A Cucumber DELLA Homolog CsGAIP May Inhibit Staminate Development through Transcriptional Repression of B Class Floral Homeotic Genes

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Abstract

In hermaphrodite Arabidopsis, the phytohormone gibberellin (GA) stimulates stamen development by opposing the DELLA repression of B and C classes of floral homeotic genes. GA can promote male flower formation in cucumber (Cucumis sativus L.), a typical monoecious vegetable with unisexual flowers, and the molecular mechanism remains unknown. Here we characterized a DELLA homolog CsGAIP in cucumber, and we found that CsGAIP is highly expressed in stem and male flower buds. In situ hybridization showed that CsGAIP is greatly enriched in the stamen primordia, especially during the hermaphroditic stage of flower development. Further, CsGAIP protein is located in nucleus. CsGAIP can partially rescue the plant height, stamen development and fertility phenotypes of Arabidopsis rga-24/gai-t6 mutant, and ectopic expression of CsGAIP in wide-type Arabidopsis results in reduced number of stamens and decreased transcription of B class floral homeotic genes APETALA3 (AP3) and PISTILLATA (PI). Our data suggest that monoecious CsGAIP may inhibit staminate development through transcriptional repression of B class floral homeotic genes in Arabidopsis.

Introduction

Gibberellins (GAs) are one class of tetracyclic diterpenoid phytohormones that play essential roles in diverse aspects of plant growth and development, including seed germination, hypocotyl elongation, root growth, stem elongation, leaf expansion, trichome formation, floral induction, flower development, and fruit development [1], in which, floral induction and flower development are the most important events regulated by GA [2]. GA content has been shown to increase dramatically before anthesis in flowers of the most important events regulated by GA [2]. GA content has been shown to increase dramatically before anthesis in flowers of monocotyledonous and dicotyledonous species, such as rice and tomato (Solanum lycopersicum), whereas stimulate pistillate development in castor bean (Ricinus communis), Hymenocallis and maize (Zea mays) [3].

Several key enzymes have been identified to be involved in GA biosynthesis, such as copalyl diphenyl sulfate synthase (CPS), ent-kaurene synthase (KS), ent-kaurene oxidase (KO) and ent-kaurenio acid oxidase (KAO) [4], and their activity is critical for GA-dependent growth and floral organ development [5–9]. Similarly, GA signal transduction factors play important roles in flower development. The GA receptors are encoded by three homologous GIBBERELLIN-INSENSITIVE DWARF1 (GID1) genes (AtGID1a, AtGID1b and AtGID1c) in Arabidopsis [10]. Despite single mutant or double mutants of gid1 display no or partial GA-deficient phenotypes respectively, triple mutant showed severe GA-deficient abnormality, including extremely dwarfism, delayed flowering, incomplete floral organs and GA-insensitivity [11]. Similarly, in rice, mutation of the GA receptors leads to GA-insensitive and dwarf phenotypes, while overexpression of GID1 results in early flowering [12]. Another key player in GA signaling pathway is DELLA repressors [13,14]. Binding of GA to GID1 promotes the interaction between GID1 and DELLA proteins, which leads to rapid degradation of DELLA proteins through the SCFCOI1/GID2 (Skp1, Culin, F-box complex) ubiquitin-proteasome pathway, and the proteolysis of DELLA proteins releases their inhibitory effect on GA-responsive genes and allows plant growth and development [1,15–19]. DELLA proteins belong to a subfamily of the GRAS family and have five members in Arabidopsis: RGA (REPRESSOR OF ga1-3), GAI (GIBBERELLIN INSENSITIVE), RGL1 (RGA-LIKE 1), RGL2 (RGA-LIKE 2), and RGL3 (RGA-LIKE 3) [1,20]. RGA and GAI are negative regulators for stem elongation [21–23]. RGA and RGL2 coordinately inhibit the development of petal, stamen and anther, while RGL1 exacerbates this repression [24–26]. Transient induction of RGA greatly downregulates the transcription of floral homeotic genes APETALA3 (AP3), PISTILLATA (PI) and AGAMOUS (AG), while removing the RGL2 and RGA DELLA activities in ga1-3 mutant (rga1-3 rgl2-1 rga+2) can...
rescue the phenotypes of flower development, including delayed flowering time, aberrant petal, stamen and anther development, suggesting that GA regulates flower development via degradation of DELLA proteins, especially RGA and RGL2, thus allows the transcription of floral homeotic genes [21,24,27]. GsMIB, on the other hand, acts as a positive regulator for GA signaling pathway [28–30]. Mutation of the GsMIB in Arabidopsis (myb33myb65) results in shorter filaments, pollen abortion and male sterility, similar to the GA-insensitive phenotype [31]. In rice, GsMIB is involved in programmed cell death (PCD) of tapetal cells, exine and ubisch body formation, as well as in the GA-induced anther development [32].

