberry, *FaGAST2* expression was not affected by MeJA (Moyano-Cañete et al. 2013). However, in *Arabidopsis*, the expression of *AtGASA4* and *AtGASA6* was downregulated by JA (Qu et al. 2016). The *JcGAST1* promoter contains a CGTCA motif, and both qPCR and GUS activity assays proved that MeJA plays a negative role in *JcGAST1* transcription. Using a Y1H screen, we found that the three transcription factors JcIAA9, JcSAP8 and JcAHL19 interacted with the CGTCA motif. IAA9 has been reported to act as a transcriptional repressor of auxin signaling in tomato (Wang et al. 2005). AHL19 and SAP8 have been described to be involved in stress resistance in *Arabidopsis* and barley, respectively (Yadeta et al. 2011; Baidyussen et al. 2021). Due to the promotional effect of *JcGAST1* on plant growth, there may be some regulatory factors that repress GAST1 to maintain energy to ensure that the organism can survive in adverse environments. Significantly, JcIAA9 and JcAHL19 were also found in the Y1H screen of the pyrimidine box, suggesting that they may cross-link GA and MeJA signaling to regulate *JcGAST1*. Additionally, we found that four other transcription factors, JcbZIP11, JcERF106, JcRF2a and JcPAT1, all interact with the pyrimidine box. JcbZIP11 and JcRF2a are members of the bZIP transcription factor family, and their homologs in other species have been described to be involved in pathogen resistance (Dai et al. 2004; Prior et al. 2021). Homologs of JcERF106 and JcPAT1 have been reported to be involved in ethylene and JA signaling (Feng et al. 2020; Wang et al. 2021). However, the regulatory relationship between these transcription factors and *JcGAST1* requires further investigation.

In summary, our results indicate that JcGAST1 plays an important role in the regulation of plant vegetative growth and inhibition of the development of female organs. GA and MeJA act as inducers and inhibitors of the expression of *JcGAST1*, respectively. Our present research provides valuable insight into the potential regulatory mechanisms of JcGAST1 during floral development. Further studies will focus on the upstream transcriptional regulators of *JcGAST1*.

Declarations

Acknowledgments

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Author contributions

GX designed and managed this study; SL, LZ and YC performed the experiments; LZ and YC analyzed the data; SL and GX wrote the manuscript; all authors read and approved the manuscript.

Conflict of interest

The authors have no conflicts of interest to declare.

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Figures

Figure 1

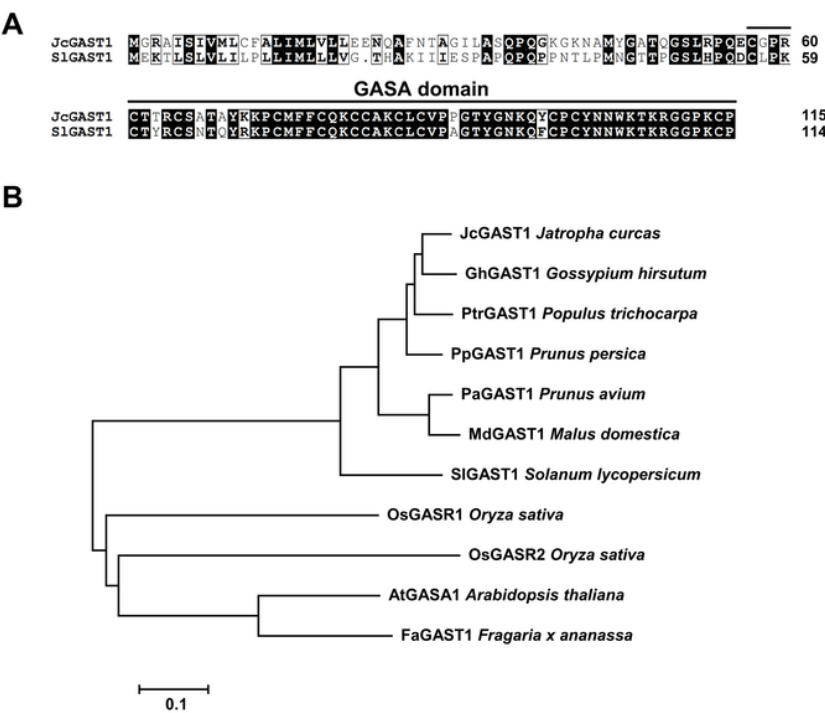


Figure 1

Bioinformatics analysis of JcGAST1

(A) Amino acid sequence alignment of JcGAST1 and SIGAST1. Identical and similar amino acids are indicated by black and white boxes, respectively. The GASA domain is highlighted via a black line. (B) Phylogenetic analysis of JcGAST1 and its homologs in plants. The neighbor-joining tree was constructed using MEGA software (version 6.0). Scale bar: 0.1 estimated amino acid substitutions per site.

