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Identification of a 1-Aminocyclopropane-1-Carboxylic Acid Synthase Gene Linked to the *Female* (*F*) Locus That Enhances Female Sex Expression in Cucumber[1]

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Sex determination in cucumber (*Cucumis sativus* L.) is controlled largely by three genes: *F*, *m*, and *a*. The *F* and *m* loci interact to produce monoecious (*M_f_*) or gynoecious (*M_F_*) sex phenotypes. Ethylene and factors that induce ethylene biosynthesis, such as 1-aminocyclopropane-1-carboxylate (ACC) and auxin, also enhance female sex expression. A genomic sequence (*CS-ACS1*) encoding ACC synthase was amplified from genomic DNA by a polymerase chain reaction using degenerate oligonucleotide primers. Expression of *CS-ACS1* is induced by auxin, but not by ACC, in wounded and intact shoot apices. Southern blot hybridization analysis of near-isogenic gynoecious (*MMFF*) and monoecious (*MMff*) lines derived from diverse genetic backgrounds revealed the existence of an additional ACC synthase (*CS-ACS1G*) genomic sequence in the gynoecious lines. Sex phenotype analysis of a segregating F_2 population detected a 100% correlation between the *CS-ACS1G* marker and the presence of the *F* locus. The *CS-ACS1G* gene is located in linkage group B coincident with the *F* locus, and in the population tested there was no recombination between the *CS-ACS1G* gene and the *F* locus. Collectively, these data suggest that *CS-ACS1G* is closely linked to the *F* locus and may play a pivotal role in the determination of sex in cucumber flowers.

Sex determination in flowering plants is a developmentally regulated process that has been the topic of much research (Dellaporta and Calderon-Urrea, 1993; Grant et al., 1994). Dioecious and monoecious species of flowering plants present an excellent opportunity to study the diverse, developmental pathways that give rise to unisexual flowers. In monoecious wild-type cucumber (*Cucumis sativus* L. var *sativus*), flowers are produced in a preset, developmental sequence along the main stem, with a first phase of staminate flowers, followed by a mixed phase of staminate and pistillate flowers, and terminated by a pistillate flower phase (Galun, 1961; Shifriss, 1961). The development state and length of a phase producing only a single flower type, as opposed to producing both staminate and pistillate flowers, is genetically determined.

The current genetic model proposes that sex expression in cucumber is determined largely by three major genes. Female sex expression is regulated by a single dominant gene, *Female* (*F*), which is modified by *In-F* (*Intensifier of female sex expression*) and *gy* (*gynoecious*) to enhance gynoecious sex expression (Kubicki, 1969; Pierce and Wehner, 1990). The *F* locus interacts with two other recessive loci, *m* (*andromonoecious*; staminate and hermaphrodite flowers) and *a* (*androecious*; all staminate flowers), the latter of which determines the degree of maleness expressed in plants (Pierce and Wehner, 1990). Interactions between sex-determining loci give rise to a wide variety of sex phenotypes that range from gynoecious plants (only female) and androecious plants (only male) to plants bearing only hermaphrodite (bisexual) flowers (Pierce and Wehner, 1990).

Phenotypic expression of these sex-determining genes is strongly modified by the environment and hormones. Long days, high temperature, and GA_3 promote the formation of staminate flowers, whereas short days, low temperature, auxin, and ethylene promote the formation of pistillate flowers (Galun, 1961; Rudich, 1985; Malepszy and Niermirowicz-Szczytt, 1991). A high correlation exists between ethylene evolution and female sex expression (George, 1971; Rudich et al., 1972; Takahashi and Suge, 1982; Trebitsh et al., 1987), and inhibitors of ethylene biosynthesis or action suppress pistillate flower development (Atsmon and Tabbak, 1979; Takahashi and Jaffe, 1984). Auxins have also been found to enhance female sex expression (Galun, 1959), possibly through the induction of ethylene biosynthesis (Takahashi and Jaffe, 1984; Trebitsh et al., 1987). Thus, sex expression in cucumber is determined by a combination of genetic, environmental, and hormonal factors (Galun, 1961; Kubicki, 1969; Rudich, 1985; Malepszy and Niermirowicz-Szczytt, 1991). The great diversity of breeding sex types as well as the extensive literature on the genetic and physiological factors involved in sex determi-

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Abbreviations: CS-ACS1, *Cucumis sativus* ACC synthase; CS-ACS1G, gynoecious *Cucumis sativus* ACC synthase; LOD, log of the odds.

nation in this species makes it ideal for studying the molecular basis of sex expression (Galun, 1961; Rudich, 1985; Malepszy and Niermirowicz-Szczytt, 1991).

In recent years significant breakthroughs have been made in our understanding of the biochemistry and the molecular regulation of ethylene production (Adams and Yang, 1979; Sato and Theologis, 1989; Kende, 1993). Genes encoding ACC synthase (EC 4.4.1.14), the key regulatory enzyme in the ethylene biosynthetic pathway, have been cloned from many species (Huang et al., 1991; Nakagawa et al., 1991; Olson et al., 1991; Liang et al., 1992; Botella et al., 1993; Kende, 1993; O'Neill et al., 1993; Destefano-Beltran et al., 1995). ACC synthase is encoded by a multigene family, and the expression of its members is differentially regulated by a wide variety of factors (Huang et al., 1991; Olson et al., 1991; Rottmann et al., 1991; Liang et al., 1992; Zarembinski and Theologis, 1993; Destefano-Beltran et al., 1995). Expression of ACC synthase genes may be developmentally regulated, tissue-specific, auxin-induced, or induced by a variety of environmental and internal signals (Nakagawa et al., 1991; Olson et al., 1991; Botella et al., 1992; O'Neill et al., 1993; Zarembinski and Theologis, 1993; Abel et al., 1995; Olson et al., 1995).

Although there is considerable knowledge about female sex determination in cucumber, the precise relationship between ethylene and auxin as well as their involvement in the regulation of female sex determination at the molecular level remains unexplored. Due to the key role of ACC synthase in the ethylene biosynthetic pathway (Yang, 1980; Kende, 1993), we were interested in understanding its regulation at the molecular level in relation to sex determination in cucumber. In this paper we report the identification of an ACC synthase genomic sequence in cucumber (*CS-ACS1*) that is specifically and transiently induced by auxin. Genetic analysis revealed the presence of an additional copy of ACC synthase that is present in gynoecious cucumber lines only (*CS-ACS1G*) and which co-segregates with the *F* locus.