However, so far, most GA-regulated flower development studies were performed in hermaphroditic species, and rarely in monoecious plants. Cucumber (Cucumis sativus L.) is a typical monoecious vegetable with individual male and female flowers, and has been served as a model system for sex determination in planta [33]. GA can promote male flower formation in cucumber, and the molecular mechanism remains unknown. In this study, we found that cucumber homologs of GA signal transduction factors GI, DELLA and GAMYB have much higher expression than those of GA synthesis genes during male flower development, and the cucumber DELLA homolog CsGAIP has the highest expression. We cloned the CsGAIP and characterized its spatial and temporal expression patterns. CsGAIP is mainly expressed in stems and male flower buds, and CsGAIP protein is located in nucleus. Ectopic expression of CsGAIP can partially rescue the phenotypes of gai-24/gai-16 double mutant in Arabidopsis, and overexpression of CsGAIP in wild type resulted in reduced number of stamens and decreased transcripion of B class floral homeotic genes. Our results suggested that CsGAIP inhibits stamen development through transcriptional repression of B class floral homeotic genes in Arabidopsis.

Results

Cucumber DELLA homolog GAIP may have prominent role during male flower development

GA has been shown to promote male flower development in cucumber [3], but the underlying mechanism remains elusive. As the first step to uncover this mystery, we explored the expression patterns of cucumber homologs of GA biosynthesis genes CPS, KS, KO, KAO and GA signal transduction factors GI, DELLA and GAMYB during different stages of male flower development. Using the sequence information in Arabidopsis, we performed BLAST analysis in Cucumber Genome Database [34], and defined the best hit as the corresponding cucumber homolog and the relative unique region of each gene was designed for quantitative real-time RT-PCR (qRT-PCR) analyses.

The developmental process of cucumber male flower can be divided into 12 stages [35], in which, five stages including hermaphrodite stage (stage 5), microsporocyte stage (stage 9), meiosis stage (stage 10), uninuclear pollen stage (stage 11) and mature pollen stage (stage 12) were identified based on morphological indications [35,36] (Figure 1A) and the lengths of cucumber male floral buds for each stage was calculated (Table 1). Then, RNA samples were collected from at least three independent male flower buds and qRT-PCR was performed using these samples. As shown in Figure 1B, GA signal transduction factors GI, GAIP (the best hit for DELLA homolog) and GAMYB have much higher expression than those of GA synthesis genes CPS, KS, KO, KAO during cucumber male flower development. In which, GAIP has the highest expression among all, particularly in the hermaphrodite stage (stage 5), for example, expression of GAIP is more than 20 fold and 6 fold higher than GA synthesis genes and other GA signal transduction factors, respectively. Further, expression of GAIP decreases as the male flower develop, suggesting that cucumber GAIP may play a key role during male flower development and promote male determination in the hermaphrodite stage.

Cloning and phylogenetic analysis of cucumber DELLA homolog CsGAIP

Through BLAST analysis, we found four DELLA homologs in cucumber, CgGAIP (Csa015258), CgGAI (Csa0153919), CgGAI2 (Csa008181) and CgGAI3 (Csa015258), in which CgGAIP has the highest similarity to DELLA in Arabidopsis, so we chose CgGAIP for further analysis in this study. CgGAIP was cloned using cdNA derived from cucumber leaves through PCR technology. The full-length CgGAIP cdDNA consists of 1761 bp and encodes 587 amino acids. Consistent with the five DELLA genes of Arabidopsis, CgGAIP gene also has no introns [13,14]. Previous studies showed that DELLA proteins belong to a GRAS subfamily that contains two highly conserved domains named as DELLA and VHIYNP in their N-terminal regions [14,22,37]. Sequence alignment of the N-terminal 150 amino acid residues of CsGAIP using ClustalW indicated that CsGAIP also has the DELLA and VHIYNP domains, which may be essential for GID1-DELLA interaction [11,38–42] (Figure 2A). Full-length CsGAIP is 89.25%, 64.72%, 64.91%, 53.28%, 51.96%, 52.53%, 52.9% identical to CmGAIP, AtRGA, AtGAI, ZmD8, TaRHT1, HvSLN1, OsSLR1, respectively. To understand the evolutionary relationship between CsGAIP and other DELLA proteins, we constructed phylogenetic tree using the neighbor-joining (NJ) method [43] (Figure 2B). cucumber CsGAIP, CgGAI2 and CgGAI3 are placed in the same cluster as other DELLA homologs, while CsGAI1 is distantly related, suggesting that CsGAIP, CgGAI2 and CgGAI3 are more likely to be the DELLA homologs in cucumber. Phylogenetic tree divides DELLA homologs into two clades: dicotyledon (green line) such as Arabidopsis, cucumber, pumpkin (Cucurbita maxima), lettuce (Lactuca sativa), pea (Pisum sativum), bean (Phaseolus vulgaris), and monocotyledon (red line) such as maize, rice, barley and wheat. Within dicotyledon clade, CsGAIP and CmGAIP, which belong to the cucurbitaceae family with unisexual flowers, fall into the same clade that is distinct from those of CgGAI2, CgGAI3 and other DELLA homologs in hermaphroditic species, such as Arabidopsis, lettuce, pea and bean, implying that CsGAIP may be involved in the unisexual flower development in cucumber.

