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A putative positive feedback regulation mechanism in *CsACS2* expression suggests a modified model for sex determination in cucumber (*Cucumis sativus* L.)

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Abstract

It is well established that the plant hormone ethylene plays a key role in cucumber sex determination. Since the unisexual control gene *M* was cloned and shown to encode an ethylene synthase, instead of an ethylene receptor, the 'one-hormone hypothesis', which was used to explain the cucumber sex phenotype, has been challenged. Here, the physiological function of *CsACS2* (the gene encoded by the *M* locus) was studied using the transgenic tobacco system. The results indicated that overexpression of *CsACS2* increased ethylene production in the tobacco plant, and the native cucumber promoter had no activity in transgenic tobacco (PM). However, when PM plants were treated with exogenous ethylene, *CsACS2* expression could be detected. In cucumber, ethylene treatment could also induce transcription of *CsACS2*, while inhibition of ethylene action reduced the expression level. These findings suggest a positive feedback regulation mechanism for *CsACS2*, and a modified 'one-hormone hypothesis' for sex determination in cucumber is proposed.

Introduction

In angiosperms, the mechanisms of sex determination, which have been extensively studied in recent years, involve a number of different genetic and epigenetic factors (Tanurdzic and Banks, 2004). As a result of its diversity in sex types, cucumber (*Cucumis sativus* L.) has been researched as a model for more than half a century (Atsmon, 1968; Tsao, 1988; Perl-Treves, 1999). Cucumber plants generally produce male and female flowers separately on the same individual (monoecious); however, certain lines (hermaphrodite and andromonoecious) also produce bisexual flowers (Galun, 1961; Shifriss, 1961; Kubicki, 1969). Morphologically, all floral buds are initially hermaphroditic with staminate and pistillate primordia

(Kubicki, 1969). A systematic morphogenetic analysis of male and female flower development revealed that the origin of floral reproductive organ differentiation and sex determination occurs after the carpel primordia initiation stage (Bai *et al.*, 2004). The selective arrest of either the staminate or pistillate organs results in female or male flowers, respectively. Furthermore, if the arrest does not happen, hermaphroditic flowers are formed (Atsmon and Galun, 1960; Goffinet, 1990; Perl-Treves, 1999).

Early genetic studies indicated that three major genes are responsible for sex expression and segregation in cucumber: *F/f*, *M/m*, and *A/a*. The *F* gene promotes femaleness, while the recessive *m* gene regulates the appearance of bisexual

flowers on the plant. Furthermore, in combination with the homozygous recessive *f* gene, the recessive *a* gene can intensify the androecious nature in cucumber plants (Galun, 1961; Kubicki, 1969; Robinson *et al.*, 1976). Additionally, sex expression can also be regulated by many environmental factors such as photoperiod, temperature, and plant hormones such as ethylene, indole-3-acetic acid, and gibberellic acid (Atsmon, 1968; Takahashi *et al.* 1983; Perl-Treves, 1999; Yamasaki *et al.*, 2005).

Preliminary physiological investigations revealed that a strong correlation exists between the plant hormone ethylene and the sex phenotype in cucumber. Elevated endogenous levels of ethylene can promote femaleness, whereas the inhibitors of ethylene biosynthesis or the ethylene response suppress the development of female reproductive organs (Atsmon and Tabbak, 1979; Takahashi and Jaffe, 1984). The arresting action of ethylene on stamen development can also be found in some other plant species. Overexpression of a cucumber ethylene synthesis gene, *CsACO2* (*Cucumis sativus* ACC oxidase gene 2), can restrain *Arabidopsis* stamen development, mimicking female cucumber flowers (Duan *et al.*, 2008).

The 'one-hormone hypothesis' posits that the *F* locus, which controls the gynoecious phenotype, regulates ethylene levels, promoting the development of the pistillate primordial state; and the dominant *M* locus, which is important in determining the monoecious condition, mediates the inhibition of stamen development by ethylene. The ethylene signal is not transmitted if the genotype is homozygous for *m*; as a result, stamen development is not inhibited, and the plant produces hermaphroditic flowers (Yin and Quinn, 1995; Yamasaki *et al.*, 2001). According to this hypothesis, the *F* locus encodes a gene for ethylene production, and the *M* locus encodes an ethylene-response factor, which might be a receptor of ethylene.

Recently, the *F* locus has been cloned and shown to encode 1-aminocyclopropane-1-carboxylic acid synthase (ACS) (Trebitsh *et al.*, 1997; Mibus *et al.*, 2004), which has been verified as being involved in ethylene biosynthesis (Li *et al.*, 2009). Furthermore, an ethylene receptor gene, *CsETR1* (*Cucumis sativus* ethylene receptor 1), has been observed to play a key role in stamen arrest (Wang *et al.*, 2010). A cucumber nuclease-encoding gene, *CsCaN* (*Cucumis sativus* calcium-dependent nuclease), has been shown to respond to the ethylene signal and is involved in the anther-specific DNA damage in the primordia of developing female flowers (Gu *et al.*, 2011). However, the cloning of the *M* locus, which was previously thought to encode an ethylene receptor, reveals that it is not part of the ethylene signalling pathway, but rather encodes an enzyme involved in ethylene biosynthesis. This gene, *CsACS2*, also encodes an ACS (Boualem *et al.*, 2009; Li *et al.*, 2009). So, the response relationship between the *CsACS2* gene and ethylene needs to be clarified.