MATERIALS AND METHODS

Plant Material and Treatments

Monoecious (*Cucumis sativus* L. var *sativus* cv Marketmore 76; *MMff*) and gynoecious (*C. sativus* L. var *sativus* cv Marketmore 76F; *MMFF*) near-isogenic cucumber lines (BC_9; backcross population for over nine generations) were used to examine the regulation of sex and gene expression. These lines were received from K. Owens (Seminis Vegetable Seeds, Woodland, CA) and were maintained by selfing (Munger, 1989). Seed of additional near-isogenic lines (Wisconsin SMR 18 versus Wisconsin 1983G; Rodgers NK 804 versus Rodgers NK 2002; and Beit-Alfa monoecious versus Beit-Alfa gynoecious) were obtained from H. Nerson (Volcani Center, Newe Ya'ar, Israel). Seeds were germinated and grown in trays containing a modified University of California standard soil mixture (sphagnum peat: sand:pumice:redwood compost, 1:1:1:1, v/v). Plants were grown in a greenhouse supplemented by metal halide lamps (100–300 mmol m^{-2} s^{-1}) under a 16-h photoperiod.

Studies on gene regulation, sex expression, and ethylene evolution were conducted. For regulation of gene expression, in vitro shoot apices were harvested when the first leaf was fully expanded. Excised apices (approximately 0.3 g) were incubated in a 30-mL flask containing 5 mL of 50 mM phosphate-citrate, pH 4.8, and 50 mM Suc with or without 100 mM IAA or 100 mM ACC. Flasks were sealed with rubber serum caps and shaken in the dark at 25°C for 2 or 5 h. After taking a 1-mL air sample for ethylene determination, the tissue was frozen in liquid nitrogen and stored at −70°C for RNA analysis. For sex expression experiments, seedlings were grown in trays and sprayed with 50 mM phosphate-citrate, pH 6.0, and 0.1% (v/v) Tween 80 with or without 5 mM IAA, 1 mM naphthalene acetic acid, or 5 mM ACC. Plants were initially sprayed when the first leaf was fully expanded, and then given two consecutive treatments at 6-d intervals. Five hours after the last treatment, six randomly chosen seedlings were selected from each treatment and transferred into 3-L pots containing the modified University of California standard soil mixture for evaluation of sex expression. For determination of ethylene evolution, shoot apices and the accompanying young leaf were excised from seedlings that were chosen at random, weighed, and incubated in a 22-mL vial with 0.5 mL of 50 mM phosphate-citrate, pH 4.8, and 50 mM Suc. Each vial contained five shoot apices or leaves. Shoot apices and the accompanying young leaf were excised from the remaining seedlings, immediately frozen in liquid nitrogen, and stored at −80°C for RNA analysis.

Determination of Ethylene

Vials containing shoot apices or young leaves were sealed with rubber serum caps and shaken slowly in the dark at 25°C. A 1-mL air sample was withdrawn from the vials with a hypodermic syringe. Ethylene production was assayed by GC as previously described (O'Neill et al., 1993).

DNA and RNA Blot Hybridization Analysis

Total RNA was isolated by a phenol-SDS method as previously described (Ausubel et al., 1993) and stored at −70°C. RNA (30 mg/lane) was fractionated by electrophoresis in formaldehyde agarose gels and blotted to Nytran Plus membranes (Schleicher & Schuell) as previously described (O'Neill et al., 1993). Genomic DNA was extracted from young leaves as described by Chetelat et al. (1995). Genomic DNA (5–10 mg) was digested with one of five restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII *Msp*I, and *Xba*I) (Promega) and separated by electrophoresis on a 0.8% agarose gel for 16 h prior to transfer onto Nytran Plus membranes following standard procedures (Sambrook et al., 1989).

Both DNA and RNA gel blots were hybridized with ^{32}P-labeled *CS-ACS1* inserts and hybridized as described previously (O'Neill et al., 1993). Equal reactivity and equal amounts of RNA in all samples were verified by hybridization with a ^{32}P-labeled actin (O'Neill et al., 1994). The inserts used as the probes were isolated from the plasmid

by electrophoresis in a low-melting-temperature agarose (Carter and Milton, 1993) and labeled with [^{32}P]dCTP by random priming (Random Primed DNA Labeling Kit, Boehringer Mannheim). Autoradiography was performed at −70°C using XAR-5 film (Kodak) and an intensifying screen (Cronex Lightning Plus, DuPont). DNA gel blots were exposed for 2 to 5 d, and RNA gel blots were exposed for 5 to 12 d.

PCR

The degenerate oligonucleotides ACS/1F and ACS/6R (courtesy of A.D. Campbell and S.F. Yang, University of California, Davis) correspond to the highly conserved amino acid sequences GVIQMG (region 1) and KMSSFG (region 6) of various ACC synthase polypeptides (Dong et al., 1991; Rottmann et al., 1991; Kende, 1993). Degenerate PCRs, using 500 ng of each primer and 50 ng of cucumber genomic DNA, were carried out using thermocycler and *Taq* DNA polymerase (Perkin-Elmer Cetus) under conditions recommended by the supplier. The reaction mixture was incubated in a thermocycler for 1 cycle: 2 min at 94°C, 2 min at 51°C, and 1.5 min at 72°C; followed by 30 cycles: 1 min at 94°C, 2 min at 56°C, and 1.5 min at 72°C; and finally, a 10-min extension at 72°C. The PCR product was purified twice by gel electrophoresis, subcloned using the TA cloning system (Invitrogen, San Diego, CA), sequenced, and designated as CS-ASC1.

Sequence Analysis

Sequencing was performed with universal and sequence-specific primers using the dideoxy-nucleotide chain reaction termination method (Sanger et al., 1977) and Sequenase version 2 as suggested by the manufacturer (United States Biochemical/Amersham). Nucleotide sequences were analyzed using the Sequence Analysis Software Package (Genetics Computer Group, Madison, WI) (Devereux et al., 1984).

Segregating Populations

Two populations of cucumber that segregated for the alleles at the *F* locus (i.e. gynoecy [*M_F_*] versus monoecy [*M_ff*]) were used for the genetic analysis of *CS-ACS1*. The first population consisted of a wide cross generated by Kennard et al. (1994) between the breeding line GY14 (*C. sativus* L. var *sativus*) as the maternal parent and PI 183967 (*C. sativus* var *hardwickii* R. Alef.) as the paternal parent.