Expression pattern of DELLA homologs in cucumber

To characterize the spatial distribution of DELLA homologs transcripts, qRT-PCR was performed in various cucumber tissues including roots, stems, leaves, male flower buds, female flower buds and fruits. As shown in Figure 3, expressions of CgGAIP and CgGAI2 are much higher than those of CgGAI1 and CgGAI3 in all the tissues we examined, and that CgGAIP and CgGAI2 display similar expression patterns, which are predominantly expressed in stems and male flower buds. CgGAI3 transcript is more enriched in roots as compared to other tissues, while CgGAI1 shows equivalent expression in all the tissues we tested. Among all the four DELLA homologs, CgGAIP displays the highest expression especially in stems and male flower buds, implicating that CgGAIP may play important roles in stem and male flower development.

Next, we examined the expression pattern of CgGAIP during male flower development of cucumber by in situ hybridization (Figure 4). CgGAIP RNA was found throughout in the inflorescence meristem (im) and floral meristem (fm) (Figure 4A), as well as in the vascular strands (arrow in Figure 4A) in stage 1 male flowers [35].
During stages 2–6 (hermaphrodite stage), CsGAIP is expressed in the developing sepals, petals, stamens and carpels, with the strongest expression in stamen primordia (arrows in Figure 4B–E). As the male flower further develops, microsporocytes initiate in stage 9, uninuclear pollen appear in stage 11 and mature pollen form by stage 12, and CsGAIP is expressed mainly in the microsporocytes (Figure 4F), anther wall and pollen grains (Figure 4G–J), despite the signal is weaker than those in hermaphrodite stage. This data is consistent with the higher expression in hermaphrodite stage as detected by qRT-PCR (Figure 1B). As negative controls, CsGAIP sense probe hybridizations show no signals in male flowers of stage 1, stage 6, stage 9 and stage 12 (Figure 4K–N).

Subcellular localization of CsGAIP

In Arabidopsis, the DELLA proteins RGA and GAI have been shown to contain putative nuclear localization signal (NLS) and...
localize in nucleus [14]. Sequence alignment of the N-terminal 200–300 amino acid residues of CsGAIP with other DELLA proteins showed that CsGAIP also has a putative NLS (Figure 5A). Subcellular localization of CsGAIP in cucumber protoplasts indicated that CsGAIP locates in nucleus as well (Figure 5B, top row), and the same result was found in epidermal cells of onion (Allium cepa) (Figure 5C, top row). As a control, signals of 35S:GFP were detected throughout the cell (Figure 5B and C, bottom row).

CsGAIP can partially rescue rga-24/gai-t6 double mutant in Arabidopsis

To explore the function of CsGAIP, we ectopically expressed the full-length CsGAIP cDNA under the control of 35S promoter of Cauliflower mosaic virus (CaMV) in Arabidopsis rga-24/gai-t6 double mutant, and 13 independent transgenic lines were obtained. Previous study reported that rga-24/gai-t6 double mutant displayed higher plant height, reduced number of pollens, shorter filaments and thus decreased seed numbers per silique [21]. As showed in Figure 6 and Table 2, all the transgenic lines display partial rescue of the rga-24/gai-t6 phenotypes. The average plant height of rga-24/gai-t6 plants is 38% taller than that of Ler, while in the transgenic lines, the average plant height is only 8% taller than that of Ler (Fig. 6A; Table 2), suggesting that CsGAIP can greatly rescue the plant height phenotype in Arabidopsis. Further, flowers in the CsGAIP transgenic plants display increased filaments length and amount of pollen as compared to those in rga-24/gai-t6 (Fig. 6B, C). Consequently, the silique length and the seed number per silique

Figure 2. Sequence alignment and phylogenetic analyses of CsGAIP and related DELLA proteins. (A) Sequence alignment of the 150 amino acid residues of CsGAIP N-terminal with other DELLA proteins. The identical and similar residues are shown in black and gray, respectively. The highly conserved DELLA and VHYNP domains are indicated in black lines. At, Arabidopsis thaliana; Cm, Cucurbita maxima; Cs, Cucumis sativus; Zm, Zea mays; Os, Oryza sativa; Hv, Hordeum vulgare; Ta, Triticum aestivum. (B) Phylogenetic analyses of CsGAIP and related DELLA proteins using MEGA5 software based on the neighbor joining method. Homologs of DELLA from six dicotyledon species (green line) and four monocotyledon species (red line) were used for the analyses and formed distinct clade (dicotyledon group and monocotyledon group). The four DELLA homologs from cucumber are indicated in red boxes. Gene ID for each of the DELLA protein used for this analysis is listed in the “accession numbers”. Ls, Lactuca sativa; Ps, Pisum sativum; Pv, Phaseolus vulgaris.

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increase in the transgenic plants (Figure 6D–I). For example, there are around 8 seeds per siliques in the rga-24/gai-t6 plant, while ectopic expression of CsGAIP in rga-24/gai-t6 background results in 43 seeds/silique, which is close to that in Ler (56 seeds/silique) (Table 2). These data suggested that cucumber CsGAIP can partially replace the function of RGA and GAI in Arabidopsis with respect to plant height, stamen development and plant fertility.