Previous work indicated that the *CsACS2* gene product is enzymatically active in *Escherichia coli* and can synthesize ACC (1-aminocyclopropane-1-carboxylic acid), which is a precursor of the ethylene molecule (Li *et al.*, 2009). In this study, the function of this gene was studied using the transgenic tobacco system. The results indicate that the overexpression of *CsACS2* increases ethylene production in tobacco, and these transgenic plants (P35) display short internodes and delayed flowering. Moreover, the original cucumber promoter of *CsACS2* was not active in transgenic tobacco (PM), and did not promote transcription of the gene. However, when the PM plants were treated with exogenous ethylene, expression of *CsACS2* could be detected. In cucumber plants, a certain amount of ethylene treatment could induce the transcription of *CsACS2*, while inhibition of ethylene action could reduce expression levels to varying degrees. These findings suggest a positive feedback regulation mechanism for *CsACS2*, which could be involved in flower development in cucumber.

Materials and methods

Plant materials

Sterile-grown seedlings of tobacco *Nicotiana tabacum* pv. *Bairihong*, which were provided by Professor Lingxia Zhao (Shanghai Jiaotong University, Shanghai), were used for *Agrobacterium*-mediated transformation. A gynoecious cucumber inbred line G06 (*FFMM*) was used for isolation of the wild-type *CsACS2* gene and its promoter. A monoecious cucumber line M06 (*ffMM*), which was the near-isogenic line (NIL) with G06 for the *F* locus, was used to study the exogenous ethylene-inducing expression of *CsACS2* in cucumber. A second gynoecious line, G34 (*FFMM*), together with the gynoecious line G06, was used to analyse the level of transcription of the *CsACS2* gene during inhibition of ethylene action. A hermaphroditic cucumber line H34 (*FFmm*), together with its NIL G34 for the *M* locus, was used to study the promoters sequence and compare the relative expression of *CsACS2* in near genotypic backgrounds, except for *M/m*. A traditional andromonoecious line 'Lemon' (*ffmm*) was used to analyse the gene expression and response to the ethylene-related chemical treatments. All cucumber lines were maintained by inbreeding at the Plant Department of Shanghai Jiaotong University and the College of Horticulture at Northwest A&F University. Conditions for germination and growth were according to Li *et al.* (2009).

Cloning of CsACS2 and its promoter

Cucumber apical shoots without any immature leaves were excised from the shoot apices with an Olympus SZ61 stereomicroscope (Olympus, Japan). As described previously (Li *et al.*, 2009), apical shoot total RNA was isolated from G06 cucumber plants at the four-leaf stage (~20 d after sowing). Reagents for RNA extraction were purchased from TIANGEN (China). DNase I (TaKaRa, China) was used to remove contaminating DNA, and first-strand cDNA was synthesized with the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). The CDS (coding sequence) of the *CsACS2* gene was amplified using a Long-Distance PCR Kit (Takara, China) with the appropriate primers (Supplementary Table S1 available at *JXB* online).

To isolate the *CsACS2* promoter, genomic DNA was extracted as described previously (Li *et al.*, 2008). The promoter fragment of the *CsACS2* gene from G06 was amplified using a Long-Distance PCR Kit (Takara, China). The promoter regions from the NILs G34 and H34 were also amplified to compare the homologous *cis*-acting elements. Putative *cis*-acting elements in the *CsACS2* promoter were identified by searching the PLACE database (http://www.dna.affrc.go.jp/PLACE/) (Higo *et al.*, 1999).

All the DNA fragments were subcloned into the T/A-cloning vector pUCm-T (Fermentas) and sequenced as previously described (Li *et al.*, 2008).

Generation of transgenic tobacco plants

The CDS of *CsACS2* was inserted into the *pBI121* plasmid in the sense orientation under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter to give the *pBI121-P35S-CsACS2* construct (Fig. 1A), which resulted in the transgenic tobacco plants designed P35. Using the restriction sites (*Cla*I and *Bam*HI) flanking the CaMV 35S promoter, the cucumber promoter fragment was inserted to produce the *pBI121-PCsACS2-CsACS2* plasmid (Fig. 1A), which resulted in the transgenic tobacco plants designed PM. The two expression cassettes were introduced into tobacco *N. tabacum* pv. *Bairihong* by *Agrobacterium*-mediated transformation (Hoekema *et al.*, 1983).

DNA was isolated from leaves of kanamycin-resistant regenerated plants to assay for positive transformation events by PCR using gene-specific primers (Supplementary Table S1 at *JXB* oline). PCR-positive transgenic plants were subjected to dot blotting analysis. Five P35 and three PM transgenic tobacco plants were chosen and self-pollinated to obtain homozygous T_2 offspring.

Dot blotting and quantitative RT-PCR

Genomic DNA and total RNA of transgenic, wild-type tobacco and cucumber plants were extracted using the method described previously (Li *et al.*, 2008, 2009).

Dot blotting: Tobacco genomic DNA samples (40 μg) were blotted onto Hybond-N$^+$ nylon membranes with the Minifold I™ Dot-Blot System (Amersham, China). Hybridizations were performed using the protocol provided by the manufacturer (Gene Images random priming labeling module and Gene Images CDP-Star detection module from Amersham, China).

Semi-quantitative RT-PCR: First-strand cDNA was synthesized according to a method described previously(Li *et al.*, 2009). Using the appropriate gene-specific primers (Supplementary Table S1 at *JXB* online), PCR was performed for 30 cycles (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s). The products were separated on 1% agarose gels stained with ethidium bromide. The results were analysed using Gene Tools software from Syngene (CA, USA). The reverse transcription-PCRs (RT-PCRs) for two housekeeping genes (tobacco *Actin* gene AY189972 and cucumber *Actin3* gene DQ115883) were performed under the same conditions as described above to estimate whether equal amounts of RNA had been used in the RT-PCRs.

Quantitative RT-PCR: Cucumber RNA extraction and first-strand cDNA synthesis were described previously. PCR was performed in a 96-well plate using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA), with SYBR Green Realtime PCR Master Mix (TaKaRa, China). The amplification was initiated by heating to 94 °C for 10 min, followed by 40 cycles at 94 °C for 5 s and 62 °C for 34 s. The amplification specificity was tested by a dissociation curve (65–90 °C). To compare results from different reactions and samples, *CsACS2* amplification was normalized to *CsActin3* using the CT values.