Leaf tissue from 10 individuals from each of the 73 F_3 lines was bulked to determine the F_2 genotypes for genetic linkage analysis. Linkage and orders of markers were estimated with Mapmaker version 2.0 for a Macintosh computer (Lander et al., 1987). Scoring errors were detected by checking for double crossovers. A LOD of 3.0 and a recombination frequency of 0.3 were chosen as thresholds for the identification of linkages. The LOD corresponds to the 95% confidence level against false detection of spurious linkages (Lander and Botstein, 1986).

Marketmore 76F (*MMFF*) was crossed with pollen from its near-isogenic counterpart Marketmore 76 (*MMff*) for segregation and correlation analysis of the *CS-ACS1* and the presence of the *F* locus. Leaf tissue was collected from 100 F_2 plants for DNA analysis, and these plants were evaluated for gynoecy (i.e. the number and pattern of pistillate nodes present on the main stem).

Sex Expression Measurements

The effect of ACC and auxin on sex expression was evaluated by determining the number of nodes bearing pistillate and staminate flowers in the first 15 nodes on the main stem. In the segregating population that was generated from the Marketmore 76 near-isogenic lines, gynoecy of an individual F_2 plant was determined by recording the sex type along the first 30 main-stem nodes. Genotypes were assigned according to designations previously described by Shifriss (1961): *FF* = gynoecious (no staminate nodes), *ff* = monoecious (predominantly staminate nodes for at least 30 main-stem nodes), and *Ff* = subgynoecious (intermediate grades between the monoecious and gynoecious parents with no staminate flower above the first 15 main-stem nodes). For the mapping population GY14 (*C. sativus* L. var *sativus*) × *C. sativus* var *hardwickii* cucumber line PI 183967, the percentage of the first 10 flower-bearing nodes along the main stem were rated for the F_2 individuals in the greenhouse as previously described (Kennard et al., 1994). Genotypes were assigned on the basis of percentage of female nodes, where *F_* ≥ 50% and *ff* ≤ 50% pistillate flowers (Kennard et al., 1994).

RESULTS AND DISCUSSION

Experimental System

To elucidate the developmental processes involved in female sex determination in cucumber, we performed a comparative study using a pair of near-isogenic monoecious and gynoecious cucumber lines (cv Marketmore 76 and 76F, respectively) under controlled environmental conditions. Two criteria were used to accurately assess the sex phenotype: the number of leaves produced until the appearance of the first pistillate flower (Fig. 1, T1) and the number of leaves produced until the female phase (Fig. 1, T2) (Shifriss, 1961). Gynoecious cv Marketmore 76F produced only pistillate flowers, whereas plants of the monoecious cv Marketmore 76 exhibited a short staminate phase followed by a mixed phase of staminate and pistillate flowers, which did not revert to a pistillate phase (Fig. 1, T2). The F_1 progeny resulting from these two lines exhibit heterozygosity at the *F* locus and a phenotype of accelerated transition from the staminate to the pistillate phase (Fig. 1, T2). An array of intermediate flowering habits resulting from crosses between gynoecious and strong monoecious lines has been previously defined as subgynoecious (Shifriss, 1961).

Isolation of an ACC Synthase Genomic Sequence

An ACC synthase gene (1025 bp) was amplified by PCR using degenerate oligonucleotides as primers and genomic DNA isolated from cv Marketmore 76F. The coding se-

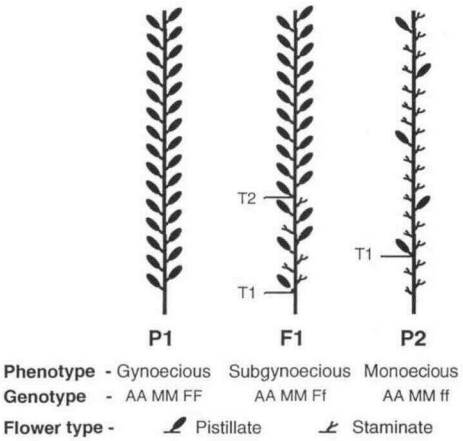

Figure 1. Pattern of sex expression along the main stem of *C. sativus* L. var *sativus* cv Marketmore. T1 and T2 represent points for estimation of sex tendencies. T1, Number of leaves until the first pistillate flower; T2, number of leaves until a solely pistillate phase is reached; P1, maternal parent (*C. sativus* L. var *sativus* cv Marketmore 76F); P2, paternal parent (*C. sativus* L. var *sativus* cv Marketmore 76); F1, the progeny resulting from a cross between P1 and P2.

quence of this PCR fragment is interrupted by two short introns of 111 and 118 bp. By comparing the structure of the gene with that of *LE-ACS2* (Rottmann et al., 1991), it is evident that the first and second introns interrupt the coding sequence at the same positions as the first and third introns in the tomato gene. The intron-exon junctions have been established by reference to splice sites of known ACC synthase cDNAs (Nakagawa et al., 1991; Rottmann et al., 1991; Zarembinski and Theologis, 1993). A similar structure has been reported for the potato *ST-ACS2* gene (Destefano-Beltran et al., 1995) and the mung bean *VR-ACS4* and *VR-ACS5* genes (Botella et al., 1993). The polypeptide encoded by *CS-ACS1* is 264 amino acids long and contains the active site and 10 of the 11 amino acids conserved in all published ACC synthases (data not presented) (Nakajima et al., 1990; Rottmann et al., 1991; Kende, 1993). The *CS-ACS1* gene bears a high sequence homology (82% identity) to the winter squash *CM-ACS2* gene, another member of the Cucurbitaceae (Nakagawa et al., 1995). The PILEUP program (Devereux et al., 1984) was used to align protein sequences for their degree of similarity (Fig. 2). The results indicate that the CS-ACS1 is most closely related to the winter squash CM-ACS2 polypeptide (93% identity; Nakagawa et al., 1991), and that they belong to a group of ACC synthases that are induced by auxin in vegetative tissues (Fig. 2) (Kim et al., 1992; Zarembinski and Theologis, 1993; Abel et al., 1995; Destefano-Beltran et al., 1995; Olson et al., 1995).