Figure 2

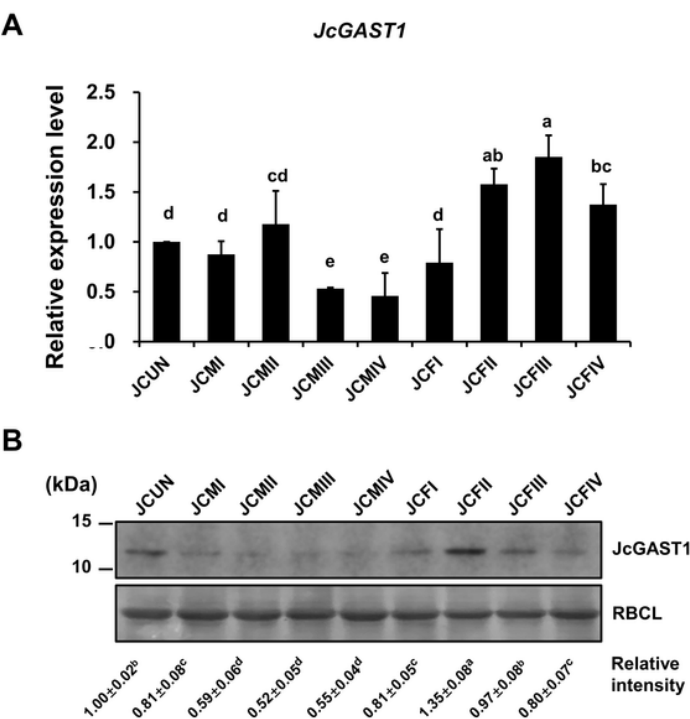


Figure 2

Expression pattern of *JcGAST1* and protein levels during flower development

(A) qPCR analysis of *JcGAST1* transcripts at different flower development stages (JCUN, undifferentiated stage; JCMI, the formation of ten stamen primordia; JCMII, from the occurrence of sporogenous cells to the occurrence of microspore mother cell; JCMIII, from the meiosis of microspore mother cell to the formation of meiotic tetrads; JCMIV, from free microspore stage to the maturation of pollen grain; JCFI, the growth of three carpels; JCFII, from the occurrence of sporogenous cells to the occurrence of macrospore mother cell (MMC); JCFIII, from the meiosis of MMC to the formation of mononuclear embryo sac; JCFIV, from the formation of MES to the maturation of embryo sac). *JcTUB* served as an internal control. (B) Protein levels of *JcGAST1* at different flower developmental stages. Immunoblot analysis was performed using an anti-*JcGAST1* antibody. Coomassie blue-stained Rubisco large subunit

(RBCL) was used as the loading control. Relative protein intensity was quantified by ImageJ software. The protein intensity at the JCUN stage was set to 1.0. Error bars represent the standard deviation (SD). Different letters above the columns indicate significant differences at $P < 0.05$.