Ethylene production in transgenic tobacco and cucumber cultivars

The time course of ethylene evolution was examined in transgenic tobacco and cucumber cultivars according to Yamasaki *et al.* (2001). To examine ethylene production from the two transgenic tobacco lines, 10 g samples of leaf tissue from each of three P35 plants (T3-8, T3-22, and T3-26) and one PM plant (TM-3) were cut into 1 cm^2 pieces and enclosed individually in 5 ml vessels sealed with rubber stoppers. After incubation at 25 °C for 1 h, 1 ml

of head gas was withdrawn from each vessel using a gas-tight syringe. The gas samples were collected once every hour during a 3 h incubation period. To examine ethylene production from the five cucumber cultivars G06, M06, G34, H34, and 'Lemon', shoot apices were excised at the four-leaf stage. Head gas was sampled after incubation at 25 °C for 16 h. All samples were injected into a gas chromatograph (GC-2010, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and an activated alumina column. The instrument was calibrated with standard ethylene gas. Each result was derived from three independent biological replicates.

Chemical treatments

To investigate the effect of ethylene on *CsACS2* expression, ethephon (an ethylene-releasing agent) was applied to transgenic tobacco and cucumber plants (M06) at various concentrations. AgNO$_3$ (ethylene action inhibitor) and aminoethoxyvinyl glycine (AVG, ethylene biosynthesis inhibitor) were also applied to two cucumber lines, G06 and G34. Ethephon and AgNO$_3$ were purchased from Sigma (China). AVG was provided by Professor Qiguang Wen (IPPE, CAS, Shanghai).

Ethephon treatment of transgenic plants: Briefly, 50 μl aliquots of 0.5 mM and 1.0 mM ethephon in water containing 0.1% (v/v) Tween-20 were applied to the apex of flower buds of the PM transgenic plant line TM-3 using a micropipette once a day for 3 d. The same treatment with water containing 0.1% (v/v) Tween-20 was the control (and was also used in the experiment below). The apex buds, leaves adjacent to the buds, and untreated lower leaves were then excised for RNA extraction.

Ethephon treatment of cucumber plants: As the expression of *CsACS2* was not detected in apical shoot, the monoecious cucumber line M06 was selected to analyse the effect of ethylene on *CsACS2* transcription. The apical shoots of four-leaf-stage M06 seedling were soaked in water containing 0.1, 0.5, and 1.0 mM ethephon with 0.1% (v/v) Tween-20 for 2, 4, and 6 h, respectively. The apices were then excised for RNA extraction.

AgNO$_3$ and AVG treatment of cucumber plant: The cucumber lines G06 and G34, which both express *CsACS2* at high levels, were selected to analyse the inhibitory effect of ethylene on *CsACS2* expression. Aliquots of 50 μl of 1.0 mM AgNO$_3$ and 1.0 mM AVG in H$_2$O containing 0.1% (v/v) Tween-20 were applied to the apices of the two cucumber lines at the four-leaf stage using a micropipette once a day for 3 d. The apices were then excised for RNA extraction.

To study the effects of chemical application on the sexual expression of cucumber plants, several of the treated plants were allowed to grow to maturity. These included G06 and G34 treated with AgNO$_3$ (1.0 mM), M06 treated with ethephon (0.5 mM and 1.0 mM), H34 treated with AgNO$_3$ (1.0 mM) and ethephon (0.5 mM and 1.0 mM), and 'Lemon' treated with AgNO$_3$ (1.0 mM) and ethephon (0.5 mM and 1.0 mM). The application method was as given above for AgNO$_3$ and AVG treatment of cucumber plants. Every treatment employed five plants, and the number of male, female, and bisexual flowers was recorded up to the 30th node on the main stem.