CsGAIP suppresses stamen development by down-regulating floral homeotic genes AP3 and PI in Arabidopsis

We further explore the function of CsGAIP by overexpression of CsGAIP in Arabidopsis wide-type Ler, and 25 independent transgenic lines were obtained. As shown in Figure 7A, ectopic expression of CsGAIP in Arabidopsis led to reduced number of stamens. In contrast to the six stamens in Ler flowers, the flowers in 35S::CsGAIP plants only display 4.6 ± 0.5 stamens (Table 3). Given that the floral homeotic genes, including APETALA1 (AP1), APETALA2 (AP2), APETALA3 (AP3), PISTILLATA (PI) and AGAMOUS (AG), are involved in floral patterning in Arabidopsis [44], and that B genes (AP3 and PI) and C gene (AG) are down-regulated by RGA activity [27], we examined the expression of floral homeotic genes in 35S::CsGAIP plants by qRT-PCR and semi-quantitative RT-PCR. We found that the expression of A class (AP1 and AP2) and C class of gene (AG) were not substantially changed in the transgenic plants, but transcripts of B function genes (AP3 and PI) were significantly decreased (Fig. 7B). For example, the transcripts of AP3 and PI in the 35S::CsGAIP plants were reduced by around 80% and 50% respectively as compared to those in the Ler background. These data suggested that CsGAIP can suppress the expression of B function genes in Arabidopsis, which may be the cause for reduced number of stamens as observed in the ectopic expression lines.

Discussion

Cucumber (Cucumis sativus L.) is a monoecious species with individual male and female flowers. During the early stage of flower development, both stamen primordia and carpel primordia are initiated, male or female flower is generated upon the arrestment of carpel or stamen development, respectively [33,35]. Due to the agricultural importance, extensive studies have been performed in the mechanism of female flower formation, while the molecular regulation of male flower generation is largely unknown [45–51]. GA can regulate flower development in both hermaphroditic and monococious species [3]. In Arabidopsis, GA promotes stamen development by antagonizing the function of DELLAs [24]. In monococious cucumber, how GA stimulates male flower development remains elusive. Here we found that the cucumber DELLAs homolog may play important roles during male flower development in cucumber (Figure 1, Table 1), and we cloned this DELLAs homolog CsGAIP (Figure 2) and investigated the expression pattern and subcellular localization (Figure 3–5). Further, we explored the function of CsGAIP through ectopic overexpression of CsGAIP in Arabidopsis (Figure 6 and 7, Table 2 and 3). Our data suggested that monococious CsGAIP may repress staminate development through transcriptional downregulation of B class floral homeotic genes in Arabidopsis.

CsGAIP may be the homolog for both RGA and GAI in cucumber

In Arabidopsis, DELLAs family has five members: RGA, GAI, RGL1, RGL2, and RGL3 [20], which coordinate function in stem
expression of CsGAIP showed no signals in K–N. The arrow in A indicated the vascular expression of CsGAIP was shown in H and J, respectively. 12 (I and N). The pollen morphology in the framed regions of G and I (A and K, early stage 1) and male flower buds at stage 2 (B), stage 4 (C), stage 5 (D), stage 6 (E and L), stage 9 (F and M), stage 11 (G) and stage 12 (I and N). The pollen morphology in the framed regions of G and I was shown in H and J, respectively. CsGAIP sense probe hybridizations showed no signals in K–N. The arrow in A indicated the vascular expression of CsGAIP, and the arrows in C–J showed the strong expression of CsGAIP in developing stamen or pollen. im, inflorescence meristem; fm, floral meristem; s, sepal; p, petal; st, stamen; c, carpel. Bar = 200 μm.

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Figure 4. In situ hybridization of CsGAIP during male flower development in cucumber. Longitudinal sections of the shoot apex (A and K, early stage 1) and male flower buds at stage 2 (B), stage 4 (C), stage 5 (D), stage 6 (E and L), stage 9 (F and M), stage 11 (G) and stage 12 (I and N). The pollen morphology in the framed regions of G and I was shown in H and J, respectively. CsGAIP sense probe hybridizations showed no signals in K–N. The arrow in A indicated the vascular expression of CsGAIP, and the arrows in C–J showed the strong expression of CsGAIP in developing stamen or pollen. im, inflorescence meristem; fm, floral meristem; s, sepal; p, petal; st, stamen; c, carpel. Bar = 200 μm.

Unisexual CsGAIP displays conserved as well as divergent functions with its bisexual homologs

Loss of function of RGA and GAI in Arabidopsis results in higher plant height and earlier flowering [21], while lack of DELLA homologs REDUCED HEIGHT and DWARF8 leads to dwarfism in wheat and maize, respectively [37,57–59], indicating that DELLA homologs have conserved role in stem elongation, but the specific role may even opposite in different species. In this study, CsGAIP is highly expressed in cucumber stems (Figure 3) and that CsGAIP can rescue the plant height phenotype of rga-24/gai-t6 (Fig. 6, Table 2), suggesting that CsGAIP may also function as a suppressor for stem elongation as those of Arabidopsis RGA and GAI. Similarly, transcripts of CsGAIP are enriched in stamen primordia, and ectopic expression of CsGAIP can rescue the stamen development and plant fertility phenotypes in rga-24/gai-t6 (Figure 6, Table 2), and lead to reduced number of stamens and decreased expression of B function genes AP3 and PI upon ectopic expression in Ler (Figure 7, Table 3), supporting that CsGAIP has a conserved role in flower development, specifically, inhibits staminate development via repressing B class of floral homeotic genes. However, unlike the down-regulating of both B and C function genes upon RGA induction in Arabidopsis [27], the transcription of C class gene AG remains unchanged upon ectopic expression of CsGAIP (Figure 7B), similarly, flowering time appeared to be undisturbed upon overexpression of CsGAIP in Arabidopsis (data not shown), suggesting that monococious CsGAIP has divergent functions from RGA and GAI in hermaphroditic Arabidopsis.

