Sex determination in cucumber (Cucumis sativus L.) plants is genetically controlled by the F and M loci. These loci interact to produce three different sexual phenotypes: gynoecious (M-F-), monoecious (M-ff), and andromonoecious (mmff). Gynoecious cucumber plants produce more ethylene than do monoecious plants. We found that the levels of ethylene production and the accumulation of CS-ACS2 mRNA in andromonoecious cucumber plants did not differ from those in monoecious and gynoecious plants and were lower than the levels measured in gynoecious plants. Ethylene inhibited stamen development in gynoecious cucumbers but not in andromonoecious ones. Furthermore, ethylene caused substantial increases in the accumulation of CS-ETR2, CS-ERS, and CS-ACS2 mRNA in monoecious and gynoecious cucumber plants, but not in andromonoecious one. In addition, the inhibitory effect of ethylene on hypocotyl elongation in andromonoecious cucumber plants was less than that in monoecious and gynoecious plants. These results suggest that ethylene responses in andromonoecious cucumber plants are reduced from those in monococious and gynoecious plants. This is the first evidence that ethylene signals may influence the product of the M locus and thus inhibit stamen development in cucumber. The andromonoecious line provides novel material for studying the function of the M locus during sex determination in flowering cucumbers.

Key words: Cucumber (Cucumis sativus) — CS-ACS2 — CS-ETR2 — CS-ERS — Ethylene — M locus — Sex expression.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; RT-PCR, reverse transcriptional polymerase chain reaction.

Introduction

Various patterns of sexual expression in cucumber (Cucumis sativus L.) plants are advantageous for investigating the general mechanism of sex determination in higher plants (Kubicki 1969a, Kubicki 1969b, Malepszy and Niemirowicz-Szczyt 1991). The gynoecious type of cucumber plant produces only female flowers. The monoecious type (the most common type of sex expression) produces male and female flowers on the same plant. The hermaphroditic type of cucumber plant produces bisexual flowers with both staminate and pistillate organs, while the andromonoecious type of cucumber plant produces bisexual and male flowers on the same plant.

Morphologically, all cucumber floral buds that later develop into male, female, or bisexual flowers have primordia of stamens and pistils at the early stage of their differentiation. Afterwards, pistil development is arrested in floral buds destined to develop into male flowers, whereas stamen development is arrested in floral buds that develop into female flowers (Kubicki 1969d). The continued development of stamens and pistils in floral buds leads to the formation of bisexual flowers (Kubicki 1969e). The sexuality of cucumber plants is genetically controlled, largely, by the F and M loci (Kubicki 1969a, Pierce and Wehner 1990). The plants are gynoecious if the genotype is M-F-, monoecious if the genotype is M-ff, hermaphroditic if the genotype is mmF-, and andromonoecious if the genotype is mmff (Pierce and Wehner 1990). To date, the functions of the F and M loci have been investigated by comparing the phenotypic expressions of female, male, or bisexual flowers (Kubicki 1969a, Pierce and Wehner 1990). The F gene is believed to regulate the degree of female flower expression, while the M gene is considered to regulate bisexual flower expression (Galun 1961, Shifriss 1961, Shifriss and George 1964, Kubicki 1969b, Frankel and Galun 1977, Pierce and Wehner 1990).

Although sexual expression in cucumber plants is genetically controlled, it can be easily modified by plant hormones and environmental conditions (Atsmon and Galun 1960, Galun 1961, Shifriss 1961, Shifriss and George 1964, Kubicki 1969b, Frankel and Galun 1977, Takahashi et al. 1983, Durand and Durand 1984). Production of the plant hormone ethylene is highly correlated with femaleness in cucumber. For example, gynoecious cucumber plants produce more ethylene than monoecious types (George 1971, Rudich et al. 1972, Trebitsh et al. 1987), and inhibitors of ethylene biosynthesis, or its downstream action, suppress the development of female flowers and induce male flowers (Beyer 1976, Atsmon and Tabbakh 1979, Takahashi and Suge 1980, Takahashi and Jaffe 1984). Furthermore, the application of ethylene to monoecious cucumber plants promotes the formation of female flowers (MacMurray and Miller 1968, Iwahori et al. 1970, Takahashi and Suge 1980, Takahashi and Suge 1982, Saito and Takahashi 1987). These
results suggest that ethylene is a regulator of sex determination in cucumber plants. The relationship between the molecular regulation of endogenous ethylene and the expression of female flowers in cucumber plants was recently demonstrated by the detection of the CS-ACS2 gene encoding 1-aminoacyclopropane-1-carboxylate (ACC) synthase. The accumulation of CS-ACS2 mRNA is highly correlated with female flower expression (Kamachi et al. 1997). We have previously isolated three cDNAs of putative ethylene receptors, CS-ETR1, CS-ETR2, and CS-ERS from cucumber plants (Yamasaki et al. 2000). The accumulation of CS-ETR2 and CS-ERS mRNA was induced by ethylene in monoecious cucumber plants. This accumulation correlated with the accumulation of CS-ACS2 mRNA, ethylene production, and female flower expression in monoecious and gynoecious cucumber plants (Yamasaki et al. 2000). These results suggest that ethylene production as well as the ethylene responses are involved in sex expression of flowers in cucumber plants.

