A GAMYB homologue CsGAMYB1 regulates sex expression of cucumber via an ethylene-independent pathway

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Received 27 December 2013; Revised 12 March 2014; Accepted 18 March 2014

Abstract

Cucumber (Cucumis sativus L.) is a typical monoecious vegetable with individual male and female flowers, and has been used as a model plant for sex determination. It is well known that sex differentiation of cucumber can be regulated by phytohormones, such as gibberellic acid (GA) and ethylene. The molecular mechanism of female sex expression modulated by ethylene has been widely understood, but how GA controls male sex expression remains elusive. In hermaphroditic Arabidopsis and rice, GA can regulate stamen and anther development via the transcriptional regulation of GAMYB. Here we characterized a GAMYB homologue CsGAMYB1 in cucumber. We found that CsGAMYB1 is predominantly expressed in male flower buds, where its expression is upregulated by GA3 treatment. CsGAMYB1 protein is localized in the nucleus. CsGAMYB1 can partially rescue stamen development and fertility phenotypes of an Arabidopsis myb33 myb65 double mutant. However, constitutive overexpression of CsGAMYB1 in wild-type Arabidopsis resulted in male sterility, which mimics the effect of GA overdose in flower development. Knockdown of CsGAMYB1 in cucumber decreases the ratio of nodes with male and female flowers, and ethylene is not involved in this process. Our data suggest that CsGAMYB1 regulates sex expression of cucumber via an ethylene-independent pathway.

Key words: CsGAMYB1, cucumber, ethylene, GAMYB, gibberellin, sex expression.

Introduction

Gibberellins (GAs), one kind of endogenous growth regulator, play an essential role in reproductive development of plants, especially in staminate development (Aya et al., 2009; Bai and Xu, 2013; King and Evans, 2003; Pharis and King, 1985; Plackett et al., 2011; Song et al., 2013). For example, GA application can promote development of male flowers in cucumber (Cucumis sativus L.) (Pike and Peterson, 1969; Wittwer and Bukovac, 1962). Additionally, GA-deficient mutants of Arabidopsis and tomato (Solanum lycopersicum) display abnormal stamen, and anther and pollen development leading to male sterility (Cheng et al., 2004; Goto and Pharis, 1999; Jacobsen and Olszewski, 1991; Koornneef and van der Veen, 1980; Nester and Zeevaart, 1988). Recently, several studies have demonstrated that GA regulates staminate development via the GA signalling pathway (Aya et al., 2009; Cheng et al., 2004; Fleet and Sun, 2005; Sun, 2010; Sun, 2011). In this pathway, GA first binds the GID1 receptor and promotes binding of DELLA proteins (repressors of GA action, and plant growth and development) to GID1; this GA–GID1–DELLA complex then enables the rapid degradation of DELLA proteins by the proteasome, which releases the inhibitory effect of the DELLA proteins and allows GA action to occur (Fleet and Sun, 2005; Harberd et al., 2009; Murase et al., 2008; Plackett et al., 2014; Sun, 2010; Sun, 2011; Ueguchi-Tanaka et al., 2007). GAMYB, a positive regulator involved in the GA signalling pathway,
has been known to act as an important downstream component in the degradation of DELLA proteins (Achard et al., 2004; Fleet and Sun, 2005; Olszewski et al., 2002). GAMYB was first identified in barley (Hordeum vulgare) aleurone cells, where its expression is upregulated by GA treatment (Gubler et al., 1995). HvGAMYB can bind specifically to GA-response elements in promoter regions of an α-amylase gene and other GA-regulated genes encoding hydrolytic enzymes, and constitutive expression of HvGAMYB mimics the positive effects of exogenous GA application on the transcriptional activation of these genes (Cercos et al., 1999; Gubler et al., 1999). GAMYB has also been demonstrated to play an important role in flower development, especially in anther development. For example, HvGAMYB shows high expression levels in barley anthers, where it is also upregulated by GA3. Overexpression of HvGAMYB in barley results in decreased anther length and male sterility (Murray et al., 2003), which phenocopies those plants treated with excessive exogenous GA (Colombo and Favret, 1996). In rice, loss-of-function mutations of GAMYB lead to abnormal anther and pollen development (Kaneko et al., 2004; Liu et al., 2010). In addition, OsGAMYB is also involved in GA-mediated programmed cell death (PCD) of tapetal cells, exine, and Ubisch body formation, and microarray analysis revealed that OsGAMYB can modulate most GA-regulated gene expression in rice anthers (Aya et al., 2009).