Results

Overexpression of CsACS2 *in transgenic tobacco plants*

Transgenic tobacco plants were generated to analyse the biological function of *CsACS2* under the control of two
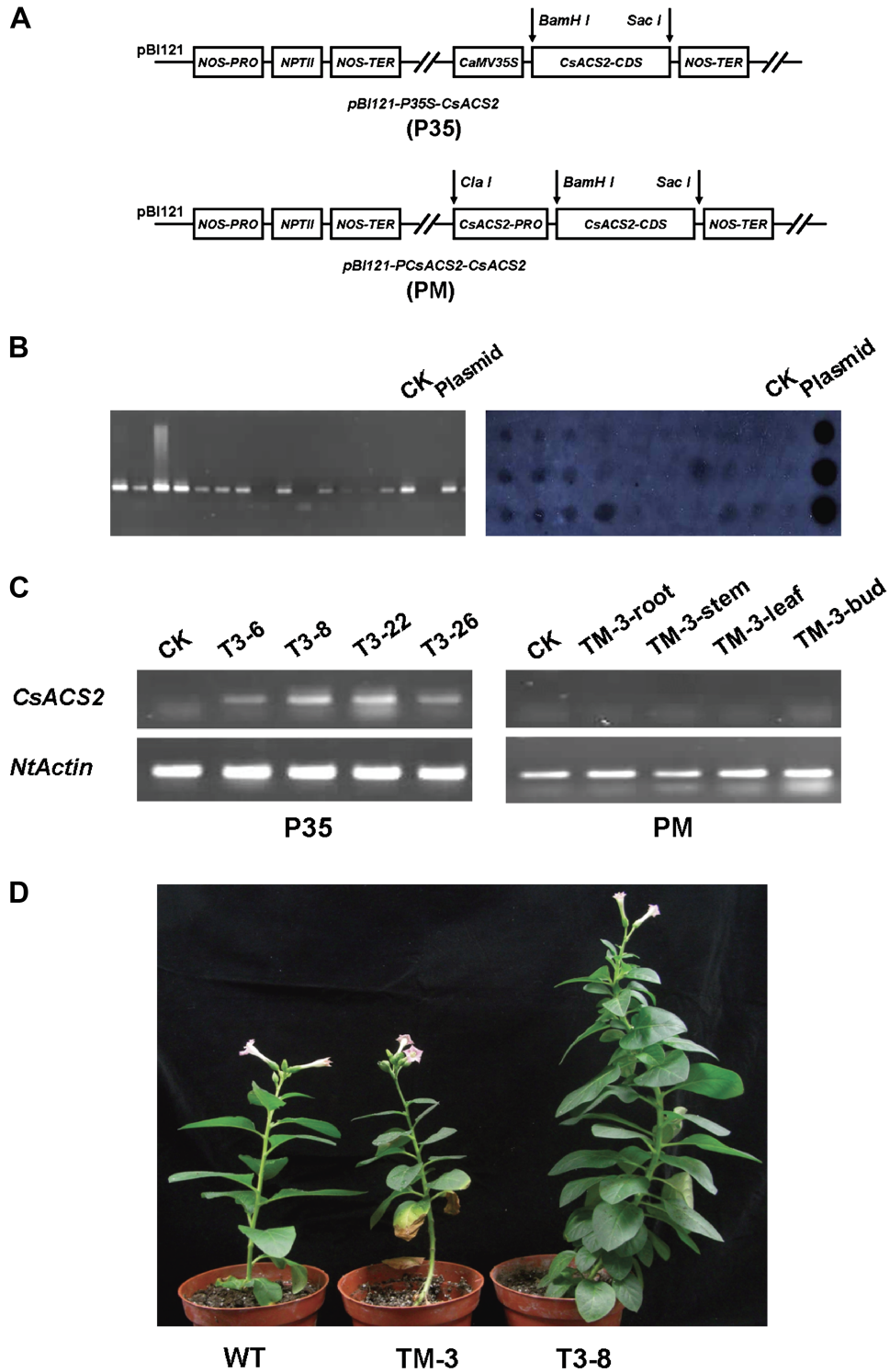
Fig. 1. Transgenic *CsACS2* tobacco plants. (A) The two plasmid vectors used for tobacco transformation. The *CsACS2* coding sequence was digested with *Bam*HI and *Sac*I, and ligated into *pBI121* to produce the *pBI121-P35S-CsACS2* construct. The *CsACS2* promoter fragment was then digested with *Cla*I and *Bam*HI to give the *pBI121-PCsACS2-CsACS2* vector. (B) PCR and dot blotting analysis of transgenic tobacco plants. A total of 36 regenerated plants (27 P35 plants and nine PM plants) were assayed to find the positive transgenic events. Wild-type tobacco is indicated as CK, and this figure revealed part of the positive results. (C) Analysis of *CsACS2* expression in transgenic plants. The left panel shows the expression pattern in four P35 plants (T3-6, T3-8, T3-22, and T3-26). No expression was detected in any positive PM transgenic tobacco plants, and the right panel shows the analysis of root, stem, leaf, and bud tissues from the TM-3 plant. (D) Phenotypes of the transgenic tobacco plants. TM-3 was a plant derived from the PM transformation (*CsACS2* under control of the cucumber native promoter), and T3-8 was from the transformation of P35 (*CsACS2* under control of the 35S promoter). (This figure is available in colour at *JXB* online.)

different promoters. After identification by PCR and dot blotting (Fig. 1B), five P35 and three PM transgenic tobacco plants were chosen for self-pollination to produce the homozygous T_2 offspring.

RT-PCR revealed a high level of expression of *CsACS2* in the P35 transgenic lines, T3-6, T3-8, T3-22, and T3-26 (Fig. 1C), and the plants showed delayed flowering compared with wild-type tobacco (Fig. 1D). The overexpression lines also had a shorter internode distance and increased leaf number before flowering (Fig. 1D). These phenotypes might have a relationship with an endogenous hormone balance disorder.

The P35 plants also showed higher ethylene production. Using gas chromatography analysis, it was found that leaves of three overexpression lines, T3-8, T3-22, and T3-26, produced more ethylene than did the wild-type (Fig. 2). This result indicated that the *CsACS2* gene is involved in ethylene biosynthesis *in planta*, and it could lead to more ethylene production in the transgenic tobacco system.

Induction of CsACS2 *expression in transgenic tobacco*

Using RT-PCR, it was not possible to detect any expression of *CsACS2* in the PM lines; and the right-hand part of Fig. 1B shows this result in root, stem, leaf, and flower bud from the TM3 line. This is consistent with the fact that there was no visible change in phenotype of transgenic PM lines (Fig. 1D), and indicated that the native *CsACS2* promoter from cucumber had no activity in tobacco. Moreover, ethylene production in TM3 was not significantly difference from that in wild-type tobacco (Fig. 2). However, after ethephon treatment, some differences in the expression level could be detected in the flower bud and its adjacent leaf (Fig. 3). It was supposed that treatment with 1.0 mM ethephon could induce more accumulation of *CsACS2*-specific mRNA due to transcription than 0.5 mM ethephon treatment, and that induction in flower bud was more sensitive than in leaf.
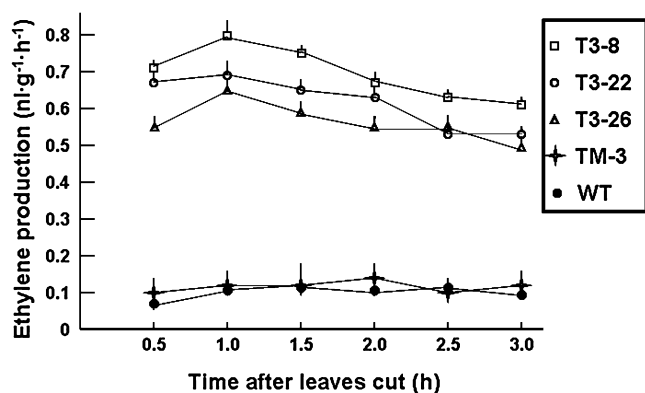