Although comparisons between gynoecious and monoecious cucumber plants have been well studied, not much attention has been paid to the andromonoecious cucumber plants. In melon (Cucumis melo L.) plants, whose common sex expression pattern is andromonoecious, it is known that ethylene application induces the formation of bisexual flowers but not female flowers (Beyer 1976). This fact suggests that the ethylene response of the stamen differs between monoecious and andromonoecious cucumber plants. That is, in monoecious cucumber plants, ethylene induces the development of pistils and inhibits the development of stamens in floral primordia, causing the formation of female flowers. In andromonoecious cucumber plants, which carry a loss-of-function of the M locus, ethylene induces the development of stamens but does not inhibit the development of stamens in floral primordia, causing the formation of bisexual flowers. In the present study, we tested this hypothesis by first comparing the accumulation of CS-ACS2 mRNA and ethylene production among gynoecious, monoecious, and andromonoecious cucumber plants. We then analysed the effect of ethylene on ethylene-inducible gene expression, sex expression of flowers, and hypocotyl elongation among gynoecious, monoecious, and andromonoecious cucumber plants. Based on our results, the function of F and M loci in the regulation of sex expression in cucumber plants is discussed.

Materials and Methods

Plant materials

Monoecious (cv. Otone No. 1), gynoecious (cv. Higan-fushinari), and andromonoecious (cv. Lemon and 94–64–62–10M) lines of cucumber (Cucumis sativus L.) were used in this study. Sex expression conditions for monoecious and gynoecious cucumber plants have been reported elsewhere (Takahashi and Suge 1980, Takahashi et al. 1983). In brief, most cucumber plants, including Otone No. 1, prefer short-day conditions for production of female flowers and long-day conditions for production of male flowers. Higan-fushinari, however, becomes completely gynoecious under long-day conditions and produces male flowers on the lower nodes of the main stem under short-day conditions. Lemon and 94–64–62–10M cucumber plants produce bisexual and male flowers on the main stem. Seeds of Otone No. 1 cucumber plants were purchased from Watanabe Seed Co., Kogota, Japan. Higan-fushinari cucumber seeds were provided by Dr. Takashi Saito (Tokyo University of Agriculture), and Lemon cucumber seed were purchased from Ferry-Morse Seeds Co. (Mountain View, CA, U.S.A.). All seeds were maintained by inbreeding at our laboratory. Seeds of 94–64–62–10M cucumber were provided by Tohoku Seed Co., Ltd., Hiroko, Japan. All seeds were germinated on wet filter paper in a Petri dish at 28°C in the dark for 1 to 2 d. Resulting seedlings were transferred to plastic pots containing a soil composite, Kureha-Engei-Baido (0.4 g N, 1.9 g P, 0.6 g K per kg; Kureha Chemical Co., Tokyo). Plants were grown under a 24-h photoperiod with daylight supplemented by fluorescent lamps in a greenhouse. Plants were adequately watered and supplied with fertilizer, 0.002% (v/v) Hypoxen (Hypoxen-Japan, Osaka).

Northern blot analysis

Total RNA was isolated from shoot apices and flower buds. The shoot apices of cucumber plants were used when the leaf blade of the fourth leaf was approximately 2 cm long (defined as the 4-leaf stage). The apical shoot, including immature leaves shorter than 2 cm in length, was excised as the shoot apex. Male, female, and bisexual flower buds in each cucumber plant were excised when the length of each flower bud was approximately 1 cm long. Chemicals were applied to shoot apices and excised 6, 12, and 24 h after treatment. These samples were frozen immediately in liquid nitrogen, and stored at −80°C prior to the extraction of nucleic acids. Total RNA was extracted from those samples using ISOGEN (Nippongene, Tokyo), according to the manufacturer’s instructions. For Northern blot analysis, 10 µg of total RNA was fractionated by electrophoresis on 1% agarose gel after denaturation with glyoxal at 50°C for 1 h. The gel was stained with ethidium bromide, and the quantity of RNA in each lane was verified by comparing rRNA levels. The fractionated RNA was then transferred to a nylon membrane (Nytran NY13N; Schleicher and Schuell, Dassel). The membrane was hybridized with DIG (digoxigenin)-labelled RNA probes specific for CS-ACS2, CS-ETR1, CS-ETR2, and CS-ERS genes (Yamasaki et al. 2000). Post-hybridization washes were performed twice successively for 5 min each in 2× SSC/0.1% SDS at room temperature and twice successively for 20 min each in 0.2× SSC/0.1% SDS at 65°C. Then the washed membrane was exposed to X-ray film (Hyperfilm-MP, Amersham International plc, Amersham, Bucks., U.K.).

Quantitative analysis by RT-PCR

Total RNA was extracted from shoot apices of monoecious (Otone No. 1), gynoecious (Higan-fushinari), and andromonoecious (Lemon and 94–64–62–10M) cucumbers as described above. The concentration of total RNA was measured using a spectrophotometer (DU-65 Spectrophotometer, Beckman Instruments, Inc., Tokyo). The cDNA was synthesized from total RNA using M-MLV Reverse Transcriptase RNAseH Minus (Toyobo, Tokyo) and random hexamer in a 20 µl volume. PCR was performed with Premix Taq (TaKaRa Ex Taq™, Version, Takara Shuzo Co., Ltd., Shiga) using 1 µl of the cDNA reaction and a specific primer set. The specific primers were designed as follows: ACS2-F, 5'-TTT AAG GTT GGA TCT GTC TGC CTT G-3' and ACS2-R, 5'-GAA TGT CTT CGA TTG TGG ACC G-3' for CS-ACS2 (Kamachi et al. 1997); ACS3-F, 5'-GTA CGG ACC AGG TTC GCC TG-3' and ACS3-R, 5'-ATT CAA GGA ATT GCC GC-3' for CS-ACS3 (accession number AB006803); ETR2-3F, 5'-CAC GAA
Table 1  Effects of ethylene on the sexual expression of andromonoecious (Lemon) and monoecious (Otone No. 1) cucumber plants