In Arabidopsis, there is a small family of GAMYB-like genes (Stracke et al., 2001), in which AtMYB33, AtMYB65, and AtMYB101 were identified to be able to substitute for barley and rice GAMYB in transactivating the α-amylase promoter (Gocal et al., 2001). Expression pattern analysis found that AtMYB33, AtMYB65, and AtMYB101 have a predominant expression in floral shoot apices and flowers, and the expression of AtMYB33 can be induced by exogenous GA (Achard et al., 2004; Gocal et al., 2001; Millar and Gubler, 2005). To further understand the function of AtGAMYBs, the deficient mutants for AtMYB33 and AtMYB65 were isolated, and the double mutant myb33 myb65 displays the phenotypes of shorter filaments, pollen abortion, and male sterility (Millar and Gubler, 2005). Furthermore, neither myb33 nor myb65 showed an abnormal phenotype compared with wild-type plants, suggesting that AtMYB33 and AtMYB65 are functionally redundant (Millar and Gubler, 2005). Taken together, these observations suggest that GAMYB is involved in GA-regulated stamen and anther development.

Cucumber is a typical monoecious vegetable with unisexual flowers, and has been served as a model plant for sex determination and differentiation (Malepszy and Niemirowicz-Szczytt, 1991). In young floral buds of cucumber, both stamen primordia and carpel primordia are initiated, and sex determination occurs just after the bisexual stage; subsequently, male or female flowers are formed and enlarged owing to the selective arrestment of carpel or stamen development, respectively (Bai et al., 2004; Malepszy and Niemirowicz-Szczytt, 1991). In this process, ethylene treatment can produce increased numbers of female flowers in cucumber (Iwahori et al., 1969; MacMurray and Miller, 1968), and the mechanism has been widely understood. Two major genes encoding ACC synthase (a key enzyme of ethylene biosynthesis), F (CsACS1G), and M (CsACS2) control female sex expression in cucumber; the F gene governs the development of female flowers (Knopf and Trebitsh, 2006; Mibus and Tatliloglu, 2004; Trebitsh et al., 1997), whereas the M gene inhibits stamen development in flower buds (Bie et al., 2013; Li et al., 2012; Saito et al., 2007; Wang et al., 2010; Yamazaki et al., 2001; Yamazaki et al., 2003). In addition, GA application can promote the male tendency (Pike and Peterson, 1969), but the molecular regulation remains elusive. Previous studies have confirmed that GA can modulate stamen and anther development via the transcriptional regulation of GAMYB in hermaphroditic plants, such as Arabidopsis and rice. However, in monoecious species cucumber whether GAMYB is involved in GA-regulated male tendency in the process of sex differentiation or not is still unknown. Therefore, in this study, a GAMYB orthologous gene in cucumber, designated as CsGAMYB1, was identified, and its spatial and temporal expression patterns were characterized. CsGAMYB1 is predominantly expressed in male flower buds, where its expression is upregulated by exogenous GA3 application, and CsGAMYB1 protein is localized in the nucleus. Ectopic expression of CsGAMYB1 can partially rescue the phenotypes of myb33 myb65 double mutant in Arabidopsis; however, constitutive overexpression of CsGAMYB1 in wild type resulted in male sterility. Furthermore, we generated CsGAMYB1-RNAi transgenic plants in cucumber and found that reduced transcript levels of CsGAMYB1 can result in decreased ratio of nodes with male and female flowers, but no effect on ethylene production and expression of F and M genes. Our results indicate that CsGAMYB1 can regulate ethylene-independent sex expression of cucumber.

Materials and methods

Plant materials and growth conditions

Monoecious cucumber (Cucumis sativus L.) line 3407 was used in this study. The seeds were germinated on wet filter paper in a Petri dish at 28 °C in dark overnight. Then the resulting seedlings were grown in a growth chamber under 16h/8h with 25 °C/18 °C in day/night. Upon the two true-leaf stage, plants were transferred to a greenhouse. The Arabidopsis mutant myb33 myb65 (Columbia background) was provided by Millar’s lab (Millar and Gubler, 2005), and Columbia (Col) was used as a wild-type control. Arabidopsis seeds were germinated on Murashige-Skoog (MS) medium, which contains 1% sucrose and 0.2% phytagar, at 4 °C for 3 d and then moved to 22 °C under a regime of 16 h light/8 h dark. Seedlings were transferred to soil 7–10 d after germination. For GA3 treatment, male flower buds of cucumber were sprayed with 200 μm GA3 (and mock-sprayed with 0.1% ethanol). Expression analyses were done after 4 h of treatment.