Given that Arabidopsis DELLA s have specific as well as partially overlapping roles, it would be interesting to explore the specificity of the function for each DELLA homologue in cucumber. The four cucumber DELLA s display distinct expression patterns (Figure 3), in which CsGA1 has low transcript accumulation in all the tissues we examined, CsGA3 is predominantly expressed in roots, whereas CsGA2 and CsGA3 are highly enriched in stems and male flower buds, suggesting that CsGA2 and CsGA3 may play important and probably partially redundant roles in stem and...
male flower development in cucumber. However, for elucidating the functional similarities and differences among these four DELLAs, cucumber transformation, a currently difficult technique, is the best way to uncover the mystery in future studies. In addition, given that DELLA can regulate the cross-talks between GA and other signaling pathways through protein-protein interactions with regulatory factors such as PIF3/PIF4 (PHYTOCHROME-INTERACTING FACTOR 3/4), SCL3 (SCARECROW-LIKE 3), ALC (ALCATRAZ) and JAZs (JA ZIM-domain proteins) [18,60], identifying the DELLA interacting proteins will greatly advance our knowledge of the diverse functions of DELLA homologs in cucumber development.

**Materials and Methods**

**Plant materials and growth conditions**

A monoecious cucumber (*Cucumis sativus* L.) line 3461 was used in this study. The plants were grown in a growth chamber under...
Functional Analysis of Cucumber GAIP

Cloning of CsGAIP, sequence alignment and phylogenetic analysis

Total RNA was extracted from cucumber leaves using the Promega’s SV Total RNA Isolation System, and cDNA was synthesized using MultiScribe reverse transcriptase (Applied Biosystems). The cDNA was amplified with primers CsGAIP-F (5’-ATGAAGAGGGAGCATCACATCTTC-3’) and CsGAIP-R (5’-TCACCTTAGGACCCGCAGGFTT-3’) at 95°C for 5 min; 30 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 2.5 min; and then 72°C for 10 min. The amino acid sequence of related DELLAs was performed using ClustalW in the MEGA5 software package, and the boxes were drawn using the BoxShade web site (http://www.ch.embnet.org/software/BOX_form.html). The phylogenetic tree was constructed using the neighbor-joining (NJ) method [43] through MEGA5 software using the bootstrap analysis with 1000 replications.

Gene expression analysis

Total RNA was extracted from cucumber leaves using the Promega’s SV Total RNA Isolation System, and cDNA was synthesized using MultiScribe reverse transcriptase (Applied Biosystems). Quantitative real-time RT-PCR (qRT-PCR) was performed using SYBR Premix Ex Tag from TaKaRa (China) on an Applied Biosystems 7500 real-time PCR system. The cucumber α-tubulin gene (TU14) and Arabidopsis β-tubulin gene (TUB2) were used as internal references. For semi-quantitative RT-PCR, the β-tubulin gene (TUB2) was used as a control. Both qRT-PCR and semi-quantitative PCR were repeated in three independent samples. The gene primers for qRT-PCR were as follows: CPS-F (5’-GCTGAGGCTCAATG-GACGATG-3’) and CPS-R (5’-TATTTCTGATGTCGATGTTTGGGC-3’); CsGAI1-F (5’-CCCATCGCTTAAGAGTAAGAACAC-3’) and CsGAI1-R (5’-ATGAAGAGGGAGCATCACATCTTC-3’); KO-F (5’-AAGAGGCTATGGTGACGAGGTA-3’) and KO-R (5’-ACATGAGCAAACAACTCCCGTATA-3’); KAO-F (5’-CACTCAAGGCTCGGAAGAATCT-3’) and KAO-R (5’-CAAGCATCATACTAGGGCTCCAT-3’); CsGAIP-F (5’-GCTCAAAGGCCGATGAAAG-3’) and CsGAIP-R (5’-GCTATGAGTGGGCGAGTGTG-3’); CsGAI2-F (5’-TAAAGAGCAGCAAGGCGAGATA-3’) and CsGAI2-R (5’-TAACCTACCTCCGACAAACACC-3’) and CsGAI3-F (5’-GGAGGAAGCCGACGACTAC-3’) and CsGAI3-R (5’-CGGAGTTTATTTGATGTTTGGGCA-3’); GAMYB-F (5’-TCAATTCTACCACCAAAAGAC-3’) and GAMYB-R (5’-TGCTGAGGTCAATG-3’); TUA-F (5’-TTCACGAGAGAACTCCACATCC-3’) and TUA-R (5’-ATCCAGACCTTCCTCCAT-3’); TTAATACGACTTATACACATCT-3’); GID1-F (5’-ATCCGAGCATGTAATCCCCGCTGCATG-3’) and GID1-R (5’-CAATGACATTTCGCCCTCC-3’); TAC1-F (5’-CCAACCAAGCCGACAACTCAC-3’) and TAC1-R (5’-GTCTGAGGGAAGACAAAGCATC-3’); AP1-F (5’-GTTGCTGACAACATTGTAGAAG-3’) and AP1-R (5’-CTTTCTGTAGGCTACATCAG-3’); AP2-F (5’-GAGAGGGGTAAACAGTGAATC-3’) and AP2-R (5’-GAGAGGGGTAAACAGTGAATC-3’); API-F (5’-GGTGAGGATGGTTTGGTG-3’) and API-R (5’-GGTGAGGATGGTTTGGTG-3’); GAMYB-F (5’-TCAATTCTACCACCAAAAGAC-3’) and GAMYB-R (5’-TGCTGAGGTCAATG-3’); TUA-F (5’-TTCACGAGAGAACTCCACATCC-3’) and TUA-R (5’-ATCCAGACCTTCCTCCAT-3’); TTAATACGACTTATACACATCT-3’); GID1-F (5’-ATCCGAGCATGTAATCCCCGCTGCATG-3’) and GID1-R (5’-CAATGACATTTCGCCCTCC-3’); TAC1-F (5’-CCAACCAAGCCGACAACTCAC-3’) and TAC1-R (5’-GTCTGAGGGAAGACAAAGCATC-3’); AP1-F (5’-GTTGCTGACAACATTGTAGAAG-3’) and AP1-R (5’-CTTTCTGTAGGCTACATCAG-3’); AP2-F (5’-GAGAGGGGTAAACAGTGAATC-3’) and AP2-R (5’-GAGAGGGGTAAACAGTGAATC-3’); API-F (5’-GGTGAGGATGGTTTGGTG-3’) and API-R (5’-GGTGAGGATGGTTTGGTG-3’).
ACTTTTGTTCTTTTTCTTGGTGGT-3; PI-F (5'-TGATCTCCAT-CTGGTGTTCTCG-3'); AG-F (5'-ATAATCAGCATACAA-ACTCCAAC-3') and AG-R (5'-ATACTTCTCTCTAATCTGCCTTCC-3'); TUB2-F (5'-ATCCGTGAAGAGTACCCA-GAT-3') and TUB2-R (5'-AAGAACCATGCACTCATCAGC-3').