Fig. 2. Ethylene production in transgenic tobacco plants. Cut leaf sections from three P35 lines (T3-8, T3-22, and T3-26) and one PM line (TM-3) were used to analyse ethylene production by gas chromatography. Vertical bars indicated the standard deviation of the means for triplicate samples.
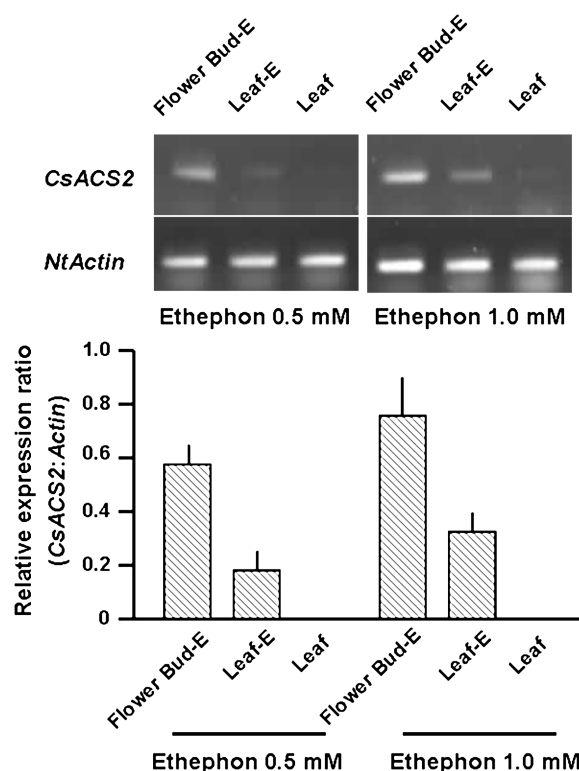

Fig. 3. RT-PCR gel images (upper) and relative expression ratio (bar graph) of *CsACS2* normalized to the *Actin* gene internal control in an ethephon-treated PM transgenic plant (TM-3). Leaf, untreated lower leaf; Flower Bud-E, ethephon-treated flower bud; Leaf-E, leaf adjacent to the ethephon-treated flower bud. Significance at $P \leqslant 0.05$ is indicated by mean standard error bars ($n=3$).

Induced expression of CsACS2 *in cucumber*

To analyse the effects of ethylene on *CsACS2* expression in cucumber, the basal level of transcription needed to be determined initially. As a result of the different expression patterns of *CsACS2* (Fig. 4), the cucumber line M06 was selected for the ethylene induction experiments, while lines G34 and G06 were used to study the inhibitory effect of ethylene on *CsACS2* expression.

In M06, after 2 h treatment with 1.0 mM ethephon, the expression of *CsACS2* could be detected; the transcription level peaked at 4 h after treatment, and then declined rapidly at 6 h. With 0.1 mM and 0.5 mM ethephon treatment, transcription was first detected at 4 h, and reached the highest level at 6 h (Fig. 5). This result indicated that certain concentrations of exogenous ethylene can induce the expression of *CsACS2* in cucumber, and that higher concentrations result in more rapid induction and accumulation of *CsACS2*-specific mRNA. Possible causes for the observed decline in *CsACS2* expression after treatment with 1.0 mM ethephon for 6 h will be discussed below.

Reduced expression of CsACS2 *in cucumber in response to chemical treatment*

After 3 d of treatment with $AgNO_3$ and AVG, a decline in *CsACS2* expression was observed in cucumber shoot apices.
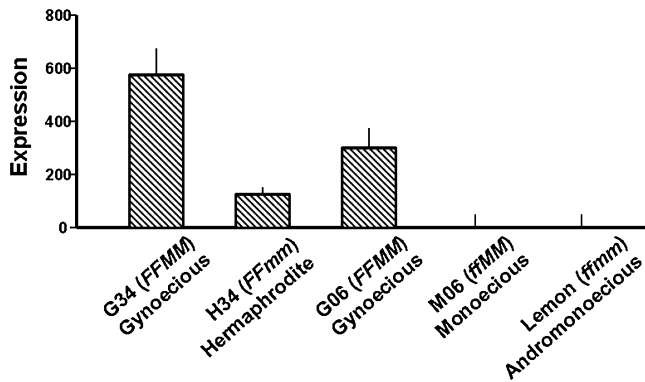
Fig. 4. Quantitative RT-PCR of *CsACS2* expression in five cucumber lines: G06 (*FFMM*), M06 (*ffMM*), G34 (*FFMM*), H34 (*FFmm*), and 'Lemon' (*ffmm*). Total RNA was extracted from shoot apices of cucumber plants at the four-leaf stage. Each result was the average of three independent biological replicates, and the significance at $P \leqslant 0.05$ is indicated by mean standard error bars ($n=3$).
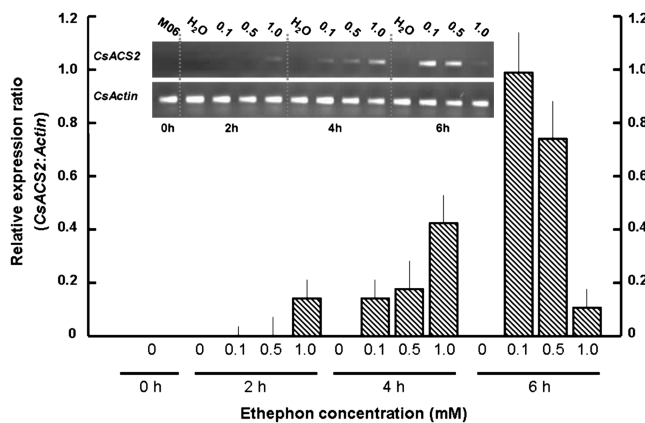

Fig. 5. RT-PCR gel images (upper) and relative expression ratio (bar graph) of *CsACS2* normalized to the *Actin* gene control in shoot apices of cucumber line M06 (*ffMM*). Apical shoots of four-leaf stage seedlings were soaked in water containing 0, 0.1, 0.5, and 1.0 mM ethephon for 2, 4, and 6 h, respectively. The apices were then excised for RNA extraction. Data analysis was as given in Fig. 3.