Cloning of CsGAMYB1

Total RNA was extracted from cucumber leaves using Promega’s SV Total RNA Isolation System, and cDNA was synthesized using MultiScribe™ reverse transcriptase (Applied Biosystems). The cDNA samples were amplified by PCR: 95 °C for 5 min, 30 cycles of 95 °C for 30s, 52 °C for 30s, and 72 °C for 2.5 min, and then 72 °C for 10 min. The primers are listed in supplementary material Table S3 available at JXB online.
Sequence alignment and phylogenetic analysis

Through BLAST analysis in Phytozone (http://www.phytozone.net/search.php) or the Arabidopsis Information Resource (http://www.Arabidopsis.org) using the sequence information of CsGAMYB1 protein, the amino acid sequence of related GAMYB proteins in various species were obtained. The multiple sequence alignment of CsGAMYB1 and related GAMYB proteins 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 (Saitou and Nei, 1987) with Poisson model and 1000 bootstrap replicates test through MEGA5 software.

Expression analysis by qRT-PCR

Total RNA was extracted using 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 Taq™ from TaKaRa (China) on an Applied Biosystems 7500 real-time PCR system. The cucumber α-TUBULIN (TUA) and Arabidopsis actin2 were used as internal controls in analysing gene expression in cucumber and Arabidopsis, respectively. And three biological replicates were performed for these experiments. The gene specific primers for qRT-PCR are listed in Supplementary Table S3 available at JXB online.

In situ hybridization

Shoot apex of 10-day-old seedlings, and male and female flower buds from 45-day-old cucumbers grown in the greenhouse were fixed, embedded, sectioned, and hybridized as described (Zhang et al., 2013). Digoxigenin-labelled sense and antisense RNA probes were generated using SP6 and T7 RNA polymerase (Roche) through PCR amplification respectively. The primer pairs are listed in Supplementary Table S3 available at JXB online.

Subcellular localization in onion epidermal cells

For transient expression in onion epidermal cells, the full-length coding region of CsGAMYB1 was cloned and fused to the upstream of the green fluorescent protein (GFP) between the EcoRI and BamHI sites in the pEZS-NL vector (http://deepgreen.stanford.edu) to generate 35S::GFP–CsGAMYB1; the empty pEZS-NL vector was used as a control. The onion epidermal layers were prepared and bombarded, as previously described (Varagona et al., 1992), with gold particles containing the plasmid using a Bio-Rad PDS-1000/He particle delivery system. After bombardment, the onion epidermal layers were prepared and bombarded, as previously described (Varagona et al., 1992), with gold particles containing the plasmid using a Bio-Rad PDS-1000/He particle delivery system. After bombardment, the onion epidermal layers were placed on MS medium and incubated in darkness at 22 ºC for 24h. Fluorescence signals were detected using Olympus BX 51 fluorescence microscopy. The primer sequences used for vector construction are listed in Supplementary Table S3 available at JXB online.

Transformation of Arabidopsis

To make the CsGAMYB1 overexpression construct, full-length CsGAMYB1 cDNA was cloned and inserted into the pCAMBIA1305.1 vector between the BglII and SpeI sites. The construct was then introduced into A. thaliana by electroporation and transformed into Col or myb65 myb85 plants as described (Clough and Bent, 1998). The transgenic plants were screened on MS medium with 25 mg l⁻¹ hygromycin. The primers for overexpression construct are listed in Supplementary Table S3 available at JXB online.

Transformation of cucumber

To generate the CsGAMYB1-RNAi transgenic plants of cucumber, two fragments of CsGAMYB1 were amplified using specific primers containing AseI (3' end) and SvaI (3' end) sites, and SpeI (5' end) and BamHI (3' end) sites. The two fragments were inversely inserted into the pFGC1008 vector, and the resulting CsGAMYB1-RNAi construct was then introduced into Agrobacterium by electroporation and transformed into the monococious cucumber line 3407 using the cotyledon transformation method as described previously (Wang et al., 2014). The primers for RNAi construct are listed in Supplementary Table S3 available at JXB online.

Quantification of ethylene

To examine the ethylene production from cucumber, shoot apices were excised at the 4-leaf stage. The samples were enclosed in a 10 ml vessel after weighing, and sealed with a rubber stopper. After incubation at 25 ºC for 0.5 h, 1 ml of gas was extracted using a syringe and injected into a gas chromatograph (GC-9A, Shimadzu, Japan). Ethylene was quantified using an activated alumina column and hydrogen flame ionization detector (FID). Standard ethylene gas was used for calibrating the instrument.