<table>
<thead>
<tr>
<th>Cultivar and treatment</th>
<th>Male flowers (%)</th>
<th>Bisexual flowers (%)</th>
<th>Female flowers (%)</th>
<th>Abortion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lemon (muff)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>12.3±0.3 (81.3±2.2)</td>
<td>2.7±0.3 (18.7±2.2)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ethephone</td>
<td>8.1±0.5 (54.0±3.4)</td>
<td>6.3±0.4 (42.0±2.5)</td>
<td>0.0</td>
<td>0.6±0.4 (4.0±2.7)</td>
</tr>
<tr>
<td>Otone No. 1 (M-ff)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.1±0.2 (73.9±1.5)</td>
<td>0.0</td>
<td>3.9±0.2 (26.1±1.5)</td>
<td>0.0</td>
</tr>
<tr>
<td>Ethephone</td>
<td>4.9±0.5 (32.8±3.0)</td>
<td>0.0</td>
<td>9.5±0.2 (63.3±1.3)</td>
<td>0.6±0.3 (3.9±2.2)</td>
</tr>
</tbody>
</table>

Ethephone at the concentration of 0.35 mM was applied to the shoot apices of cucumber plants at the 4-leaf stage. The number, and percentage in parentheses, of male, female, or bisexual flowers were calculated up to the 15th node on the main stem (Lemon, n = 10; Otone No. 1, n = 12). Nodes with no flowers were defined as “abortion”. All values represent the means ± SE.

GCA TGG CAC TTG TTC-3’ and ETR2-3R, 5’-CAC CGT TCA TCC CAA TCT GC-3’ for CS-ETR2; ERS-3F, 5’-CGA TCA AAC AAA ACA AAT AGT GGC-3’ and ERS-3R, 5’-TGG TGG GCT GCA CAT TTG GC-3’ for CS-ERS; and CS-actin-F, 5’-GAC ATT CAA TGT GCC TGC TAT G-3’ and CS-actin-R, 5’-CAT ACC GAT GAG AGA TGG CTG-3’ for actin (accession number AB010922). The PCR conditions were 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min. The PCR products were analysed on a 1.2% agarose gel by electrophoresis and blotted onto a nylon membrane. The membrane was hybridized with either DIG-labelled cDNA or DIG-labelled oligonucleotides. CS-AC2 and CS-ACS3 cDNAs were amplified by RT-PCR from total RNA isolated from the shoot apices of Otone No. 1 at the 4-leaf stage with the primer pairs ACS2-F/ACS2-R and ACS3-F/ACS3-R, respectively. The amplified cDNAs were directionally ligated into pGEM®-T vector (Promega Inc., Madison), sequenced, and used to make DIG-labelled cDNA probes. The cloned cDNAs were labelled according to the instructions provided with the DIG DNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany). For detection of CS-ERS, CS-ETR2, and actin cDNA, oligonucleotides labelled with the DIG at both the 5’ and 3’ ends were synthesized as follows: CS-ERS3F3R, 5’-GAA CAC GGC TGT CGT TCC TTT ATC GGC GGC-3’ for CS-ERS; CS-ETR23F3R, 5’-TTC AGG CAT GGC CAC TGC TAT GGA GGG TCG-3’ for CS-ETR2; and CS-actin3F3R, 5’-ACA CCA TCA CCA GAA TCC AGC ATA CCA-3’ for actin. The hybridization conditions, post-hybridization washes, and DIG-detection were carried out exactly as described above. The density of the band exposed to X-ray film was measured with imaging densitometer (Model GS-700, BIO-RAD, Tokyo).

Chemical application
To determine differences in the effects of ethylene on hypocotyl elongation among cucumber cultivars, we first germinated the seeds of gynoecious (Higan-fushinari), monoecious (Otone No. 1), and andromonoecious (Lemon) cucumber on wet filter paper in a Petri dish at 28°C in the dark. When the roots were approximately 5 mm long, the seedlings were transferred to plastic chambers containing ethylene gas at various concentrations. After incubation in the dark for an additional 5 d, the length of the hypocotyl was measured. Seven plants were used for each treatment.

To determine the effect of ethylene on sex expression, we applied 50 μl of 0.35 mM 2-chloroethylphosphonic acid (ethephone), an ethylene releasing agent (Iwahori et al. 1970), in H2O containing 0.1% (v/v) Tween 20 to the shoot apices of cucumber at the 4-leaf stage using a micropipette once a day for 3 d. The sex of each flower up to node 15 on the main stem was determined and classified as male, bisexual, or female. Then, the percentages and the numbers of nodes with male, female, or bisexual flowers on the main stem were calculated.

To investigate the effects of ethylene on the development of stamens and pistils among cultivars, we applied 50 μl of 0.35 mM ethephone in H2O containing 0.1% (v/v) Tween 20 to the shoot apices of andromonoecious cucumbers. We also applied 50 μl of 1.0 mM AgNO3, an ethylene action inhibitor (Beyer 1976, Atsum and Tabak 1979, Takahashi and Jaffe 1984, Takahashi and Suge 1980), in H2O containing 0.1% (v/v) Tween 20 to the shoot apices of gynoecious cucumbers. These solutions were applied to the shoot apices of both andromonoecious (Lemon, n = 3 per experiment) and gynoecious (Higan-fushinari, n = 3 per experiment) cucumbers as described above. The sex of each flower up to node 15 on the main stem was determined and classified as male, bisexual, or female. We measured the total lengths of anthers and filaments (defined the length of stamens) and those of stigmas and ovaries (defined the length of pistils) of all flowers (n = 15±3). We also applied 50 μl of H2O containing 0.1% (v/v) Tween 20 to the shoot apices of both andromonoecious and gynoecious cucumbers as controls.