For the $AgNO_3$ treatment, expression decreased by 42% and 18% in G34 and G06, respectively. However, treatment with AVG had a more pronounced inhibitory effect than was observed for $AgNO_3$; *CsACS2* expression decreased by 60% and 95% in G34 and G06, respectively (Fig. 6). Therefore, the inhibitory effects of the endogenous ethylene response and biosynthesis negatively controlled *CsACS2* expression.

Effects of chemical treatment on sex determination in cucumber

Besides the effects on the expression of *CsACS2*, ethylene has a significant correlation with sex determination in cucumber plants, and it can induce femaleness. Table 1 shows the effect of ethylene-related treatments on the gynoecious (G06 and G34), monoecious (M06), hermaphrodite (H34), and andromonoecious ('Lemon') cucumber lines. $AgNO_3$ is known to interfere with ethylene action, and its application promoted the conversion of female/hermaphrodite flowers into male flowers, which increased male flower number in all four cucumber cultivars tested (G06, from 0.5 to 13.5; G34, from 0 to 10.4; H34, from 0 to 13.5; and 'Lemon', from 16.6 to 28.4). Moreover, ethephon could induce development of female flowers in M06, with female flowers produced on 5.6 and 7.4 nodes after treatment with 0.5 mM and 1.0 mM ethephon, respectively. In bisexual lines, 0.5 mM ethephon treatment resulted in more bisexual flowers (16.5) in 'Lemon'. Unexpectedly, when the concentration was increased to 1.0 mM, some female flowers (8.9) were produced, along with a further reduction of both male and bisexual flowers. A similar phenomenon was found with line H34, in which female flowers were first produced at 0.5 mM ethephon (3.2 nodes), and increased to 11.3 nodes at 1.0 mM. The number of bisexual flowers on H34 plants decreased to 24.3 and 12.6 nodes, respectively, at 0.5 mM and 1.0 mM ethephon. These results confirm the action of ethylene in arresting stamen development, which was expressed as the conversion of male/hermaphrodite into female flowers in cucumber plants with ethephon treatment, and suggest that there is a significant correlation between ethylene content and sex expression in cucumber.

CsACS2 promoter sequence analysis

The 1958 bp promoter fragments derived from G34 and H34 genomic DNA were sequenced, and no DNA polymorphisms were identified between them (GenBank accession no. FJ529216). Additionally, analysis of the conserved *CsACS2* promoter sequence indicated that there were two EREs (ethylene-responsive elements; AWTTCAAA) at positions −154 to −147 and −1334 to −1327, respectively (Table 2). The EREs were identified as an ethylene-responsive enhancer element in *Solanum lycopersicum* (Montgomery *et al.*, 1993), *Dianthus caryophillus* (Itzhaki *et al.*, 1994), and *Lycopersicon chilense* (Tapia *et al.*, 2005). These two *cis*-acting elements might take part in the ethylene-regulated expression of *CsACS2*. Moreover, one cytokinin (CTK) response element and one LEAFY element were also found in the *CsACS2* promoter. These findings could be in agreement with the spatial expression pattern of *CsACS2* in the cucumber shoot apical meristem (Saito *et al.*, 2007).

Discussion

There is a long history of research into the mechanisms of sex determination in cucumber, and extensive physiological studies indicate that the plant hormone ethylene is the key point in the regulatory pathways. Based on the studies of Yin and Quinn (1995) and Yamasaki *et al.* (2001), the 'one-hormone hypothesis' was proposed, in which the core view
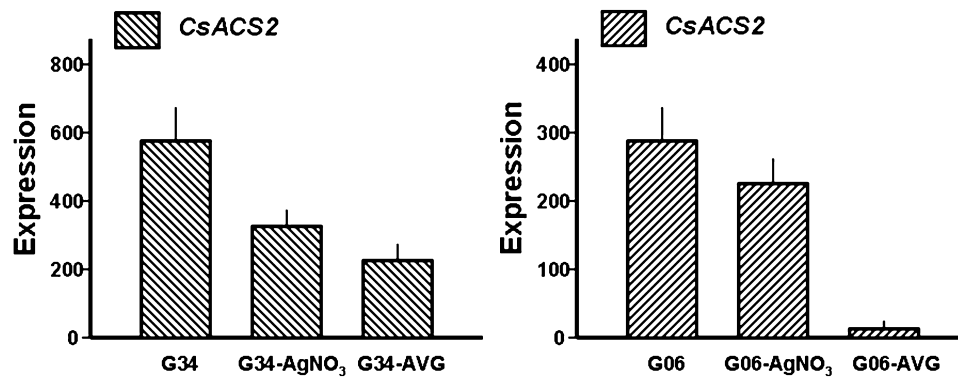
Fig. 6. Quantitative RT-PCR analysis of *CsACS2* expression in two cucumber lines homozygous for the *F* locus after AgNO$_3$ or AVG treatment. Left panel: expression of *CsACS2* in cucumber line G34 (*FFMM*) resulting from different chemical treatments. Right panel: *CsACS2* gene expression in G06 (*FFMM*). AgNO$_3$ treatments are marked with '-AgNO$_3$', and AVG treatments with '-AVG'. Plant materials, RNA extraction, and expression analysis were as described in Fig. 4 and in the Materials and methods.