Figure 3

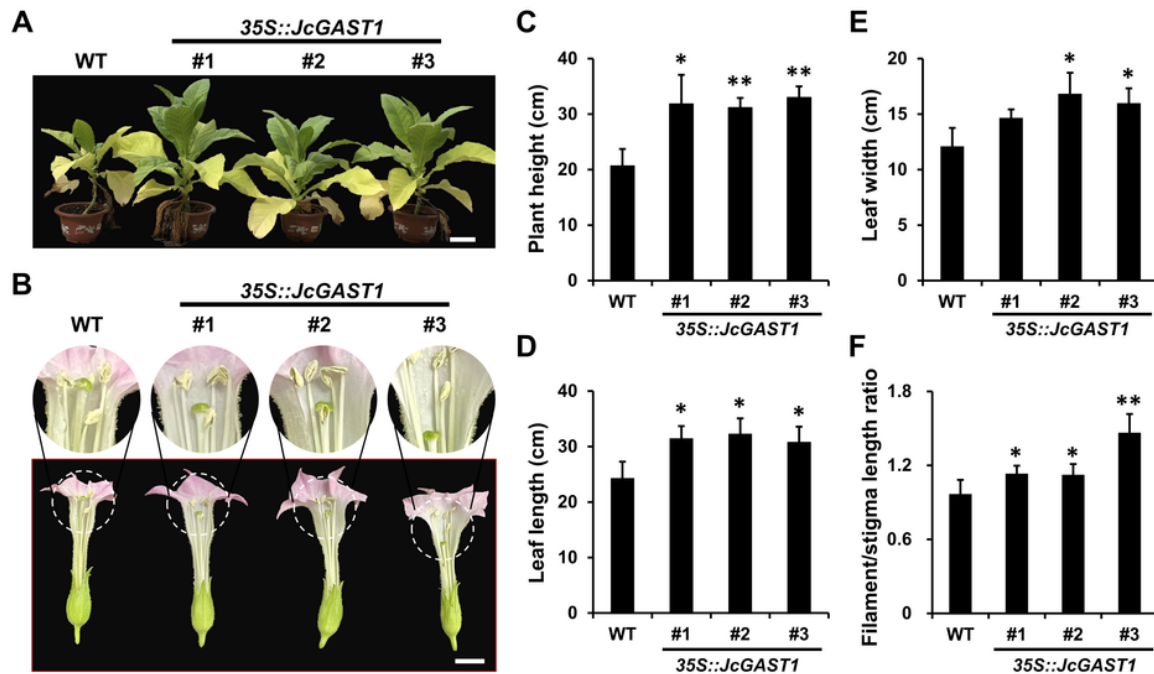


Figure 3

Phenotypic assay of *35S::JcGAST1* transgenic tobacco plants

(A) Representative images of 2-month-old WT and *JcGAST1* transgenic tobacco plants (#1, #2 and #3). (B) Morphology of transgenic tobacco plants flowers. The statistical analysis of plant height (C), leaf length (D), leaf width (E) and filament/stigma ratio (F) of WT and transgenic tobacco plants. Error bars represent the standard deviation (SD; $n \geq 3$, ttest: * $P < 0.05$, ** $P < 0.01$). Scale bar = (A) 5 cm; (B) 1 cm.