Results

Identification of the CsGAMYB1 gene from cucumber

Through BLAST analysis in the Cucumber Genome Database (Huang et al., 2009), we discovered three GAMYB-like genes named as CsGAMYB1 (Csa090914), CsGAMYB2 (Csa019830), and CsGAMYB3 (Csa013555). The CsGAMYB1 gene shows the highest similarity compared with other GAMYB orthologues, so CsGAMYB1 was chosen and analysed in this study. CsGAMYB1 was cloned using cDNA derived from cucumber leaves. Consistent with three GAMYB orthologues in Arabidopsis (Achard et al., 2004; Gocal et al., 2001; Millar and Gubler, 2005), CsGAMYB1 also contains three exons and two introns (Fig. 1A), encoding 552 amino acids. The sequence alignment of the amino acid residues of CsGAMYB1 compared with other members of the GAMYB family was performed using ClustalW in the MEGA5 software package (Tamura et al., 2011). CsGAMYB1, HvGAMYB, AtMYB33, and AtMYB65 share an R2R3 repeat DNA-binding domain in their N-terminal regions (Kranz et al., 1998; Romero et al., 1998; Stracke et al., 2001); over this sequence, CsGAMYB1 shows high identity to HvGAMYB, AtMYB33, and AtMYB65, with 88.46%, 87.5%, and 87.5% identity, respectively. In addition, these proteins also contain three conserved motifs Box 1, Box 2, and Box 3, which are typical structures in the GAMYB family (Supplementary Fig. S1 available at JXB online) (Gocal et al., 2001).

To further understand the evolutionary relationship between CsGAMYB1 and other GAMYB homologues, phylogenetic analysis was performed using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987) (Fig. 1B). CsGAMYB1 (shown in the box in Fig. 1B) is placed in the same clade as other GAMYB proteins, whereas CsGAMYB2 and CsGAMYB3 are distinct from this clade, suggesting that CsGAMYB1, but not CsGAMYB2 and CsGAMYB3, belongs to the GAMYB family in cucumber. A phylogenetic tree of the GAMYB family can be divided into two main groups: dicotyledon and monocotyledon. Within the dicotyledon group, GAMYB proteins in cucumber, which belong to the cucurbitaceae family; Arabidopsis of the cruciferae
family; kidney bean (*Phaseolus vulgaris*) and soybean (*Glycine max*) of the leguminosae family; and several woody plants such as apple (*Malus domestica*), cassava (*Manihot esculenta*), cotton (*Gossypium raimondii*), *Prunus persica*, and *Populus trichocarpa* all fall into the same clade, indicating that these plants may share a common origin. However, CsGAMYB1 is placed in a distinct group with GAMYB homologues in hermaphroditic species such as *Arabidopsis*, tomato, potato (*Solanum tuberosum*), kidney bean, and soybean, and it is also different from GAMYB groups in other monoecious plants such as cassava, *Populus trichocarpa*, and castor bean (*Ricinus communis*). These observations demonstrated that CsGAMYB1 is a GAMYB homologue in cucumber.

**Expression pattern of CsGAMYB1 in cucumber**

To obtain insights into the biological function of CsGAMYB1, we investigated its spatial and temporal expression patterns in cucumber. Quantitative real-time RT-PCR (qRT-PCR) was performed in various cucumber tissues including roots, stems, leaves, male flower buds, female flower buds, and fruits. CsGAMYB1 was expressed in all examined tissues, and the highest expression was detected in male flower buds (Fig. 2A), where its expression was increased almost two-fold by GA3 application (Fig. 2B). These data suggested that CsGAMYB1 may play an important role in cucumber male flower development.

Further, we examined the detailed expression patterns of CsGAMYB1 during cucumber flower development by *in situ* hybridization (Fig. 3). CsGAMYB1 RNA was found throughout the inflorescence meristem (im) and floral meristem (fm) in stage 1 of cucumber flower development (*Bai et al.*, 2004) (Fig. 3A). When flowers developed into the bisexual stages 4–5, the crucial periods that stamen primordia and carpel primordia initiate, higher expressions were detected in sepal primordia, petal primordia, stamen primordia, and carpel primordia (Fig. 3B, C). For male flowers, during the stages of microsporocytes (stage 9), meiosis (stage 10),
uninuclear pollens (stage 11), and mature pollens (stage 12), *CsGAMYB1* was predominately expressed in the microsporocytes (Fig. 3D), anther wall, and pollen grains (Fig. 3E–J). For female flowers, the expression of *CsGAMYB1* was detected in the developing ovary of stage 8 (Fig. 3K), but the signal was weak. As negative controls, *CsGAMYB1* sense probe hybridization showed no signals in the male flowers of stage 1, stage 5, stage 9, and stage 12 (Fig. 3L–O).

**Subcellular localization of CsGAMYB1 protein**

To further determine the subcellular localization of CsGAMYB1 protein, the GFP–CsGAMYB1 fusion protein was constructed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and introduced into onion epidermal cells by particle bombardment. As shown in Fig. 4, the GFP–CsGAMYB1 fusion protein is localized in the nucleus (Fig. 4A–C). As a control, the signals of 35S:GFP are detected throughout the cell (Fig. 4D–F).