Southern blot hybridization analysis of genomic DNA that was isolated from the monoecious cucumber (cv Marketmore 76) indicates the presence of a single-copy gene with the possibility of several additional copies with low homology to *CS-ACS1*, as seen in the *Eco*RI digest (Fig. 3). In contrast, the gynoecious near-isogenic counterpart has an additional copy of *CS-ACS1*, as shown by the additional restriction fragment in the *Msp*I digest (Fig. 3). Digestion of

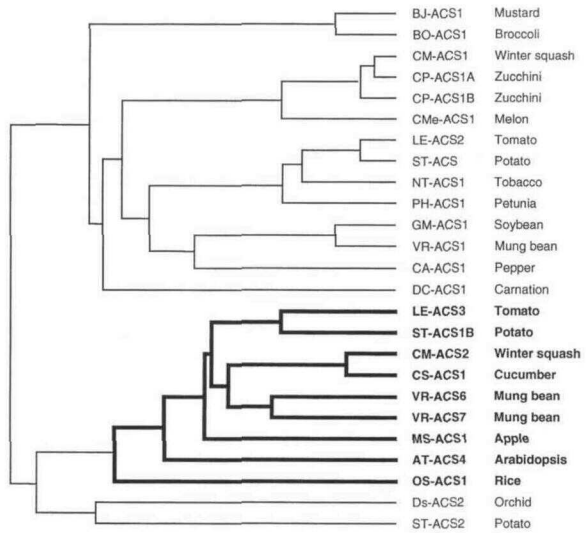

Figure 2. Similarity comparison of ACC synthases most closely related to CS-ACS1. A dendogram of a multiple alignment of the ACC synthase polypeptides was generated using the program PILEUP (Devereux et al., 1984). GenBank accession numbers are given in parentheses: AT-ACS4, Arabidopsis (U23482); BJ-ACS1, mustard (X72676); BO-ACS1, broccoli (X82273); CA-ACS1, pepper (X82265); CM-ACS1 and CM-ACS2, winter squash (D01032 and D01033, respectively); CMe-ACS1, melon (D30805); CP-ACS1A and CP-ACS1B, zucchini (M61195); CS-ACS1, cucumber (this work); DC-ACS1, carnation (Z18952); Ds-ACS2, orchid (L07883); GM-ACS1, soybean (X67100); LE-ACS2 and LE-ACS3, tomato (X59145 and L34171, respectively); MS-ACS1, apple (U03294); NT-ACS1, tobacco (X65982); OS-ACS1, rice (M96673); PH-ACS1, petunia (Z18953); ST-ACS, ST-ACS1B, and ST-ACS2, potato (L20634, Z27234, and Z27235, respectively); and VR-ACS1, VR-ACS6, and VR-ACS7, mung bean (M80554, U34986, and U34986, respectively). The polypeptides shown in boldface are auxin-inducible in plant tissues.

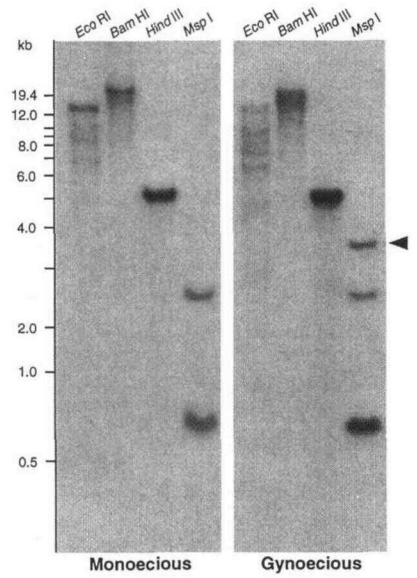

Figure 3. Southern blot hybridization analysis of genomic DNA isolated from monoecious and gynoecious cucumber (*C. sativus* L. *sativus* cv Marketmore 76). Each lane contains 10 mg of DNA. The blot was probed with CS-ACS1. The arrowhead indicates the female-specific ACC synthase genomic sequence *CS-ACS1G*.

the genomic DNA with *Bam*HI and *Hin*dIII yields a single band in both genotypes, with a stronger signal in the gynoecious DNA. This is consistent with the proposal that the gynoecious line has more than one gene copy. *CS-ACS1* has a *Msp*I restriction site (two fragments of 584 and 441 bp), and analysis of its restriction digestion pattern lends support to the hypothesis that *CS-ACS1* is a set of twin genes in the gynoecious line. The 0.8-kb fragment has a stronger hybridization signal intensity than the other two fragments, suggesting that there may be more than one copy of this fragment. Signal intensity was determined using the BioImage system (Millipore) (data not presented). Thus, it is possible that 0.8- and 2.4-kb fragments represent the monoecious *CS-ACS1*, whereas second 0.8- and 3.4-kb fragments represent the gynoecious *CS-ACS1* (Fig. 3). Southern blot analysis suggests that in the gynoecious line, the two *CS-ACS1* genes may be two highly homologous genes that have resulted from a gene duplication event, such as in ACC synthase genes of potato (Destefano-Beltran et al., 1995) and zucchini (Huang et al., 1991). Hereafter, we refer to the gynoecious-specific ACC synthase genomic sequence as *CS-ACS1G*.

These results suggest that the role of the additional ACC synthase gene in female sex determination of cucumber might be viewed as a dose-response effect similar to that observed for the *F* locus (Shifriss et al., 1964). A female-inducing hormone such as auxin may induce ACC synthase activity and, in doing so, may promote the level of ethylene beyond a critical threshold level, thereby inducing female flower development. Alternatively, expression of *CS-ACS1G* may be developmentally regulated by signals other than auxin, resulting in enhanced female sex expression.

Regulation of Sex and Gene Expression

Ethylene and auxin are known to induce female sex expression in cucumber (Takahashi and Suge, 1982; Rudich, 1985). The relative abundance of *CS-ACS1* mRNA was examined in excised apices that were treated with auxin or the ethylene precursor ACC. Although ACC induced ethylene production in the excised apices (Table I), the *CS-ACS1* mRNA transcript level was barely detectable even at the end of a 5-h incubation period (Fig. 4). In contrast, auxin induced the production of ethylene as well as higher levels of *CS-ACS1* mRNA within 2 h (Fig. 4). The auxin-induced mRNA transcript level increased at 2 h, but the transcript level decreased and was no greater than that of the control at 5 h. A similar expression pattern of *CS-ACS1* was observed in both genotypes (Fig. 4).