To study the ethylene-induced accumulation of CS-ETR1, CS-ETR2, and CS-ERS mRNA, the shoot apices were cut by razor blade 6, 12, and 24 h post-ethephone application for 3 d.

Quantitation of ethylene
To examine the ethylene production from three cucumber cultivars, Otone No. 1, Higan-fushinari, and Lemon, shoot apices were excised at the 4-leaf stage. These samples were each enclosed in a 18.2-ml vessel and sealed with a rubber stopper. After incubation at 25°C for 16 h, 1 ml of head gas was withdrawn from each vessel using a gas-tight syringe and injected into a gas chromatograph (GC-4CMF, Shimadzu, Kyoto) equipped with a flame ionization detector and an activated alumina column for the measurement of ethylene. The instrument was calibrated with standard ethylene gas.

Results
Effects of ethylene on the sexual expression of monoecious, gynoecious, and andromonoecious cucumber plants
Table 1 shows the effects of ethylene (ethephone) on sexual expression in monoecious and andromonoecious cucumber plants. Control plants of monoecious cucumber had male ten-
Ethylene responses and sex expression in cucumber

dendencies, producing male flowers on 73.9% of the nodes and female flowers on 26.1% of the nodes when observed up to node 15 on the main stem (Table 1). When ethephone was applied to the shoot apices of monoecious cucumber plants at the 4-leaf stage, female flowers were produced on 63.3% of the nodes, suggesting a 37.2% greater femaleness than the control ($p < 0.0001$, Table 1). In this experiment, the andromonoecious cucumber plants treated with ethephone increased the number of bisexual flowers but produced no female flowers (Table 1).

We next examined the effect of ethylene on the development of stamens and pistils in andromonoecious and gynoecious cucumber plants. The relationship between the length of stamens and that of pistils in each flower is shown in Fig. 1A, and those representative features are shown in Fig. 1B. Andromonoecious cucumber plants were treated with 0.35 mM ethephone to increase the ethylene level, and gynoecious cucumber plants were treated with 1.0 mM AgNO$_3$ to block the action of endogenous ethylene. Bisexual flowers with pistils at various developmental stages and complete stamens appear in the ethephone-treated andromonoecious cucumbers. Male flowers and bisexual flowers with pistils and stamens at various developmental stages appear in the AgNO$_3$-treated gynoecious cucumbers.
flowers (Fig. 1A, B). Also, the lengths of male flower pistils in gynoecious cucumber plants were shorter than those in bisexual or female flowers (Fig. 1A, B). Thus, ethylene did not inhibit stamen development in andromonoecious cucumber plants, but it did inhibit stamen development in gynoecious cucumbers. In contrast, ethylene induced pistil development in both andromonoecious and gynoecious cucumber plants.

**Ethylene production and the accumulation of CS-ACS2 and CS-ACS3 mRNA in the shoot apices of gynoecious, monoecious, and andromonoecious cucumber plants**

It has been reported that ethylene production and CS-ACS2 mRNA accumulation in gynoecious cucumber plants are greater than those in monoecious ones at the 4- and 5-leaf stage (Kamachi et al. 1997). In the present study, ethylene production and the accumulation of CS-ACS2 mRNA in the shoot apices of andromonoecious cucumber plants were compared to those of gynoecious and monoecious plants. Fig. 2A shows the ethylene production in the shoot apices of these cucumber plants at the 4-leaf stage. The levels of ethylene production in monoecious, gynoecious, and andromonoecious plants were approximately 0.15, 0.26, and 0.13 μl kg⁻¹ h⁻¹, respectively (Fig. 2A).

Shoot apices of gynoecious cucumber plants produced more ethylene than those of monoecious (p < 0.004) or andromonoecious (p < 0.002) plants at the 4-leaf stage (Fig. 2A). There was little difference in ethylene production between shoot apices of monoecious and andromonoecious cucumber plants (p > 0.1; Fig. 2A). Northern blot analysis showed a greater accumulation of CS-ACS2 mRNA in the shoot apices of gynoecious cucumber plants (Fig. 2B). However, Northern blot analysis did not detect any accumulation of CS-ACS2 mRNA in the shoot apices of monoecious or andromonoecious cucumber plants (Fig. 2B). Additionally, there was no detectable CS-ACS2 mRNA in the flower bud RNA isolated from any of the three cultivars (Fig. 2B). These results are consistent with sexual expression, which is determined at an early developmental stage of flower buds in the shoot apices of cucumber plants.