Figure 4

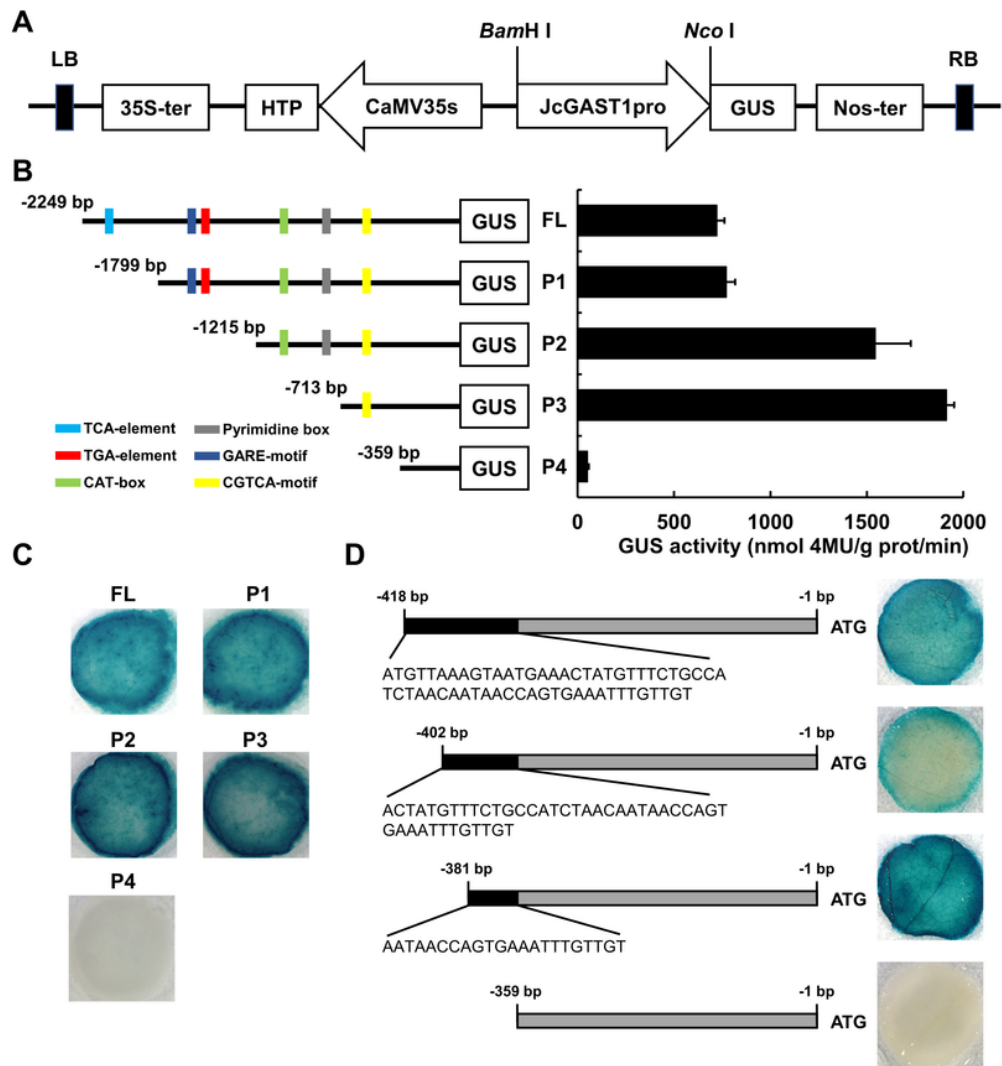


Figure 4

Deletion analysis of the *JcGAST1* promoter

(A) Schematic diagram of *proJcGAST1::GUS* constructs. (B) Schematic representation of various *JcGAST1* promoter deletions (left panel) and the fluorometric assay of GUS activity in the anthers (right panel). (C) Histochemical GUS staining of tobacco leaf discs expressing deleted constructs. (D) Identification of the *JcGAST1* promoter core region. Error bars represent the standard deviation (SD).

Figure 5

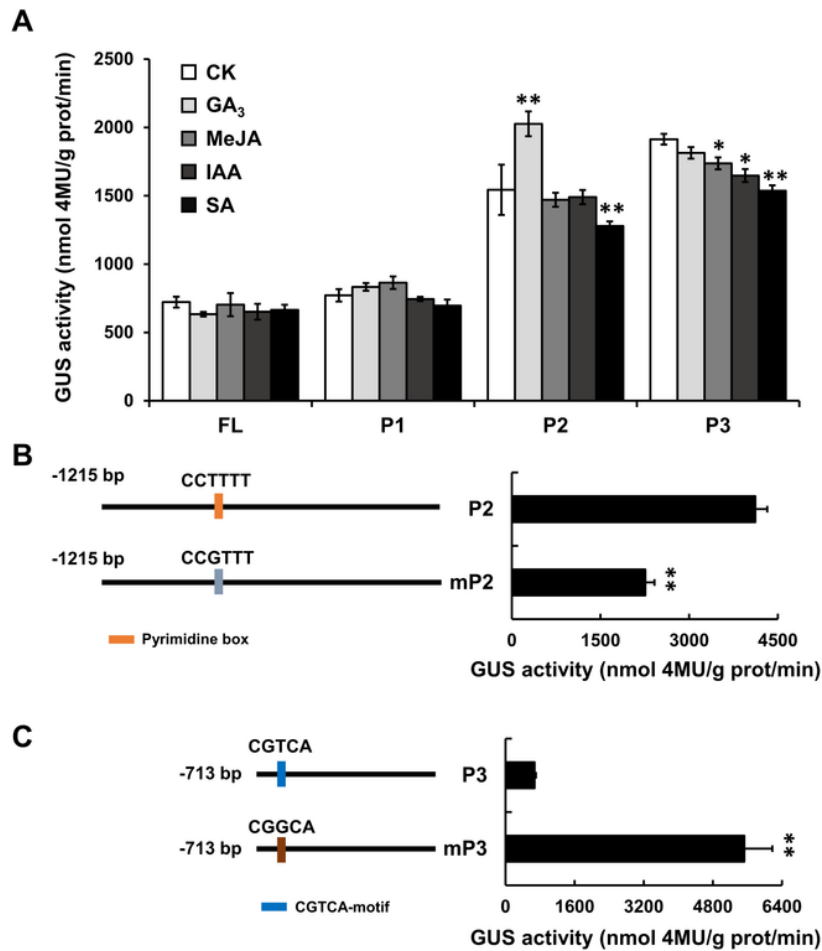


Figure 5

Promoter activity analysis of *JcGAST1* upon phytohormone treatment

(A) GUS activity of *JcGAST1* promoter deletions in response to GA₃, MeJA, IAA and SA. Identification of cis-acting GA (B) and MeJA (C) responsive elements in the *JcGAST1* promoter. Error bars represent the standard deviation (SD; n ≥ 3, *t* test: ** *P* < 0.01).