**CsGAMYB1 can partially rescue myb33 myb65 double mutant phenotypes in Arabidopsis**

To investigate the biological role of *CsGAMYB1*, we ectopically expressed the full-length *CsGAMYB1* cDNA under the control of a 35S promoter in an Arabidopsis *myb33 myb65* double mutant, which displayed shorter filaments, pollen abortion, and male sterility (Millar and Gubler, 2005). A total of 23 independent transgenic lines were obtained, all of which could partially rescue the phenotypes of the *myb33 myb65* double mutant, and all of which displayed similar phenotypes. As shown in Fig. 5, flowers in the transgenic plants had increased filaments length and pollen numbers as compared with those in the *myb33 myb65* double mutant (Fig 5A, B). Consequently, fertility increased in the *CsGAMYB1* transgenic plants (Fig 5C–F). For example, the *myb33 myb65* plants displayed much smaller siliques (Fig 5C), which failed to set any seeds (Fig 5D), whereas ectopic expression of *CsGAMYB1* resulted in normal siliques (Fig 5C), which set seeds with similar shape and numbers, compared with those in wild-type plants (Fig 5E, F). We chose eight transgenic lines to analyse expression of *CsGAMYB1* and plant fertility. The fertility is defined as the percentage of siliques that set seeds per plant. The expression of *CsGAMYB1* displayed different levels in these lines (Fig 5G), although the lines with the higher *CsGAMYB1* mRNA levels showed the higher increase in fertility compared with the *myb33 myb65* plants (Fig 5H). For example, the average fertility of *myb33 myb65* plants was only 2.8%, whereas in the six lines (lines 1, 2, 3, 4, 5, and 6) which had lower *CsGAMYB1* mRNA expression, the average fertility increased to 28.6%. However, in line 7, which had higher *CsGAMYB1* mRNA expression, the fertility reached...
46.0%, and the highest \textit{CsGAMYB1} mRNA level in line 8 resulted in 77.3% fertility, which was close to that in wild-type (92.2% fertility; \textit{Supplementary Tables S1} and \textit{S2} available at \textit{JXB} online), suggesting that \textit{CsGAMYB1} can partially rescue the fertility of the \textit{myb33 myb65} double mutant, but the recovery relies on the levels of \textit{CsGAMYB1} expression. These
results implied that the function of CsGAMYB1 in cucumber, which has unisexual flowers, is conserved with AtMYB33 and AtMYB65 in Arabidopsis, which has complete flowers with respect to stamen development and plant fertility.

Constitutive overexpression of CsGAMYB1 results in male sterility in Arabidopsis

To further explore the function of CsGAMYB1, we also generated transgenic lines overexpressing CsGAMYB1 in Arabidopsis wild-type Columbia (Col); a total of 24 independent transgenic lines were obtained. In the transgenic plants, stamens were shorter than those in wild type and failed to fully extend to the pistil (Fig. 6A, B), and anthers were smaller than their wild-type counterparts and failed to generate pollen (Fig. 6C), leading to much smaller siliques, which failed to set any seeds (Fig. 6D, E).

Among the 24 transgenic lines, 8 lines were chosen for further analysis. Of these lines, five lines (lines 4, 5, 6, 7 and 8) were sterile and three other lines (lines 1, 2 and 3) exhibited partial fertility. For example, in these five sterile lines, few siliques that set seeds were present, from which few seeds were obtained, and the average fertility was only 3.1%. However, there were some normal siliques in lines 1, 2, and 3, characterized by the similar seeds number per silique compared with that of wild-type, resulting in partial fertility, with 51.2%, 21.7%, and 23.4%, respectively (Fig. 6F, Supplementary Table S2 available at JXB online). The expression of CsGAMYB1 in these eight transgenic plants was also analysed (Fig. 6G). We found that CsGAMYB1 mRNA expressions in the five sterile lines were more than 3.4-fold higher than those in the three partial sterile plants. Line 8, which had only 1.9% fertility, displayed the highest level of CsGAMYB1 mRNA. In line 8, CsGAMYB1 mRNA level was more than 35-fold higher than that in line 1, in which the fertility reached 51.2%. This correlation between high levels of CsGAMYB1 mRNA and sterility in multiple transgenic lines suggests that constitutive overexpression of CsGAMYB1 results in male sterility in Arabidopsis in a dose-dependent manner.

CsGAMYB1 can regulate sex expression of cucumber via an ethylene-independent pathway

To further determine the biological function of CsGAMYB1 in cucumber, a double-strand RNAi construct containing the specific sequence of CsGAMYB1 under the control of
35S promoter was introduced into the monoecious cucumber plants. A total of seven independent RNAi lines were obtained. In these RNAi lines, the transcript levels of \(CsGAMYB1\) were significantly reduced, whereas the expressions of the other two \(GAMYB\)-like genes, \(CsGAMYB2\) and \(CsGAMYB3\), had no change as compared with those in the wild-type plants (Fig. 7A). This suggests that \(CsGAMYB1\) expression was effectively knocked down by RNAi, and this process had no effect on the expression of other \(GAMYB\)-like genes that show high sequence similarity with \(CsGAMYB1\).