Monoecious plants were treated with either auxin or ACC to induce sex reversal, and the levels of *CS-ACS1* mRNA in relation to sex expression were examined. Both auxin and ACC enhanced the production of pistillate flowers and reduced the number of nodes bearing staminate flowers (Table I). The high level of auxin- and ACC-induced ethylene resulted in delayed flowering, as manifested by the number of barren nodes or nodes bearing aborted flowers (Table I). These results are consistent with those of previous studies indicating that the early stage of flower development is sensitive to ethylene (George, 1971). This sensitivity may initiate senescence processes that result in floral bud abortion. Ethylene mimics the effect of the *F* gene as reported by Shifriss et al. (1964), who found that the percentage of leaf axils with impaired floral bud formation increased appreciably as the dosage of *F* increased from zero to two, i.e. from monoecious to gynoecious phenotypes (Shifriss et al., 1964). Despite the fact that in monoecious plants ACC induced a stronger feminizing effect as well as higher ethylene production than did auxin (Table I), no *CS-ACS1* message was detected (Fig. 5). Only auxin-induced *CS-ACS1* transcript was detected in apices and leaves in planta (Fig. 5). This result agrees with those obtained with shoot apices treated in vitro (Fig. 4). Since no *CS-ACS1* message was detected in the control (Fig. 5), it can be assumed that the low message level apparent in excised cucumber apices after 2 h (Fig. 4) is due to some cross-reaction of the *CS-ACS1* probe with a different ACC synthase transcript, possibly a wound-induced one. Since the *CS-ACS1* probe used was not gene-specific, differentiation between the transcripts of the *CS-ACS1* and *CS-ACS1G* genes could not be assessed (Fig. 3).

Genetic Analysis and Gene Mapping

Southern-blot-hybridization analysis showed the presence of a second copy of *CS-ACS1* in gynoecious cv Marketmore 76F (Fig. 3). To exclude the possibility that this was a result unique to this cultivar, we compared three additional pairs of near-isogenic breeding lines of diverse, genetic backgrounds: Wisconsin SMR 18 versus Wisconsin 1983G, Rodgers NK 804 versus Rodgers NK 2002, and Beit-Alfa monoecious versus Beit-Alfa gynoecious. Southern blot hybridization analysis confirmed the presence of

Table I. *Effect of various treatments on sex expression and ethylene evolution of monoecious cucumber plants*

Observations were made up to the 15th node of the main stem. All values represent means ± SE; $n = 6$.

Treatment	Number of Nodes Per Plant			Ethylene Production	
	Pistillate	Staminate	Aborted	Apices	Leaves
				$nl\ h^{-1}g^{-1}$	
Control	3.2 ± 0.1	10.7 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.7 ± 0.0
IAA, 5 mM	5.2 ± 0.3	7.7 ± 0.2	2.3 ± 0.2	7.8 ± 2.0	9.0 ± 2.1
NAA,[a] 1 mM	4.8 ± 0.2	7.7 ± 0.3	2.5 ± 0.1	10.1 ± 0.5	17.7 ± 5.8
ACC, 5 mM	7.4 ± 0.4	2.0 ± 0.3	5.6 ± 0.2	243.6 ± 30.7	305.0 ± 11.5

[a] NAA, Naphthalene acetic acid.

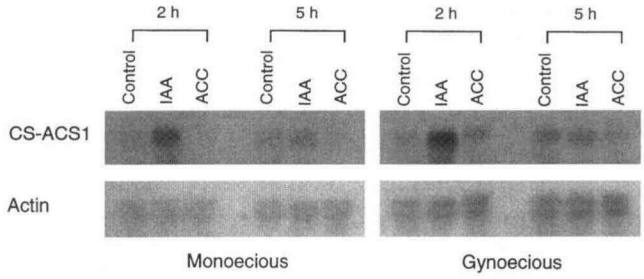

Figure 4. Effect of female-inducing treatments on the level of CS-ACS1 mRNA in apices treated in vitro. Excised cucumber apices were incubated with IAA (0.1 mM) or ACC (0.1 mM). RNA was isolated at the times indicated. Each lane contains 30 mg of total RNA. Blots were probed with CS-ACS1 and actin.

an additional ACC synthase gene (*CS-ACS1G*) of an identical size in all of the gynoecious cultivars that we examined (Fig. 6). These observations are consistent with the fact that the source of femaleness in all U.S. cucumber cultivars, and possibly all other cultivars, is derived from the Japanese cultivar Shogoin female PI 220860 (Galun, 1961; Shifriss, 1961; Pierce and Wehner, 1990).

Since near-isogenic lines were used, it is possible that *CS-ACS1G* corresponds to the *F* locus itself. To examine this hypothesis, two segregating populations were used. One population consisted of F_3 families that were used to genotype F_2 individuals for map construction using the gynoecious GY14 (*C. sativus* L. var *sativus*) and the monoecious *C. sativus* var *hardwickii* cucumber line PI 183967 (Kennard et al., 1994). The other population consisted of F_2 progeny that were generated from a mating between two near-isogenic lines of Marketmore 76 with subsequent selfing. F_2 plants were characterized phenotypically for the degree of gynoecy (*F*) and the occurrence of *CS-ACS1G*.

The population generated from the Marketmore 76 near-isogenic lines segregated into three classes, with gynoecious, subgynoecious, and monoecious individuals fitting a 1:2:1 ratio (Table II). These results indicate that gynoecy is controlled by a single dominant gene, as previously reported (Galun, 1961; Kubicki, 1969). Southern blot hybridization analysis of the same population showed that *CS-ACS1G* segregated in a 3:1 ratio. Moreover, a 100%

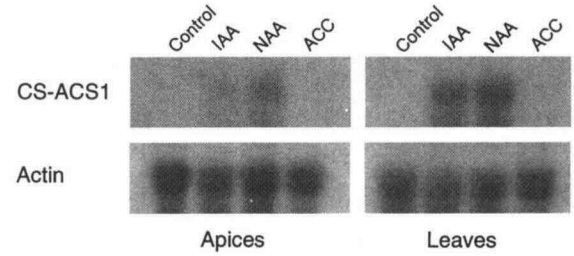

Figure 5. Abundance of CS-ACS1 mRNA in monoecious cucumber plants in response to female-inducing treatments. Monoecious plants at the two-leaf stage were treated as described in "Materials and Methods." Leaves and plant apices were excised for 5 h following treatment, and tissue was frozen in liquid nitrogen for total RNA isolation. Each lane contains 30 mg of total RNA. Blots were probed with CS-ACS1 and actin.