To clarify the differences in the accumulation of CS-ACS2 mRNA between the shoot apices of monoecious (M-ff) and andromonoecious (mmff) cucumber plants, we used RT-PCR expression analysis, which has a higher detection sensitivity than Northern blot analysis. RT-PCR expression analysis is useful for measuring mRNA levels by determining the quantitative range of PCR cycle numbers and template amounts (Wang et al. 1989, Nomura et al. 1995). Also, we examined the accumulation of another ACS gene by RT-PCR expression analysis. It has been reported that CS-ACS1 (accession number U59813) expression in cucumber plants is induced by auxin, but not by 1-aminocyclopropane-1-carboxylate (ACC) (Trebitsh et al. 1997). On the other hand, it has been reported that expression of another ACS gene, CS-ACS1 (accession number AB006803), in cucumber plants is induced by wounding and CO₂ stress (Shiomi et al. 1998, Mathooko et al. 1999). To avoid confusion, the latter ACS gene (accession number AB006803) (Shiomi et al. 1998, Mathooko et al. 1999) was designated as CS-ACS3 in the present study, and its mRNA accumulation was analyzed. Signal intensities of the products for CS-ACS2 mRNA increased exponentially between 0.03 μg and 0.5 μg of templates in both gynoecious and monoecious cucumber plants following 20 cycles of PCR amplification (Fig. 2C). CS-ACS2 mRNA levels in gynoecious cucumber plants were almost ten times higher than those observed in monoecious ones (Fig. 2C, a b). Varying the cycle numbers from 16 to 24 cycles and using 0.5 μg of template yielded CS-ACS2 mRNA signals in monoecious cucumber plants that increased exponentially with cycle number (Fig. 2D). We next examined the signal intensities for CS-ACS3 and actin mRNA at various cycle numbers using 0.5 μg of template obtained from monoecious cucumber plants (Fig. 2E, F). The CS-ACS3 mRNA specific signal increased exponentially between 16 and 24 cycles (Fig. 2E), while the actin mRNA specific signal increased exponentially between 8 and 16 cycles (Fig. 2F). These results were compared to RT-PCR expression analyses of CS-ACS2, CS-ACS3, and actin mRNA, which were performed using 20, 20, and 12 cycle numbers, respectively, with 0.5 μg of each template (Fig. 2G). The CS-ACS2 mRNA in gynoecious cucumber plants gave the strongest signal among all cultivars examined (Fig. 2G). The trends obtained in the RT-PCR expression analysis of CS-ACS2 mRNA were similar to those obtained by Northern blot analysis (Fig. 2B, G). The RT-PCR expression analysis revealed that the level of CS-ACS2 mRNA expressed in the shoot apices of two types of andromonoecious cultivars and a monoecious cultivar was similar (Fig. 2G). In contrast, the levels of CS-ACS3 and actin mRNA expressed in the shoot apices did not differ among all cultivars investigated (Fig. 2G). Thus, andromonoecious and monoecious cucumber

Fig. 2 Ethylene production and the accumulation of CS-ACS2 and CS-ACS3 mRNA in monoecious, gynoecious, and andromonoecious cucumber plants. (A) Ethylene production from the shoot apices of monoecious (Otone No. 1), gynoecious (Higan-fushinari), and andromonoecious (Lemon) cucumber plants at the 4-leaf stage. Vertical bar indicates the standard deviation of the mean for triplicate samples. (B) Northern blot analysis of CS-ACS2 mRNA levels in the shoot apices and flower buds of monoecious (Otone No. 1), gynoecious (Higan-fushinari), and andromonoecious (Lemon) cucumber plants. Total RNA was harvested from the shoot apices at the 4-leaf stage and from 1-cm long flower buds. (C), (D), (E), and (F) Titration curves of RT-PCR products. Total RNA from the shoot apices of monoecious (C, D, E, and F) and gynoecious (C) cucumber plants were used as templates. Signal intensities of RT-PCR products are expressed in arbitrary units and plotted against template amounts (C) and cycle numbers (D, E, and F). (G) Quantitative RT-PCR analysis of CS-ACS2 and CS-ACS3 mRNA in the shoot apices of monoecious (Otone No. 1), gynoecious (Higan-fushinari), and andromonoecious (Lemon and 94–64–62–10M) cucumber plants. Total RNA was harvested from the shoot apices of four cucumber plants at the 4-leaf stage.
Ethylene responses and sex expression in cucumber plants, as expected, have similar levels of CS-ACS2 mRNA and ethylene production in the shoot apices.

The effects of ethylene on CS-ETR1, CS-ETR2, CS-ERS, and CS-ACS2 mRNA accumulation in monoecious, gynoecious, and andromonoecious cucumber plants

Ethylene-induced CS-ETR2 and CS-ERS mRNA expression has been previously shown in the shoot apices of monoecious cucumber plants (Yamasaki et al. 2000). To investigate the differences in ethylene responses between monoecious and andromonoecious cucumber plants, we examined the effect of ethylene on mRNA accumulations of ethylene receptor-related genes (CS-ETR1, CS-ETR2, and CS-ERS) in the shoot apices of cucumber plants. Fig. 3A shows the effect of ethephone, an ethylene-releasing agent, on the accumulation of CS-ETR1, CS-ETR2, and CS-ERS mRNA in monoecious and andromonoecious cucumber plants at the 4-leaf stage. Compared to the control, the mRNA levels of the CS-ETR2 and CS-ERS genes were
significantly elevated 6 and 12 h after applying 0.35 mM ethephone to the shoot apices of monoecious cucumber plants (Fig. 3A). At 12 h post-treatment with 0.35 mM ethephone, there was a 3.2- and a 7.2-fold higher level of CS-ETR2 and CS-ERS mRNA, respectively, compared to control shoot apices (Fig. 3A).

The CS-ETR1 mRNA levels increased by 1.6-fold after ethephone application, much less than that observed for CS-ETR2 and CS-ERS mRNA (Fig. 3A). The levels of CS-ETR2 and CS-ERS mRNA decreased to the levels observed for controls 24 h after applying ethephone in monoecious cucumber plants (Fig. 3A). Thus, the levels of CS-ETR2 and CS-ERS mRNA were transiently increased. It could be related to the decrease in ethylene level, although we did not measure it 24 h after ethephone application. In contrast, virtually no ethylene-induced ethylene-receptor-related gene mRNA was detected in the shoot apices of andromonoecious cucumber (Fig. 3A).