When \(CsGAMYB1\)-RNAi plants grew until anthesis of flowers on node 20, the sex of the flowers on each node of the main stem was recorded (Fig. 7B–E). The percentage of nodes with male flowers was 75.7% in wild type, and 45% in the \(CsGAMYB1\)-RNAi plants, whereas the proportion of nodes with female flowers was 24.3% and 55%, respectively. So, the ratio of nodes with male and female flowers in the transgenic RNAi plants decreased by almost 4-fold in comparison with wild type (Fig. 7A and B), suggesting that ethylene may not be involved in the pathway of \(CsGAMYB1\)-regulated sex expression of cucumber.

**Discussion**

\(CsGAMYB1\) may be the homologue for both \(AtMYB33\) and \(AtMYB65\)

In \textit{Arabidopsis}, the \textit{GAMYB} family has three members: \textit{MYB33}, \textit{MYB65}, and \textit{MYB101} (Gocal et al., 2001), in which \textit{MYB33} and \textit{MYB65} are functionally redundant in anther development (Millar and Gubler, 2005). Although in cucumber, there are also three putative \textit{GAMYB}-like genes: \(CsGAMYB1\), \(CsGAMYB2\), and \(CsGAMYB3\). \(CsGAMYB1\)
is closely related to AtMYB33 and AtMYB65, whereas CsGAMYB2 and CsGAMYB3 may not belong to the GAMYB family (Fig. 1B). Similar to AtMYB33 and AtMYB65, CsGAMYB1 also has an R2R3 repeat DNA-binding domain and three conserved motifs containing Box 1, Box 2, and Box 3 (Supplementary Fig. S1 available at JXB online). In addition, CsGAMYB1 can partially rescue the phenotypes of myb33 myb65 double mutant in Arabidopsis, with respect to stamen development and plant fertility (Fig. 5). These observations suggested that CsGAMYB1 acts as the homologue for both AtMYB33 and AtMYB65 in cucumber.

CsGAMYB1 has both conserved and divergent functions with its hermaphroditic homologues

In hermaphroditic plants, GAMYB has been shown to play an important role in stamen and anther development. For example, HvGAMYB in barley, OsGAMYB in rice, and AtMYB33 and AtMYB65 in Arabidopsis were strongly expressed in floral organs, especially in stamens and anthers, but weakly in mature pollen grains, and the expression was upregulated by exogenous GA3 (Aya et al., 2009; Gocal et al., 2001; Kaneko et al., 2004; Murray et al., 2003; Tsuji et al., 2006). Loss of function of OsGAMYB in rice, and AtMYB33 and AtMYB65 in Arabidopsis resulted in abnormal staminate specific organs, such as shorter filaments and pollen abortion caused by endless expansion of the tapetum, leading to male sterility (Alonso-Peral et al., 2010; Aya et al., 2009; Kaneko et al., 2004; Liu et al., 2010; Millar and Gubler, 2005), whereas overexpression of HvGAMYB in barley also led to decreased anther length and male sterility (Murray et al., 2003). This suggests that GAMYB homologues have a conserved role in staminate development, but the regulatory mechanism may be different in various species. In our study, CsGAMYB1 was highly expressed in male flower buds, where its expression was also up-regulated by GA3 application (Fig. 2), and...
CsGAMYB1 rescued the phenotypes of *Arabidopsis* double mutant *myb33 myb65* in stamen development and plant fertility (Fig. 5, Supplementary Table S1 available at *JXB* online), indicating that CsGAMYB1 may also function as a positive regulator for staminate development as those of *Arabidopsis MYB33* and *MYB65*. Meanwhile, ectopic expression of *CsGAMYB1* in *Arabidopsis* wild type resulted in reduced filaments, aborted pollen, and male sterility (Fig. 6, Supplementary Table S2 available at *JXB* online), similar to overexpression of HvGAMYB in barley, supporting the hypothesis that *CsGAMYB1* has a conserved role in staminate development.