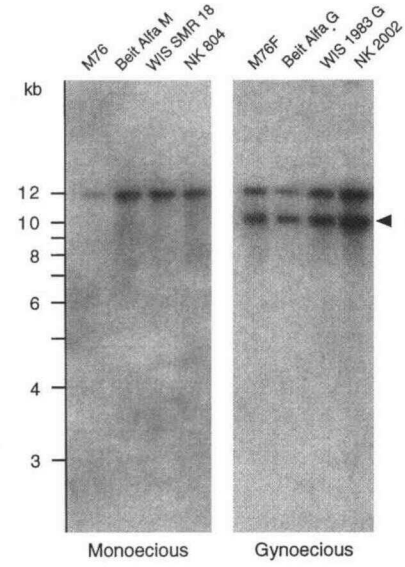

Figure 6. Southern blot hybridization analysis of several pairs of near-isogenic monoecious and gynoecious cucumber lines of diverse genetic backgrounds. Genomic DNA (10 mg/lane) was digested with *Xba*I and electrophoresed on a 0.8% agarose gel. The blot was probed with CS-ACS1. The arrowhead indicates the female-specific ACC synthase genomic sequence *CS-ACS1G*.

correlation was observed between the gynoecious phenotypes possessing at least one dominant *F* allele (i.e. gynoecious and subgynoecious phenotypes) and the presence of CS-ACS1G, as detected by Southern blot hybridization analysis. One dominant allele at the *F* locus is sufficient to induce the appearance of the female phase at an earlier developmental stage than observed in the monoecious parent, thus producing subgynoecious individuals (Table II).

These and other published results (Shifriss, 1961; Shifriss et al., 1964; Kubicki, 1969; Atsmon and Tabbak, 1979; Takahashi and Suge, 1982; Takahashi and Jaffe, 1984; Rudich, 1985; Trebitsh et al., 1987; Kende, 1993) suggest that a striking similarity exists between the genetics and action of the *F* gene in cucumber to ethylene action and the genetics of the ACC synthase gene. For instance, the intensity of the female phase increases as the dosage of dominant alleles at the *F* locus increases from zero to two (Fig. 1) (Shifriss, 1961; Shifriss et al., 1964), similar to what is seen in *CS-ACS1G* (Table II). Ethylene, its precursor ACC, and, to a lesser degree, auxin, simulate *F* in sex expression as well as flower inhibition (Table I) (George, 1971). Additionally, a

Table II. *Segregation analysis of femaleness (F) and CS-ACS1G*

Segregation and chi-square analysis were examined in the F_2 segregating population of *C. sativus* L. var *sativus* cv Marketmore 76 × *C. sativus* L. var *sativus* cv Marketmore 76F.

Marker	(G[a]:SG[b]):M[c]	Expected	Chi-Square	*df*[d]	P
Phenotype					
F	(30:46):24	1:2:1	1.36	2	>0.50
Genotype					
CS-ACS1G	(76):24	3:1	0	1	>0.99

[a] G, *FF*. [b] SG, *Ff*. [c] M, *ff*. [d] *df*, Degrees of freedom.

high correlation exists between ethylene evolution and female sex expression (Table I) (George, 1971; Rudich et al., 1972; Takahashi and Suge, 1982; Rudich, 1985). In cucumber, auxin was found to induce female sex expression and ACC synthase activity in shoot apices (Galun, 1959; Trebitsh et al., 1987). Inhibitors of ethylene action or biosynthesis repress expression at the *F* locus and ACC- or auxin-induced female sex expression (Atsmon and Tabbak, 1979; Takahashi and Jaffe, 1984). These findings, in conjunction with the data presented here, suggest the possibility that the *CS-ACS1G* is the *F* locus itself or may be a closely linked female intensifier gene.

To obtain additional evidence for linkage of *CS-ACS1G* to the *F* locus, we mapped its chromosomal location in a previously characterized F_3 population (Kennard et al., 1994). Southern blot hybridization analysis showed that *CS-ACS1G* segregated in the expected 3:1 ratio (P = 0.95, Table III). *CS-ACS1G* was placed on linkage group B (Kennard et al., 1994), where it mapped to the same position as the *F* locus (recombination frequency = 0%) (Fig. 7).

Studies in several plant species have designated the involvement of sex-determining genes in the production of plant hormones (Irish and Nelson, 1989; Durand and Durand, 1991; Grant et al., 1994). For example, the sex of the dioecious plant *Mercurialis annua* is dependent on three loci that regulate qualitative and quantitative variation of zeatin in male and female individuals (Durand and Durand, 1991). Likewise, maize (*Zea mays* L.), a monoecious plant, has many unlinked loci that affect the sex of the flower. Some of these genes function in the metabolism of GA, a plant hormone that promotes female sex expression in maize (Irish and Nelson, 1989). Thus far, the only sex-related locus that has been cloned in any flowering plant species is *TASSELSEED2* in maize. This gene encodes a short-chain alcohol dehydrogenase that might serve as a signal molecule, controlling the selective abortion of the gynoecium early in flower development and resulting in staminate flowers (DeLong et al., 1993). We hypothesize that in cucumber an internal developmental signal, either directly or via auxin, induces expression of *CS-ACS1G*, which, in turn, induces ethylene production. The increased ethylene level initiates a developmental process, which results in the selective abortion of stamen primordia in the floral bud, allowing the development of pistillate flowers.

Table III. *Segregation analysis of CS-ACS1G in a mapping population*

Segregation and chi-square analysis for CS-ACS1G in the mapping population GY14 (*C. sativus* L. var *sativus*) × PI 183967 (*C. sativus* var *hardwickii*). Analysis of CS-ACS1G was performed on F_3 families representing F_2 individuals. For details, see "Materials and Methods" and Kennard et al. (1994).

Marker	G/SG[a]:M[b]	Expected	Chi-Square	*df*[c]	P
Phenotype					
F	54:19	3:1	0.004	1	0.95
Genotype					
CS-ACS1G	54:19	3:1	0.004	1	0.95

[a] G/SG, *F_*.* [b] M, *ff*. [c] *df*, Degrees of freedom.