The primers for AP1, AP2, AP3, PI and AG for semi-quantitative PCR were performed as previously reported [27].

**Table 2.** CsGAIP can rescue the plant height and fertility of rga-24/gai-t6 in Arabidopsis.

| Genotype              | Number of plants | Plant height (cm) | Seeds/silique ± SE |
|-----------------------|------------------|-------------------|---------------------|
| rga-24/gai-t6         | 13               | 26.1±0.8 a        | 8.0±1.6 a           |
| 35S:CsGAIP rga-24/gai-t6 | 13               | 20.5±0.8 b        | 43.4±4.0 b          |
| Ler                   | 13               | 18.9±1.1 c        | 56.4±3.2 c          |

The values shown are the means ± SE of 13 plants from rga-24/gai-t6, 13 CsGAIP transgenic T1 lines or 13 Ler plants, respectively. Different letters (a–c) in the same column indicate significant differences (P<0.05) determined by Duncan’s test.

*Fertility was counted by the number of seeds per silique. Ten siliques were measured in each plant.

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**In situ hybridization**

Shoot apex of 10-day-old seedling and male flower buds from 45-day-old cucumbers grown in the greenhouse were fixed and hybridized as described [61]. Digoxigenin-labeled probes were generated through PCR amplification of cDNA using gene specific primers containing SP6 and T7 RNA polymerase-binding sites. SP6 and T7 RNA polymerase were used for the synthesis of sense and antisense probes, respectively. The primers of cucumber CsGAIP in situ probes were as follow: 5'-GATTTAGGTGACACTATAGAATGCTATCCGATGCCTAATTTTGCGA-3' (bold

**Figure 7.** Transcription analyses of floral homeotic genes upon ectopic expression of CsGAIP in WT Arabidopsis. (A) Stamens in Ler or lines of CsGAIP overexpression. (B) qRT-PCR (top) and semi-quantitative RT-PCR (bottom) analyses of floral homeotic genes in the inflorescence apices of Ler or CsGAIP overexpression lines. The β-tubulin gene (TUB2) was used as an internal control, and three biological replicates were performed for each gene. Asterisks indicate the significant differences (P<0.01) between Ler and CsGAIP overexpression lines determined by Duncan’s test.

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represents the SP6 RNA polymerase binding sites) and 5’-TGTATACGGACTATAGGCAACGCTATGCAAGCTAGCTAT-CGGACACT-3’ (bold shows the T7 RNA polymerase binding sites).