However, unlike the weak expression of HvGAMYB, OsGAMYB, AtMYB33, and AtMYB65 in mature pollen grains, CsGAMYB1 is expressed throughout male flower development containing the mature pollen grains in cucumber (Fig. 3), but there are no significant difference in anthers and pollen development of male flowers between CsGAMYB1-RNAi and wild-type plants in cucumber (data not shown), which is divergent with the phenotypes of the GAMYB-deficient mutants in hermaphroditic species. Moreover, GAMYB genes in *Arabidopsis* might mediate GA signalling in flowering responses by activating the expression of a floral meristem identity gene, LEAFY (Gocal et al., 2001). Also, in the grass *Lolium temulentum*, the content of GAs increases in the leaves and SAM (shoot apical meristem) with exposure to long-day which is sufficient to induce flowering (King et al., 2001), and the increase in GAs level is followed by increased expression of *LtGAMYB* in the shoot apex, suggesting that *LtGAMYB* plays an important role in GA-regulated flower initiation (Gocal et al., 1999). But in cucumber, even though high expression was detected in the inflorescence meristem (im) and floral meristem (fm) (Fig. 3A), flowering time of either male or female flowers seemed to be undisturbed upon partial loss of function of *CsGAMYB1* in the transgenic RNAi plants, indicating that *CsGAMYB1* may not be involved in floral initiation of cucumber. These observations verified that monoecious *CsGAMYB1* displays conserved as well as divergent functions with its hermaphroditic homologues.

The possible roles of CsGAMYB1 in Arabidopsis

Ectopic expression of *CsGAMYB1* in *Arabidopsis* leads to reduced filament length, aborted pollen, and male sterility (Fig. 6), and it is probably caused by a number of events. For example, *CsGAMYB1* may interfere with some MYB transcription factors of *Arabidopsis* that have been shown to be needed in GA-regulated stamen development (Cheng et al., 2009; Millar and Gubler, 2005). Alternatively, *CsGAMYB1* possibly up-regulates the target genes of these MYB transcription factors and there may be feedback in this process. In addition, it could result from an accumulated level of GA signalling, which mimics the effect of GA overdose in flower development and male fertility (Colombo and Favret, 1996; Jacobsen and Olszewski, 1993). Interestingly,

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**Fig. 8.** Ethylene is not involved in CsGAMYB1-regulated sex expression of cucumber. (A) Expression of F (CsACS1G) and M (CsACS2) by qRT-PCR in WT and transgenic RNAi lines. The cucumber α-TUBULIN (TUA) was used as an internal control, and the experiments were repeated in triplicate independent samples. Error bars indicate the standard errors. (B) Quantification of ethylene released from shoot apices in WT and transgenic RNAi lines at 4-leaf stage. Amount of ethylene was measured per 1 g fresh weight and per h. Vertical bars indicate the standard errors of the mean for triplicate samples. (This figure is available in colour at *JXB* online.)
this developmental phenomenon owing to GA signalling is also observed in other plants. For instance, constitutive overexpression of HvGAMYB in barley causes male sterility (Murray et al., 2003), whereas loss of function of RGA and GAI, repressors of GA signalling and plant growth and development, results in shorter stamen filaments and reduced pollen levels as well as fertility in Arabidopsis (Dill and Sun, 2001). Overall, despite ectopic expression of CsGAMYB1 in Arabidopsis having an important role in stamen development, the regulatory mechanism is unclear, and further analysis is needed.

Moreover, CsGAMYB1 can partially rescue the fertility of a myb33 myb65 double mutant, and the recovery levels rely on the levels of CsGAMYB1 expression (Fig. 5), whereas constitutive overexpression of CsGAMYB1 results in male sterility in Arabidopsis (Fig. 6), suggesting that although CsGAMYB1 is important for stamen development, the effect is dose dependent. And in the transgenic plants overexpressing CsGAMYB1 in Arabidopsis, the expression of CsGAMYB1 in line 8 is higher than that in line 4, 5, 6, and 7, but they are all male sterile (Fig. 6F and G, Supplementary Table S2 available at JXB online), indicating that the level of expression of CsGAMYB1 in 4, 5, 6, and 7 is sufficient to lead to sterility.

**GA and ethylene may regulate sex expression of cucumber via two parallel pathways**

The sex determination of cucumber occurs owing to the selective arrestment of either carpel or stamen development at the bisexual stage (Bai et al., 2004; Malepszy and Niemirowicz-Szczytt, 1991). In the pathway of ethylene-regulated female sex expression, F gene governs female flowers formation (Knauf and Trebisch, 2006; Mibus and Tatlioglu, 2004; Trebisch et al., 1997), whereas M gene inhibits stamen development in flower buds (Yamasaki et al., 2001; Yamazaki et al., 2003). CsGAMYB1 can enhance the ratio of nodes with male and female flowers (Fig. 7), probably owing to either promotion in male tendency or repression of female sex expression. However, even though CsGAMYB1 is highly expressed in both stamen primordia and carpel primordia at the bisexual stage, its expression remains high in the male specific organs, but weak in female flowers during later stages (Figs 2 and 3), suggesting that CsGAMYB1 may promote the development of male flowers and inhibit the development of female flowers. In addition, in the CsGAMYB1-RNAi plants, the proportion of nodes with male flowers and female flowers is reduced and increased, respectively (Fig. 7), and no bisexual flowers are observed (data not shown). So, we conclude that CsGAMYB1 may modulate the sex expression of cucumber by promoting male tendency and inhibiting the formation of female flowers at the same time, which potentially is an overlapping role with both F and M genes, even though the specific functions are opposite. Moreover, in the process of male flower development, CsGAMYB1 expresses ubiquitously in the male flower, including sepal, petal, and stamen (Fig. 3), but has no effect on the male floral patterning when CsGAMYB1 is knocked down by RNAi (data not shown), which possibly results from a number of events, one of which may be partial functional redundancy of three CsGAMYBs of cucumber. Although phylogenetic analysis has suggested that CsGAMYB2 and CsGAMYB3 may not belong to the GAMYB family (Fig. 1B), they show high similarity compared with CsGAMYB1 (data not shown). Given that Arabidopsis GAMYBs have specific as well as partially overlapping roles, we speculate that CsGAMYB1 may regulate male sex expression of cucumber specifically, whereas the three CsGAMYBs are likely to be functionally redundant in the development of male floral organs. However, for elucidating the functional similarities and differences among these three CsGAMYBs, the functional analysis of CsGAMYB2 and CsGAMYB3 in cucumber is the best way to elucidate this in future studies.