We next investigated the relationship between ethylene dose and the accumulation of ethylene-induced mRNAs in monoecious and andromonoecious cucumber plants by RT-PCR expression analysis. First, we quantified cycle numbers of RT-PCR against a fixed amount of template, 0.5 μg, obtained from monoecious cucumber plants (Fig. 3B, C). The signal intensity of CS-ETR2 and CS-ERS mRNA increased exponentially between 16 and 24 cycles (Fig. 3B, C). We examined the effect of ethephone on CS-ETR2, CS-ERS, and CS-ACS2 mRNA levels 12 h after treatment with 0.014, 0.07, 0.35, or 1.75 mM ethephone in monoecious cucumber plants. After 20 cycles of PCR, the signals for CS-ETR2, CS-ERS, and CS-ACS2 mRNA were significantly elevated over the control as the concentration of ethephone increased from 0.014 mM to 1.75 mM (Fig. 3D). The greatest increase in signal was observed for the CS-ERS mRNA after applying 1.75 mM ethephone. In contrast, the ethylene-induced mRNA levels were barely detectable in andromonoecious cucumber treated with ethephone at 0.014, 0.07, and 0.35 mM (Fig. 3D). The application of 1.75 mM ethephone caused slight increases in CS-ERS and CS-ACS2 mRNA levels in andromonoecious cucumbers (Fig. 3D).

We have previously shown that the CS-ETR2 and CS-ERS mRNA levels were reduced by an inhibitor of ethylene biosynthesis, aminoethoxyvinyl glycine (AVG), in the shoot apices of gynoecious cucumber plants (Yamasaki et al. 2000). To investigate the up-regulation of CS-ETR2, CS-ERS, and CS-ACS2 mRNA expression by ethylene in gynoecious cucumber plants, we examined the effect of ethephone on their mRNA levels following the application of 1.0 mM of AVG to their shoot apices. Compared to the control, the mRNA levels of the CS-ETR2 and CS-ERS genes were significantly decreased by AVG application, which agrees with our previously published results (Yamasaki et al. 2000) (Fig. 3E). The mRNA level of the CS-ACS2 gene was also decreased by AVG application from the control level (Fig. 3E). In the AVG-treated plants, the levels of the CS-ETR2, CS-ERS, and CS-ACS2 mRNA were significantly elevated in gynoecious cucumber plants as the concentration of ethephone increased from 0.07 mM to 1.75 mM (Fig. 3E). The increase in accumulation was most noticeable for the CS-ERS gene after applying 1.75 mM ethephone (Fig. 3E).

Thus, the accumulation of CS-ETR2, CS-ERS, and CS-ACS2 mRNA in the shoot apices are up-regulated by ethylene in monoecious and gynoecious, but not in andromonoecious cucumber plants.

**The effect of ethylene gas on the hypocotyl elongation of monoecious, gynoecious, and andromonoecious cucumber seedlings**

To examine whether ethylene influenced other aspects of growth and development in andromonoecious cucumber plants, we compared the effects of ethylene on the hypocotyl elongation of monoecious, gynoecious, and andromonoecious seedlings (Fig. 4). The lengths (mean ± SE) of the control hypocotyls in andromonoecious, monoecious, and gynoecious cucumbers were 12.77±0.83, 10.93±0.42, and 6.27±1.21 cm, respectively. When seedlings were treated with 1.0, 2.0, 4.0, or 6.0 μl liter⁻¹ of ethylene, the inhibitory effect of ethylene on hypocotyl elongation in andromonoecious cucumber was much less than that observed in monoecious (p < 0.01) or gynoecious (p < 0.01) cucumbers (Fig. 4). However, the inhibitory effect of ethylene on hypocotyl elongation in gynoecious cucumbers did not differ from that in monoecious cucumbers when treated with 1.0, 2.0, 4.0, or 6.0 μl liter⁻¹ ethylene (p >
Gene encoding ACC synthase, but the gynoecious CS-ACS1 (Trebitsh et al. 1997). The monoecious cucumber genome has a single copy of this gene (Kamachi et al. 2000). The relationship to the expression of female flowers has been studied in cucumber plants (Kamachi et al. 1997). It was also reported that the accumulation of the CS-ACS2 mRNA in the shoot apices of gynoecious cucumber plants (Kamachi et al. 2000). In the present study, both ethylene production and the accumulation of CS-ACS2 mRNA in the shoot apices of andromonoecious and monoecious cucumber plants were similar. However, the levels observed in both of these plants were lower than those of gynoecious plants (Fig. 2A, B, G). Since the steady-state level of CS-ACS3 mRNA in the shoot apices did not differ among four cultivars investigated (Fig. 2G), these results suggest that the accumulation of CS-ACS2 mRNA caused the higher level of ethylene production from the shoot apices of gynoecious cucumber plants. It seems likely that expression of other ACS genes, such as CS-ACS3, contribute to a basal level of ethylene production in the four cultivars investigated. Thus, both ethylene production and accumulation of CS-ACS2 mRNA are regulated by the M locus in cucumber plants (Fig. 2A, B, G). However, it is noteworthy that the difference in the CS-ACS2 mRNA accumulation was much greater than that of ethylene production between andromonoecious/monoecious and gynoecious cucumbers. It should be noted that ethylene production was measured after incubation of the excised shoot apices for 16 h, although total RNA for Northern blot analysis was isolated from the shoot apices soon after excision. The differences in ethylene levels of the shoot apices before excision could be larger. The correlation between the mRNA levels and ethylene production need to be studied further because no such large difference as that seen in CS-ACS2 mRNA level has been reported for ethylene production among cucumber cultivars.