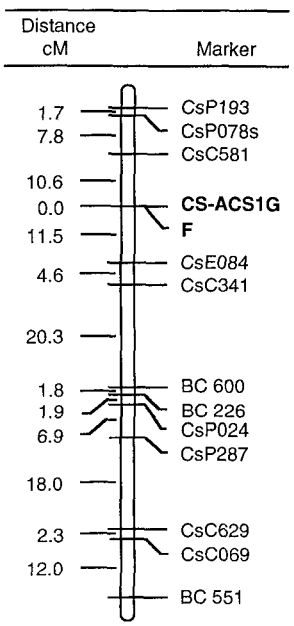

Figure 7. Linkage of *CS-ACS1G* to other known markers (LOD = 3) in the mapping population of GY14 (*C. sativus* L. var *sativus*) × PI183967 (*C. sativus* var *hardwickii*). For further details, see "Materials and Methods" (Kennard et al., 1994).

CONCLUSIONS

We isolated an ACC synthase (*CS-ACS1*) genomic sequence that is auxin-inducible in both monoecious and gynoecious cucumber apices (Figs. 4 and 5). Monoecious cucumber possesses a single copy of this gene, whereas gynoecious lines possess at least one additional copy (Figs. 3 and 6). The occurrence of the gynoecious *CS-ACS1G* was associated with acceleration of the rate of sex conversion (i.e. the pistillate phase is reached at an earlier stage during plant development). The monoecious cucumber lines examined did not possess a *CS-ACS1G* gene but contained in their genome the *CS-ACS1* gene, which is auxin-inducible (Figs. 4 and 5). This is consistent with the fact that monoecious cucumber plants retain female flowering potential and can be manipulated by auxin and ethylene to produce pistillate flowers (Table I) (Galun, 1959; George, 1971; Takahashi and Jaffe, 1984; Rudich, 1985). Additionally, subgynoecious cucumber phenotypes are by strict definition monoecious and are developmentally able to achieve a female-only phase (Fig. 1) (Shifriss, 1961). The subgynoecious phenotypes observed in our studies possessed the *CS-ACS1G* gene (Table II). Moreover, alleles at the *Female* (*F*) locus function to accelerate the rate of sex conversion in a dosage-related manner (Fig. 1) (Shifriss, 1961; Shifriss et al., 1964). Ethylene has been shown to simulate the action of the dominant alleles at the *F* locus by inducing pistillate flower development (Rudich, 1985). In addition, inhibition of ethylene biosynthesis or action has been shown to counteract gene expression at the *F* locus by postponing the pistillate phase, thus increasing the staminate tendency (Atsmon and Tabbak, 1979; Takahashi and Jaffe, 1984).

The *CS-ACS1G* gene was mapped to the *F* locus (Fig. 7). Based on the data presented here, *CS-ACS1G* is tightly

linked to the *F* locus and, based on the physiological involvement of ethylene in the determination of sex in cucumber, it is possible that *CS-ACS1G* corresponds to the *F* locus itself. Elucidation of the molecular mechanisms regulating sex determination in cucumber will allow a better understanding of the signal transduction pathway, leading to the development of a unisexual flower from a bisexual one. Furthermore, the development of molecular genetic tools useful for manipulating sex expression in the Cucurbitaceae would provide a new means for the development of hybrids with uniform, stable sex expression.

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LITERATURE CITED

Abel S, Nguyen MN, Chow W, Theologis A (1995) *ACS4*, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis thaliana*. J Biol Chem **270:** 19093–19099

Adams DO, Yang SF (1979) Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. Proc Natl Acad Sci USA **76:** 170–174

Atsmon D, Tabbak C (1979) Comparative effects of gibberellin, silver nitrate and aminoethoxyvinyl glycine on sexual tendency and ethylene evolution in cucumber plant (*Cucumis sativus* L.). Plant Cell Physiol **20:** 1547–1555

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1993) Current Protocols in Molecular Biology. John Wiley & Sons, New York

Botella JR, Arteca JM, Schlagnhaufer CD, Arteca RN, Phillips AT (1992) Identification and characterization of a full length cDNA encoding for an auxin-induced 1-aminocyclopropane-1-carboxylate synthase from etiolated mung bean hypocotyl segments and expression of its mRNA in response to indole-3-acetic acid. Plant Mol Biol **20:** 425–436

Botella JR, Schlagnhaufer CD, Arteca JM, Arteca RN, Phillips AT (1993) Identification of two new members of the 1-aminocyclopropane-1-carboxylate synthase-encoding multigene family in mung bean. Gene **123:** 249–253

Carter M, Milton I (1993) An inexpensive and simple method for DNA purifications on silica particles. Nucleic Acids Res **21:** 1044

Chetelat RT, Verna JW, Bennett AB (1995) Introgression into tomato (*Lycopersicon esculentum*) of the *L. chmielewskii* sucrose accumulation gene (sucr) controlling fruit sugar composition. Theor Appl Genet **91:** 327–333

Dellaporta SL, Calderon-Urrea A (1993) Sex determination in flowering plants. Plant Cell **5:** 1241–1251

DeLong A, Carderon-Urrea A, Dellaporta SL (1993) Sex determination gene TASSELSEED2 of maize encodes a short chain alcohol dehydrogenase required for stage-specific floral organ abortion. Cell **74:** 757–768

Destefano-Beltran LJC, Caeneghem WV, Gielen J, Richard L, Montegu MV, Straeten DVD (1995) Characterization of three members of the ACC synthase gene family in *Solanum tuberosum* L. Mol Gen Genet **246:** 496–508

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res **12:** 387–395

Dong JG, Kim WT, Yip WK, Thompson GA, Li L, Bennett AB, Yang SF (1991) Cloning of a cDNA encoding 1-aminocyclopropane-1-carboxylate synthase and expression of its mRNA in ripening apple fruit. Planta **185:** 38–45

Durand B, Durand R (1991) Sex determination and reproductive organ differentiation in *Mercurialis*. Plant Sci **80:** 49–65

Galun E (1959) The role of auxins in the sex expression of the cucumber. Physiol Plant **12:** 48–61