Sex differentiation of cucumber exists plasticity, and male and female expression can be changed by GA and ethylene, respectively (Iwashorii et al., 1969; MacMurray and Miller, 1968; Pike and Peterson, 1969; Wittwer and Bukovac, 1962); however, whether there is a crosstalk between these two pathways is unknown. In our study, we certified that GA can modulate sex expression of cucumber via the transcriptional regulation of CsGAMYB1, which acts a positive factor of GA signalling pathway (Figs 2B and 7), and this process has no effect on ethylene biosynthesis and production (Fig. 8), indicating that GA-CsGAMYB1-regulated male sex expression and ethylene-modulated female sex expression of cucumber might take two independent pathways. Our study on GA-CsGAMYB1 reveals a new model for sex expression, which enhances our understanding of sex determination in cucumber and provides the basis for molecular flower induction and high-yield cultural practices. Besides, given that the signalling pathway GA–GID1–DELLA complex play important roles in plant growth and development, particularly in staminate development (Cheng et al., 2004; Dill and Sun, 2001; Fleet and Sun, 2005; Griffiths et al., 2006; Hou et al., 2008; Sun, 2010; Sun, 2011; Tyler et al., 2004), and GAMYB acts as an important downstream component of the DELLA proteins in Arabidopsis (Achard et al., 2004; Fleet and Sun, 2005; Olszewski et al., 2002), identifying the position of CsGAMYB1 in GA response pathways and the relationship between CsGAMYB1 and the GA–GID1–DELLA complex will shed light on the molecular mechanism of male sex expression of cucumber regulated by GA signalling.

**Accession numbers**

Sequence data of GAMYB proteins in this study can be found in the Cucumber Genome DataBase, Arabidopsis Genome Initiative, Phytozone or GenBank/EMBL/Swiss-Prot databases under the following accession numbers: CsGAMYB1 (Csa009014), CsGAMYB2 (Csa019830), CsGAMYB3 (Csa013555), AtMYB3 (AT5G06100), AtMYB65 (AT3G11440), Malus domestica (MDP0000147309), Prunus persica (ppa003628m.g), Populus trichocarpa (Potri.003G189700), Manihot esculenta (cassava4.1_004600m.g), Gossypium raimondii (Gorai.009G301100), Glycine max (Glyma13g25716), Phaseolus vulgaris (Phvul.011G191300), Ricinus
Supplementary data

Supplementary data are available at JXB online

Fig. S1. Sequence alignment of amino acid residues of CsGAMYB1 with other GAMYB proteins.

Table S1. CsGAMYB1 can partially rescue the fertility of myb33myb65 in Arabidopsis.

Table S2. Overexpression of CsGAMYB1 resulted in partial sterility in Arabidopsis

Table S3. List of primers and their uses.

Acknowledgments

This work was supported by National Public Service Sectors (Agriculture) Project of China (201203003), National High Technology Research and Development Program (863) of China (2012AA100103), National Science and Technology Projects of China (2013BAD20B01), Innovative Team Program of Beijing Industrial Technology System for Fruit-vegetables and Scientific and Technological Research Project of Beijing (D13110000713001) to HR. We thank Anthony A. Millar for providing the myb33 myb65 seeds, Chunjiang Zhou (Cornell University) for revising the manuscript, and members of the Ren lab for helpful discussions and technical assistance. YZ and HR designed the experiments, YZ performed most of the experiments and wrote the paper along with XZ and XL (Xiaowei Liu). BL and WW helped with the in situ hybridization, CC helped with the qRT-PCR. XL (Xiaofeng Liu) helped the subcellular localization, and SY helped with the measurement of ethylene production.

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