Table 1. Effects of chemical treatment on sex expression in cucumber plants

Cultivar and genotype	Treatment	No. of flowers per plant[z]			
		Male flowers	**Bisexual flowers**	**Female flowers**	**Abortion**
G06 (*FFMM*)	Control	0.5±0.3 a	0.0 a	28.8±1.0 b	0.8±0.8 a
	AgNO$_3$ (1.0 mM)	13.5±2.1 b	0.0 a	15.5±2.2 a	1.0±0.9 a
G34 (*FFMM*)	Control	0.0 a	0.0a	28.5±0.8 b	1.1±0.4 a
	AgNO$_3$ (1.0 mM)	10.4±1.7 b	0.0 a	18.2±1.8 a	0.9±0.8 a
M06 (*ffMM*)	Control	28.8±0.7 b	0.0 a	0.6±0.5 a	0.6±0.5 a
	Ethephon (0.5 mM)	18.2±2.8 a	0.0 a	5.6±1.2 b	3.6±1.8 a,b
	Ethephon (1.0 mM)	15.7±5.6 a	0.0 a	7.4±3.2 b	6.9±3.2 b
H34 (*FFmm*)	Control	0.0 a	29.6±0.5 d	0.0 a	0.5±0.4 a
	AgNO$_3$ (1.0 mM)	13.5±1.0 b	16.3±1.0 b	0.0 a	0.4±0.2 a
	Ethephon (0.5 mM)	0.0 a	24.3±1.2 c	3.2±0.5 b	2.4±1.3 a
	Ethephon (1.0 mM)	0.0 a	12.6±2.0 a	11.3±0.8 c	6.1±2.1 b
'Lemon' (*ffmm*)	Control	16.6±1.7 b	13.1±2.0 c	0.0 a	0.5±0.3 a
	AgNO$_3$ (1.0 mM)	28.4±1.7 c	1.3±1.5 a	0.0 a	0.5±0.3 a
	Ethephon (0.5 mM)	12.3±3.0 a	16.5±2.5 c	0.0 a	1.8±0.5 a
	Ethephon (1.0 mM)	10.3±2.0 a	8.4±1.3 b	8.9±2.0 b	2.4±3.0 a

AgNO$_3$ and ethephon were applied to the shoot apices of cucumber plants at the four-leaf stage. The number of male, female, and bisexual flowers was recorded up to the 30th node on the main stem. Nodes with no flowers were defined as 'abortion'.
[z] Mean values of five independent trials. Means followed by different letters are significantly different at the 5% level by LSD test.

Table 2. Specific putative cis-acting elements in the promoter of *CsACS2* (negative values indicate the first nucleotide position of the *cis*-elements with respect to the transcription initiation site located at position +1)

Response	*cis*-element	Sequence (5' to 3')	Position
CTK response	CPBCSPOR [TATTAG]	TATTAG	−1407[a]
Ethylene response	ERE [A(A/T)TTCAAA]	AATTCAAA	−154,−1334
LEAFY response	LEAFYATAG [CCAATGT]	CCAATGT	−1715[a]

[a] Sequence of the complementary strand.

is that ethylene regulates expression of the three cucumber flower phenotypes (female, male, and hermaphrodite). According to this hypothesis, the *F* locus is involved in ethylene synthesis, and the *M* locus is part of the ethylene signalling pathway. It was previously shown that the products of both *CsACS1G* (the *F* locus gene) and *CsACS2* (the *M* locus gene) possess ACS enzyme activity, and that they might both be involved in ethylene biosynthesis (Li *et al.*, 2009). In the present study, overexpression of *CsACS2* in transgenic tobacco resulted in production of increased levels of ethylene gas, thus confirming that *CsACS2* encodes an ethylene synthesis-related enzyme. Therefore, the regulation pattern and the response relationship between *CsACS2* and ethylene needs to be clarified.

The present results demonstrated that the native *CsACS2* promoter from cucumber could be induced by ethephon. This effect was detected in both transgenic tobacco and cucumber. These results suggest that the ethylene-responsive elements (possibly the EREs) in the cucumber *CsACS2* promoter had a conserved function in the two plants. Unexpectedly, in cucumber plant, it was found that when

the ethephon concentration was increased to 1.0 mM, and the treatment extended to 6 h, the expression of *CsACS2* declined significantly. This is in agreement with the results of the study of Wang *et al.* (2010), in which treatment with ethephon at 1.0 mM for 6 h caused DNA damage in cucumber protoplasts.

The results of experiments in which chemical treatments interfered with endogenous ethylene action also confirmed this conclusion. To summarize, it was demonstrated that *CsACS2* could cause increased ethylene production, and that expression of the *CsACS2* gene can be induced by ethylene. Thus, a positive feedback mechanism could exist in the regulation of the *CsACS2* gene. In a previous study, *CsACS2* expression analysis indicated significant differences (>9-fold) between NILs for the *M* locus (Li *et al.*, 2009). The experiments were repeated with more accurate shoot apex materials, and it was confirmed that the transcription level was much higher in the gynoecious line, G34 (*FFMM*), than in its hermaphrodite NIL partner, H34 (*FFmm*) (Fig. 4). Considering the very close genetic relationship, the difference in expression probably derives from the *CsACS2* (*M* locus gene) itself. In this study, the allelic gene promoter fragments isolated from G34 and H34 did not show any sequence polymorphism. In previous work, it was also demonstrated that the recessive *m* allele, which is mutated at a single nucleotide, encoded an inactive product (Li *et al.*, 2009). Thus, the putative positive feedback mechanism suggested here could explain this difference between the NILs. It is proposed that the ethylene derived from the wild-type *M* locus (*CsACS2*) induced self-expression, which was absent in the *mm* genotype because of the non-functional mutant.