Galun E (1961) Study of the inheritance of sex expression in the cucumber: the interaction of major genes with modifying genetic and non-genetic factors. Genetica **32:** 134–163

George WL (1971) Influence of genetic background on sex conversion by 2-chloroethylphosphonic acid in monoecious cucumbers. J Am Soc Hortic Sci **96:** 152–154

Grant S, Houben A, Vyskot B, Siroky J, Pan W, Macas J, Saedler H (1994) Genetics of sex determination in flowering plants. Dev Genet **15:** 214–230

Huang PL, Parks JE, Rottmann WH, Theologis A (1991) Two genes encoding 1-aminocyclopropane-1-carboxylate synthase in zucchini (*Cucurbita pepo*) are clustered and similar but differentially regulated. Proc Natl Acad Sci USA **88:** 7021–7025

Irish EE, Nelson T (1989) Sex determination in monoecious and dioecious plants. Cell **1:** 737–744

Kende H (1993) Ethylene biosynthesis. Annu Rev Plant Physiol Plant Mol Biol **44:** 283–307

Kennard WC, Poetter K, Dijkhuizen A, Meglic V, Staub JE, Havey MJ (1994) Linkages among RFLP, RAPD, isozyme, disease-resistance and morphological markers in narrow and wide crosses of cucumber. Theor Appl Genet **89:** 42–48

Kim WT, Silverstone A, Yip WK, Dong JG, Yang SF (1992) Induction of 1-aminocyclopropane-1-carboxylase synthase mRNA by auxin in mung bean hypocotyls and cultured apple shoots. Plant Physiol **98:** 465–471

Kubicki B (1969) Investigation on sex determination in cucumber (*Cucumis sativus* L.). Genet Pol **10:** 69–86

Lander ES, Botstein D (1986) Mapping complex genetic traits in humans: new methods using a complex RFLP linkage map. Symp Quant Biol **51:** 49–92

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics **1:** 174–181

Liang X, Abel S, Keller JA, Shen NF, Theologis A (1992) The 1-aminocyclopropane-1-carboxylate synthase gene family of *Arabidopsis thaliana*. Proc Natl Acad Sci USA **89:** 11046–11050

Malepszy S, Niermirowicz-Szczytt K (1991) Sex determination in cucumber (*Cucumis sativus*) as a model system for molecular biology. Plant Sci **80:** 39–47

Munger H (1989) Transforming germplasm resources to varieties in use. *In* C Thomas, ed, Proceedings Cucurbitaceae 89: Evaluation and Enhancement of Cucurbit Germplasm, November 29–December 2. U.S. Department of Agriculture Agricultural Research Service, Charleston, SC, pp 4–8

Nakagawa N, Kamiya Y, Imaseki H (1995) Nucleotide sequence of an auxin-regulated 1-aminocyclopropane-1-carboxylic acid synthase gene (accession no. U37774) from *Cucurbita maxima* Duch. (PGR95–110). Plant Physiol **109:** 1499

Nakagawa N, Mori H, Yamazaki K, Imaseki H (1991) Cloning of a complementary DNA for auxin-induced 1-aminocyclopropane-1-carboxylate synthase and differential expression of the gene by auxin and wounding. Plant Cell Physiol **32:** 1153–1163

Nakajima N, Mori H, Yamazaki K, Imaseki H (1990) Molecular cloning and sequence of a complementary DNA encoding 1-aminocyclopropane-1-carboxylate synthase induced by tissue wounding. Plant Cell Physiol **31:** 1021–1029

O'Neill SD, Nadeau JA, Zhang X, Bui AQ, Halevy AH (1993) Interorgan regulation of ethylene biosynthetic genes by pollination. Plant Cell **5:** 419–432

O'Neill SD, Zhang XS, Zheng CC (1994) Dark and circadian regulation of mRNA accumulation in the short-day plant *Pharbitis nil*. Plant Physiol **104:** 569–580

Olson DC, Oetikers JH, Yang SF (1995) Analysis of *LE-ACS3*, a 1-aminocyclopropane-1-carboxylic acid synthase gene expressed during flooding in the roots of tomato plants. J Biol Chem **270:** 14056–14061

Olson DC, White JA, Edelman L, Harkins RN, Kende H (1991) Differential expression of two genes for 1-aminocyclopropane-1-carboxylate synthase in tomato fruits. Proc Natl Acad Sci USA **88:** 5340–5344

Pierce LK, Wehner TC (1990) Review of genes and linkage groups in cucumber. HortScience **25:** 605–615

Rottmann WH, Peter GF, Oeller PW, Keller JA, Shen NF, Nagy BP, Taylor LP, Theologis A (1991) 1-Aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. J Mol Biol **222:** 937–961

Rudich J (1985) *Cucumis sativus*. *In* AH Halevy, ed, Handbook of Flowering, Vol 2. CRC Press, Boca Raton, FL, pp 365–374

Rudich J, Halevy HA, Kedar N (1972) Ethylene evolution from cucumber plants as related to sex expression. Plant Physiol **49:** 998–999

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nickleu S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Sato T, Theologis A (1989) Cloning the mRNA encoding 1-aminocyclopropane-1-carboxylate synthase, the key regulatory enzyme for the ethylene biosynthesis. Proc Natl Acad Sci USA **86:** 6621–6625

Shifriss O (1961) Sex control in cucumbers. J Hered **52:** 5–12

Shifriss O, George WL, Quinones JA (1964) Gynodioecism in cucumber. Genetics **49:** 285–291

Takahashi H, Jaffe MJ (1984) Further studies of auxin and ACC induced feminization in the cucumber plant using ethylene inhibitors. Phyton **44:** 81–86

Takahashi H, Suge H (1982) Sex expression and ethylene production in cucumber plants as affected by 1-aminocyclopropane-1-carboxylic acid. J Jpn Soc Hortic Sci **51:** 51–55

Trebitsh T, Riov J, Rudich J (1987) Auxin, biosynthesis of ethylene and sex expression in cucumber (*Cucumis sativus*). Plant Growth Regul **5:** 105–113

Yang SF (1980) Regulation of ethylene biosynthesis. HortScience **15:** 238–243

Zarembinski TI, Theologis A (1993) Anaerobiosis and plant growth hormones induce two genes encoding 1-aminocyclopropane-1-carboxylate synthase in rice (*Oryza sativa* L.). Mol Biol Cell **4:** 363–373