Figure 6

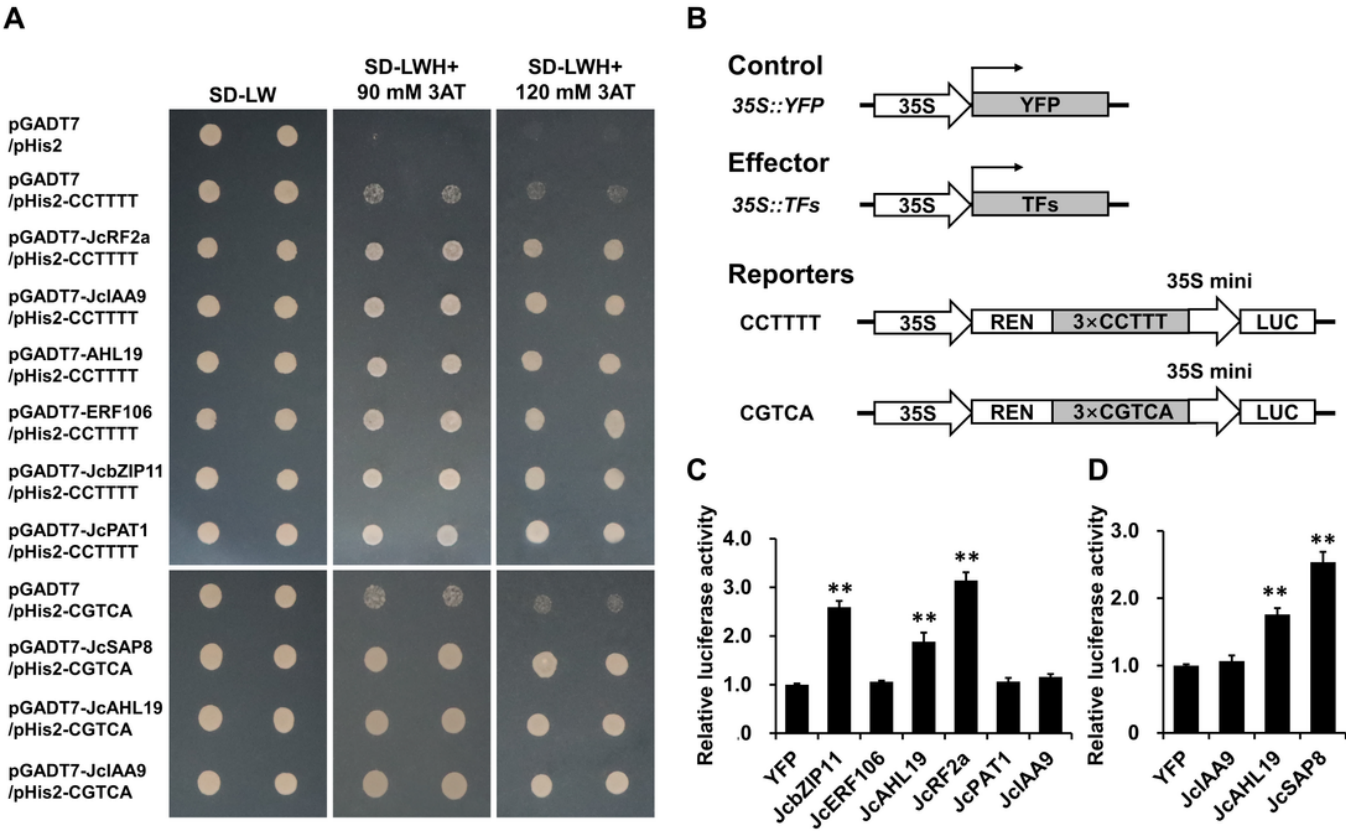


Figure 6

Identification of interactors of *J. curcas* cis-acting GA- and MeJA-responsive elements

(A) Interactions of JcRF2a, JcIAA9, JcAHL9, ERF106, JcbZIP11, JcPAT1, and JcSAP8 proteins with cis-acting GA- and MeJA-responsive elements in yeast cells. (B) Diagrams of reporter and effector constructs in transient dual-luciferase assays. Effects of JcRF2a, JcIAA9, JcAHL9, ERF106, JcbZIP11, and JcPAT1 on the activities of cis-acting GA responsive elements (C) and JcIAA9, JcAHL9 and JcSAP8 on the activities of cis-acting MeJA responsive elements in tobacco leaf cells. Error bars represent the standard deviation (SD; $n \geq 3$, t test: ** $P < 0.01$).

Supplementary Files

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