The involvement of ethylene in the development of the gynoecium has been reported in hermaphrodite plants such as *Arabidopsis* and tobacco (De Martinis and Mariani, 1999; Tsuchisaka and Theologis, 2004). Moreover, ethylene production can directly disrupt stamen development in *Arabidopsis* (Duan *et al.*, 2008). However, in the present study, relatively high concentrations of ethylene did not have an obvious arresting effect on tobacco stamen development (Supplementary Fig. S1 at *JXB* online), and ethephon treatments also did not cause any significant changes in filament length in PM transgenic plants (unpublished data). This leads to the proposal that stamen development in tobacco is less sensitive to ethylene than in cucumber and *Arabidopsis*.

The action of ethylene in arresting stamen development in cucumber was confirmed in the present study. The classical chemical application tests were repeated, and it was found that $AgNO_3$ could induce stamen development, which led to an increase in the number of male flowers in all four cucumber genotypes. It was also shown that ethylene could induce pistil development and stamen arrest in three genetic backgrounds. As observed by Yamasaki *et al.* (2001), treatment with a low concentration of ethephon (0.5 mM in the present study) could induce the production of female flowers in the monoecious line (M06), but was not able to convert hermaphrodite into female flowers in the andromonoecious

line ('Lemon'). Unexpectedly, it was found that relatively high concentrations of ethephon could induce the change from bisexual flowers to female flowers in the hermaphrodite (H34 at 0.5 mM and 1.0 mM) and andromonoecious ('Lemon' at 1.0 mM) lines. As the *M* locus encodes an ethylene synthase and caused ethylene production *in planta*, it is proposed that there could be a local ethylene threshold to control the action of ethylene in arresting stamen development in cucumber. Under natural conditions, the ethylene derived from the continuous expression of *CsACS2*, which is induced by *CsACS1G* and maintained by positive feedback regulation, stays above the threshold and arrests the stamen development from stage 5 to stage 12 shown by Bai *et al.* (2004) and Satio *et al.* (2007), leading to female flowers in cucumber. In the *mm* genotype (hermaphrodite and andromonoecious lines), the ethylene threshold is not maintained, and stamen development does not arrest. In this case, the appropriate concentration of exogenous ethylene could compensate for this threshold and arrest stamen development, which was revealed when the lines H34 and 'Lemon' were treated with 1.0 mM ethephon. Moreover, in the H34 line, 0.5 mM ethephon induced female flowers, but this concentration was ineffective in 'Lemon'. This finding was consistent with higher endogenous levels of ethylene in the H34 line than in 'Lemon' (Supplementary Fig. S2 at *JXB* online).

Saito *et al.* (2007) performed a comprehensive study on the expression pattern of *CsACS2*. Their results showed that the *CsACS2* mRNA initially accumulated just beneath the pistil primordia of flower buds at the bisexual stage, and this coincided with the sex determination stage (stage 6) in cucumbers (Bai *et al.*, 2004). It was noticed in the study that the continuous accumulation of *CsACS2* mRNA was correlated with the final establishment of female flowers. These results imply the relationship between the expression of *CsACS2* and the continuous arrest of stamen development. As has been proposed from this study, the positive feedback mechanism which leads to a constant level of transcription of *CsACS2* could supply continuous ethylene, and could therefore constantly arrest stamen development.

Combining all the previous data for *F* and *M*, the following modified model for sex determination in cucumber is proposed (Fig. 7).

(i) Both *F* (*CsACS1G*) and *M* (*CsACS2*) encode ACC synthases, and their cooperation produces female, male, or bisexual flowers.

(ii) In the primordial buds that will develop into female flowers, *F* may be the first specific gene activated, and the eventual product, ethylene, promotes pistil primordia development. At the same time, ethylene activates *M*, which then begins its positive feedback expression. The continuous accumulation of ethylene beneath the region of the pistil primordia by translation of the *M* gene mRNA constantly arrests stamen primordia development, resulting in female flowers.
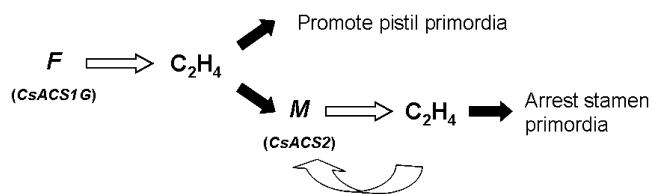
Fig. 7. Model of *F* and *M* functions during the development of female flowers in cucumber. Both *F* and *M* encode ACC synthases. *F* is the first specific gene activated and its eventual product, ethylene, promotes pistil primordia development. Concomitantly, ethylene activates *M*, and *CsACS2* then begins its positive feedback activating expression. The continuous accumulation of ethylene constantly arrests the development of stamen primordia, resulting in the production of female flowers.

(iii) In the pre-determined stamen primordial buds, *F* will not be activated, and there will not be enough ethylene to start pistil primordia development and promote the expression of *M*. As a result, the pistil primordia do not develop and the arrest of stamen development does not happen. Thus, male flowers develop.

(iv) In the *F-mm* genotype, *F* will be activated normally, and pistil primordia develop without resistance. However, there will not be enough ethylene accumulation to eliminate the development of stamen primordia because of the inactive ACC synthase mutant in the *mm* genotype. Thus, bisexual flowers develop.

The detailed mechanism of how ethylene arrests stamen development is still unknown in cucumber, and the ethylene threshold proposed here is speculative. The hypothesis stated above is probably not the final one, and it can only explain the current research results in cucumber. Identifying more mutations in the sex expression pathway and cloning the *a* gene in cucumber may help to reveal more complete information in this area.

Supplementary data

Supplementary data are available at *JXB* online.
  Figure S1. Flower phenotypes in transgenic tobacco plants.
  Figure S2. Ethylene production from shoot apices of the five cucumber cultivars.
  Table S1. Primers used in this study

Acknowledgements

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