Subcellular localization in cucumber protoplasts and onion epidermal cells

For transient expression in cucumber protoplasts and onion epidermal cells, the full length coding region of CsGAIP were cloned using primers 5’-ACGGGTCGCAATGAAGAGGGAGGAGCTACACCATCTTC-3’ (Sal I site in bold) and 5’-CGGGATCTTACGGCCAAGGGTGTGTT-3’ (BanH I site in bold), and then inserted into the pEVS-NL vector (with GFP protein driven by 35S promoter) to generate 35SGFP-CsGAIP, and the empty pEVS-NL vector was used as control. The constructs were introduced into cucumber protoplasts using Huang’s method [62]. The onion epidermal layers were prepared and bombarded, as previously described [63], with gold particles containing the plasmid using a Bio-Rad PDS-1000/He particle delivery system. After bombardment, the onion epidermis were placed on MS medium and incubated in darkness at 22°C for 24 h. Fluorescence signals were detected using Olympus BX 51 fluorescence microscopy.

Ectopic expression of CsGAIP in Arabidopsis

To make the CsGAIP overexpression construct, full length CsGAIP cDNA were cloned using primers 5’-GGACTTGAAT-GAAGGAGGAAGCTACATCCATCTTC-3’ (Spe I site in bold) and 5’-GACGGACGGTGCATTAGGCACCCAGG-3’ (Pml I site in bold), and inserted into the pCAMBIA1305.1 vector with 35S promoter. The construct was then introduced into Agrobacterium by electroporation and transformed into Ler or gai-24/gai-t6 plants as described [64]. The transgenic plants were screened on MS medium with 25 mg/L hygromycin.

Accession numbers

Sequence data in this study can be found in the Cucumber Genome DataBase, Arabidopsis Genome Initiative or GenBank/EMBL/Swiss-Prot databases under the following accession numbers: CsGAIP (Csa021618), CsGAI (Csa015919), CsGAR (Csa001811), CsGAI2 (Csa015258), AtRGA (At2g01570), AtGAI (At1g1920), AtGRL1 (At1g66350), AtGRL2 (At3g03450), AtGRL3 (At5g17490), CsGAP (Q6E1O6), znD8 (Q9ST4B), TaRHT1 (Q9ST59), HvSLN1 (Q9SK17), OsSLR1 (Q7G2J6), LdDEL1 (BAG17102), LdDEL2 (BAG71101), PdLA (ABI30654), and PdGAP (BAF62637).

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Author Contributions

Conceived and designed the experiments: YZ HR XZ. Performed the experiments: YZ BL SY JA CC. Analyzed the data: YZ. Contributed reagents/materials/analysis tools: YZ. Wrote the paper: YZ XZ.

Table 3. Reduced numbers of stamens upon overexpression of CsGAIP in Arabidopsis.

| Genotype       | Number of plants | Number of stamens1 |
|----------------|------------------|--------------------|
| Ler            | 10               | 6.0±0.0 a          |
| 35S:CsGAIP Ler | 25               | 4.6±0.5 b          |

The values shown are the means ± SE of 25 CsGAIP transgenic T1 plants, or 10 Ler plants. Different letters (a and b) in the same column indicate significant differences (P<0.05) between Ler and transgenic plants determined by Dunnet’s test.

The number of stamens were the average of 20 flowers from each line. doi:10.1371/journal.pone.0091804.t003