Discussion

Relationship of the F locus to ethylene production

The molecular regulation of endogenous ethylene and its relationship to the expression of female flowers has been studied in cucumber plants (Trebitsh et al. 1997, Kamachi et al. 1997). The monoecious cucumber genome has a single copy of CS-ACS1 gene encoding ACC synthase, but the gynoecious cucumber genome has an additional copy (CS-ACS1G) that was detected by Southern blot analysis using a CS-ACS1 probe (Trebitsh et al. 1997). The CS-ACS1G gene was closely linked to the F locus (Trebitsh et al. 1997). A CS-ACS2 cDNA encoding ACC synthase was isolated and expressed during the development of female flowers in the shoot apices of gynoecious cucumber plants (Kamachi et al. 1997). The accumulation of CS-ACS2 mRNA in the shoot apices of gynoecious cucumber plants is greater than that in monoecious ones (Fig. 2B, G; Kamachi et al. 1997). It was also reported that the accumulation of CS-ACS1 (CS-ACS1G) mRNA occurred prior to the accumulation of the CS-ACS2 mRNA in the shoot apices of gynoecious cucumber plants (Kamachi et al. 2000). In the present study, both ethylene production and the accumulation of CS-ACS2 mRNA in the shoot apices of andromonoecious and monoecious cucumber plants were similar. However, the levels observed in both of these plants were lower than those of gynoecious plants (Fig. 2A, B, G). Since the steady-state level of CS-ACS3 mRNA in the shoot apices did not differ among four cultivars investigated (Fig. 2G), these results suggest that the accumulation of CS-ACS2 mRNA caused the higher level of ethylene production from the shoot apices of gynoecious cucumber plants. It seems likely that expression of other ACS genes, such as CS-ACS3, contribute to a basal level of ethylene production in the four cultivars investigated. Thus, both ethylene production and accumulation of CS-ACS2 mRNA are regulated by the M locus in cucumber plants (Fig. 2A, B, G). However, it is noteworthy that the difference in the CS-ACS2 mRNA accumulation was much greater than that of ethylene production between andromonoecious/monoecious and gynoecious cucumbers. It should be noted that ethylene production was measured after incubation of the excised shoot apices for 16 h, although total RNA for Northern blot analysis was isolated from the shoot apices soon after excision. The differences in ethylene levels of the shoot apices before excision could be larger. The correlation between the mRNA levels and ethylene production need to be studied further because no such large difference as that seen in CS-ACS2 mRNA level has been reported for ethylene production among cucumber cultivars.

Relationship of the M locus to the ethylene response

Application of ethylene induced the formation of bisexual flowers but not of female flowers in andromonoecious cucumber plants, although it induced the formation of female flowers in monoecious cucumber plants (Table 1). Additionally, the ethylene treatment did not inhibit stamen development in andromonoecious cucumber plants, although it inhibited stamen development in gynoecious cucumber plants (Fig. 1). These results suggest that the primodia of stamens are insensitive to ethylene in gynoecious cucumber plants. On the other hand, primodia of pistils are sensitive to ethylene in andromonoecious cucumber plants in spite of the mm genotype (Fig. 1). In addition, the steady state levels of CS-ETR2, CS-ERS, and CS-ACS2 mRNA are up-regulated by ethylene in the shoot apices of both monoecious and gynoecious cucumber plants, both of which have the M- genotype, but not in the shoot apices of andromonoecious cucumber plants, which have the mm genotype. Furthermore, there was less inhibition of hypocotyl elongation by ethylene in andromonoecious cucumber plants than in monoecious and gynoecious plants (Fig. 4). These results indicate that the response of andromonoecious cucumber plants to ethylene is reduced from that of monoecious and gynoecious plants, suggesting that the product(s) of the M locus mediates ethylene signals. Accordingly, these results suggest that the product(s) of the M locus regulate not
only the development of stamens but also the accumulations of CS-ETR2, CS-ERS, and CS-ACS2 mRNA and hypocotyl elongation by the mediation of ethylene signals.

Ethylene-insensitive mutants in both Arabidopsis and tomato have a pleiotropic loss of ethylene responses; these include the triple response, leaf growth, and leaf senescence (Chang et al. 1993, Hua et al. 1995, Hua et al. 1998, Sakai et al. 1998). Similarly, the product of the M locus is believed to be involved in pleiotropic ethylene responses such as inhibition of hypocotyl elongation and stamen development in cucumber plants. In addition, the development of pistils is also regulated by ethylene in these plants (Table 1, Fig. 1). If the product of the M locus does not mediate the ethylene response in inducing pistil development, it may specifically mediate the ethylene-induced inhibition of stamen development in sex determination, although it could mediate other ethylene responses such as hypocotyl elongation. Alternatively, it is possible that the superiority or inferiority of the M locus does not affect the ethylene response in inducing stamen development, even when the product of the M locus mediates both responses of stamen and pistil to ethylene in cucumber plants.