References

1. Fleet CM, Sun TP (2005) A DELLA-acate balance: the role of gibberellin in plant morphogenesis. Curr Opin Plant Biol 8: 77–85.
2. King RW, Evans LT (2003) Gibberellins and flowering of grasses and cereals: prizing open the lid of the "florigen" black box. Annu Rev Plant Physiol Plant Mol Biol 48: 431–460.
3. Helliwell CA, Sheldon CC, Olive MR, Walker AR, Zeevaart JAD, et al. (1998) Gibberellin biosynthesis: enzymes, genes and their regulation. Annu Rev Plant Physiol Plant Mol Biol 48: 431–460.
4. Silverstone AL, Ciampaglio CN, Sun TP (1998) The Arabidopsis RGA gene regulates Arabidopsis floral development via suppression of DELLA protein catabolism, and response pathways. Plant Cell (Supplement): S61–S80.
25. Swain SM, Muller AJ, Singh DP (2004) The ga2 and rga alleles increase the growth of gibberellin-deficient pollen tubes in Arabidopsis. Plant Physiol 134: 694–703.
26. Tyler L, Thomas SG, Hu JH, Dill A, Alonso JM, et al. (2004) DELLA proteins and gibberellin-regulated seed germination and floral development in Arabidopsis. Plant Physiol 135: 1008–1019.
27. Yu H, Ito T, Zhao Y, Peng J, Kumar P, et al. (2004) Floral homeotic genes are targets of gibberellin signaling in flowering development. Proc Natl Acad Sci USA 101: 7827–7832.
28. Gocal GF, Sheldon CC, Gubler F, Moritz T, Bagnall DJ, et al. (2001) GAMYB-like genes, flowering, and gibberellin signaling in Arabidopsis. Plant Physiol 127: 1692–1693.
29. Gubler F, Kalla R, Roberts JK, Jacobsen JV (1995) Gibberellin-regulated expression of a myb gene in barley aleurone cell: evidence for Myb transactivation of a high-pI 35S-promoter gene promoter. Plant Cell 7: 1879–1891.
30. Gubler F, Raventos D, Keys M, Watts R, Mundy J, et al. (1999) Target genes and regulatory domains of the GAMYB transcriptional activator in cereal aleurone. Plant J 17: 1–9.
31. Millar AA, Gubler F (2005) The Arabidopsis GAMYB-like genes, MYB33 and MYB65, are microRNA-regulated genes that redundantly facilitate anther development. Plant Cell 17: 705–721.
32. Ay K, Ueguchi-Tanaka M, Kondo M, Hamada K, Yano K, et al. (2009) Gibberellic modulates anther development in rice via the transcriptional regulation of GAMYB. Plant Cell 21: 1453–1472.
33. Malepszy S, Niemirovicz-Szeryt K (1991) Sex determination in cucumber (Cucumis sativus) as a model system for molecular biology. Plant Sci 80: 39–47.
34. Huang S, Li R, Zhang Z, Li L, Gu X, et al. (2009) The genome of the cucumber, Cucumis sativus L. Nat Genet 41: 1275–1281.
35. Bai SL, Peng YB, Cai JX, Gu HT, Xu LY, et al. (2004) Developmental analyses reveal early arrests of the spore-bearing parts of reproductive organs in unisexual cucumber flowers (Cucumis sativus L.). Planta 220: 230–240.
36. Chen XH, Chen YP, Jin YG (2003) Study on abortion process of sex organs in cucumber flowers at cell level. Journal of Yangzhou University (Agricultural and Life Sciences Edition) 24: 68–71 (in Chinese).
37. Peng J, Richards DE, Hartley NM, Murphy GP, Devos KM, et al. (1999) ‘Green revolution’ genes encode mutant gibberellin response modulators. Nature 400: 256–261.
38. Hirano K, Asano K, Tsuji H, Kawamura M, Mori H, et al. (2010) Gibberellin modulates anther development in rice via the transcriptional activation of a high-pI amylase gene promoter. Plant Cell 7: 1879–1891.
39. Willige BC, Ghosh S, Nill C, Zourelidou M, Dohmann EMN, et al. (2007) The gibberellin-GID1-DELLA pathway mediates the interaction with the GA INSENSITIVE-related (GID1) DELLA protein, SLR1, and gibberellin. Plant Cell 19: 2120–2130.
40. Knopf RR, Trebizh T (2006) The female-specific Cs-ACS1G gene of cucumber. A case of gene duplication and recombination between the non-specific 1-aminocyclopropane-1-carboxylic acid synthase gene and a branched-chain amino acid transaminase gene. Plant Cell Physiol 47: 1217–1228.
41. Saito S, Fuji S, Miyazawa Y, Yamashita S, Matsura S, et al. (2007) Correlation between development of female flower buds and expression of the CS-ACS2 gene in cucumber plants. J Exp Bot 58: 2907–2907.
42. Wu J, Kong X, Wan J, Liu X, Zhang X, et al. (2011) Dominant and pleiotropic effects of a GAI gene in wheat results from lack of interaction between DELLA and GID1. Plant Physiol 157: 2120–2130.
43. Varagona MJ, Schmidt RJ, Raikhel NV (1992) Nuclear localization signal(s) required for nuclear targeting of the maize regulatory protein Opaque-2. Plant Cell 4: 1213–1227.
44. Lohmann JU, Weigel D (2002) Building beauty: the genetic control of floral patterning. Dev Cell 2: 135–142.
45. Hao YJ, Wang DH, Peng YB, Bai SL, Xu LY, et al. (2003) DNA damage in the early primordial anther is closely correlated with stamen arrest in the female flower of cucumber (Cucumis sativus L.). Planta 217: 888–893.
46. Wang DH, Li F, Duan QH, Han T, Xia ZH, et al. (2010) Ethylene perception is involved in female cucumber flower development. Plant J 61: 862–872.
47. Yamashita S, Fuji N, Matsura S, Mizusawa H, Takahashi H (2001) The M locus and ethylene-controlled sex determination in andromonoecious cucumber plants. Plant Cell Physiol 42: 608–619.
48. Yamashita S, Fuji N, Takahashi H (2003) Photoperiodic regulation of CS-ACS6, CS-ACS4 and CS-ERS gene expression contributes to the femaleness of cucumber flowers through diurnal ethylene production under short-day conditions. Plant Cell Environ 26: 537–546.
49. Wang DH, Li F, Duan QH, Han T, Xia ZH, et al. (2010) Ethylene perception is involved in female cucumber flower development. Plant J 61: 862–872.
50. Knopf RR, Trebizh T (2006) The female-specific Cs-ACS1G gene of cucumber. A case of gene duplication and recombination between the non-specific 1-aminocyclopropane-1-carboxylic acid synthase gene and a branched-chain amino acid transaminase gene. Plant Cell Physiol 47: 1217–1228.
51. Saito S, Fuji S, Miyazawa Y, Yamashita S, Matsura S, et al. (2007) Correlation between development of female flower buds and expression of the CS-ACS2 gene in cucumber plants. J Exp Bot 58: 2907–2907.
52. Pike LM, Peterson CE (1969) Gibberellin A1/A4, for induction of staminate flowers on the gynoecious cucumber (Cucumis sativus ssp. Sativus). Phytochemistry 8: 106–109.
53. Badshy RH, Badshy HN (1980) Changes of endogenous gibberellins in cucumber as influenced by the various gibberellins. Naturwissenschaften 47: 39–35.