Relationship between F and M loci with regard to sex expression

Our results suggest that the product of the M locus acts downstream from the product of the F locus, because the F locus regulates ethylene production while the M locus mediates ethylene signals. In addition, the superiority or inferiority of the M locus governs inhibition of stamen development, but does not affect the induction of pistil development by ethylene. Based on our results, we propose a model for sexual expression regulated by F and M loci in cucumber plants (Fig. 5A). We focused on the development of stamens and pistils rather than on the expression of male, female, and bisexual flowers, which was previously used to deduce the function of F and M loci (Kubicki 1969a, Pierce and Wehner 1990). By comparing the genotypes of gynoecious and monocious plants, we found that loss-of-function of the F locus leads to some male flowers, suggesting that the induction of stamen development and the inhibition of pistil development occur in some floral primordia. Thus, one function of the F locus might be to inhibit the development of stamens and to induce the development of pistils in some floral primordia. Comparison of the genotypes of gynoecious and hermaphroditic plants suggests that loss-of-function of the M locus leads to the disappearance of female flowers and the appearance of bisexual flowers, indicating that constitutive induction of stamen development occurs in all floral primordia. Thus, it is possible that one function of the M locus is to inhibit the development of stamens in all floral primordia. Accordingly, in hermaphroditic cucumber plants, the inhibition of stamen development might be caused by the F locus, but the constitutive induction of stamen development would also be caused by loss-of-function of the M locus. This hypothesis suggests that the function of the F locus and the loss-of-function of the M locus have opposite effects on the development of stamens. Hermaphroditic cucumber plants produce only bisexual flowers with continued development of stamens and pistils, suggesting that the M locus is epistatic to the F locus. Based on this hypothesis of the F and M loci function and their epistasis, the sexual expression of the mnnf genotype is suggested to be as follows. Loss of function of the M and F loci leads to the induction of stamen development in all floral primordia, and to the induction of stamen development and the inhibition of pistil development in some floral primordia, respectively. Thus, plants with the mnnf genotype produce the bisexual and male flowers characteristic of the andromonoecious cucumber.

![Fig. 5](image-url)

**Fig. 5** (A) A genetic model involving the F and M loci in the sexual expression of cucumbers. The function of the F locus is to inhibit the development of stamens and to induce the development of pistils in some floral primordia. A loss-of-function of the F locus does not affect all floral primordia. The function of the M locus is to inhibit the development of stamens in all floral primordia. A loss-of-function of the M locus affects all floral primordia. (B) Each sexual phenotype of cucumber plants explained by our genetic model. a. Gynoeccious (M-F-) cucumber plants produce only female flowers. b. Monoeccious (M-ff) cucumber plants produce male and female flowers. c. Hermaphroditic (mnnF-) cucumber plants produce only bisexual flowers. d. Andromonoecious (mnnff) cucumber plants produce only male flowers. If the genotype is F-, all floral primordia produce enough ethylene to induce femalelessness. Otherwise, if the genotype is ff, not all floral primordia produce enough ethylene to induce femaleness. The product of the M locus mediates the inhibition of stamen development by ethylene. If the genotype is mnn, then the ethylene signal is not transmitted, and stamen development is not inhibited.
The genetic study described above is consistent with the M locus being epistatic to the F locus; the M locus inhibits stamen development without affecting pistil development. These conclusions are fully consistent with our model (Fig. 5A).

Regulation of sexual expression by the F locus, M locus, and ethylene

By considering the relationship of the F locus to ethylene production, the relationship of the M locus to ethylene response, and the genetic model constructed in the present study, the sexual phenotypes of gynoecious, monoecious, hermaphroditic, and andromonoecious cucumber plants can be explained (Fig. 5B). In gynoecious cucumber plants, all floral primordia may produce enough ethylene to induce femaleness due to F-, and stamen development may be inhibited by ethylene due to M-. Thus, the induction of pistil development and the inhibition of stamen development occur in all floral primordia, leading to the formation of female flowers (Fig. 5B a). In monoecious cucumber plants, not all floral primordia produce enough ethylene to induce femaleness because of ff, and stamen development may be inhibited by ethylene because of M-. Thus, in some floral primordia that produce enough ethylene to induce femaleness, induction of the pistil development and inhibition of the stamen development lead to the formation of female flowers. In the others that do not produce enough ethylene to induce femaleness, neither induction of pistil development nor inhibition of the stamen development occurs, and male flowers appear. Consequently, monoecious cucumber plants produce both male and female flowers (Fig. 5B b). In hermaphroditic cucumber plants, stamen development may have a reduced response to ethylene because of mm, which would lead to the constitutive development of stamens in all floral primordia. These plants may also produce enough ethylene to induce the development of pistils because of F-. Hermaphroditic cucumber plants therefore produce only bisexual flowers (Fig. 5B c). In andromonoecious cucumber plants, stamen development in all floral primordia has a reduced response to ethylene because of mm, which leads to constitutive stamen development. Not all floral primordia produce enough ethylene to induce the development of pistils because of ff. In some floral primordia that produce enough ethylene, induction of pistil development and constitutive stamen development leads to the formation of bisexual flowers. In the others that do not produce enough ethylene, inhibition of pistil development and constitutive stamen development lead to the formation of male flowers. Consequently, andromonoecious cucumber plants produce both bisexual and male flowers (Fig. 5B d). Thus, our genetic model consistently explains the sex phenotypes of gynoecious, monoecious, hermaphroditic, and andromonoecious cucumber plants.

The model proposed in this study is the first to show that the M locus is epistatic to the F locus, and that the product of the M locus mediates the inhibition of stamen development by ethylene. Yin and Quinn (1995) proposed a mechanistic and testable model of one hormone regulating both male and female sex in cucumber. They assumed that there exist male and female cell receptors for a hormone that independently inhibits one sex and induces the other. In order to understand sex expression in cucumber, they suggested that both the range of hormone concentrations and the sensitivity of male and female cell receptors to the hormone should be taken into consideration. In cucumber, this sex hormone is likely to be ethylene (Yin and Quinn 1995). The results of the present study strongly support this hypothesis.

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