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Note

Molecular Isolation of the *M* Gene Suggests That a Conserved-Residue Conversion Induces the Formation of Bisexual Flowers in Cucumber Plants

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ABSTRACT

Sex determination in plants involves a variety of mechanisms. Here, we report the map-based cloning and characterization of the unisexual-flower-controlling gene *M*. *M* was identified as a previously characterized putative 1-aminocyclopropane-1-carboxylic acid synthase gene, while the *m* allele that mutated at a conserved site (Gly33Cys) lost activity in the original enzymatically active allele.

SEX determination in angiosperms, including crop plants, evolves a variety of mechanisms that involve a number of different genetic and epigenetic factors (Tanurdzic and Banks 2004). Due to its diversity in sex types and to the extensive physiological and genetic studies conducted on it, cucumber (*Cucumis sativus* L.; $2n = 2x = 14$) is becoming a model plant for sex-determination research (Atsmon 1968; Tsao 1988; Perl-Treves 1999; Tanurdzic and Banks 2004). In cucumber plants, male and female flowers are generally produced separately in the same individual; however, certain lines also produce bisexual flowers. Preliminary genetic studies have indicated that three major genes are responsible for sex expression and segregation in the cucumber plant*F/f*, *M/m*, and *A/a*. The *F* gene may promote femaleness, while the *m* gene regulates the appearance of hermaphroditic flowers on the plant. Furthermore, in combination with the homozygous recessive *f* gene, the recessive *a* gene can intensify the androecious nature (Galun 1961; Robinson *et al.* 1976).

Sex expression in cucumber plants can also be modified by various environmental factors and plant hormones such as ethylene (Atsmon 1968; Takahashi *et al.* 1983; Takahashi and Jaffe 1984; Perl-Treves 1999; Yamasaki *et al.* 2005). A series of studies (Kamachi *et al.* 1997, 2000; Trebitsh *et al.* 1997; Yamasaki *et al.* 2003a; Mibus and Tatlioglu 2004; Knopf and Trebitsh 2006) have been conducted to investigate the *F/f* gene. These studies have shown that *CsACS1G*, which encodes a key enzyme of the ethylene-synthesis pathway, is the candidate gene for the *F/f* locus. However, the *M/m* gene has not been studied in as much detail as the *F/f* gene. Here, we report the map-based cloning and characterization of the unisexual-flower-controlling *M* gene.

RESULTS

In the previous studies, the *M/m* locus was independently mapped into a genetic interval of 2.5 cM (Liu *et al.* 2008) and 6.1 cM (Li *et al.* 2008). In this study, we developed two larger segregating populations, which included 2830 F_2 + BC_1 (population 1983) and 2700 F_2 (population 5234) individuals and constructed a high-resolution collinear genetic map for the *M/m* locus (supporting information, Figure S1). After chromosome walking, a bacterial artificial chromosome (BAC) contig formed by two BAC clones (overlapped by an ~9.2-kb sequence) was found to be anchored to the

Sequence data have been deposited with the EMBL/GenBank Data Libraries under accession nos. FJ529216 and FJ971626–FJ971630.

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.109.104737/DC1.

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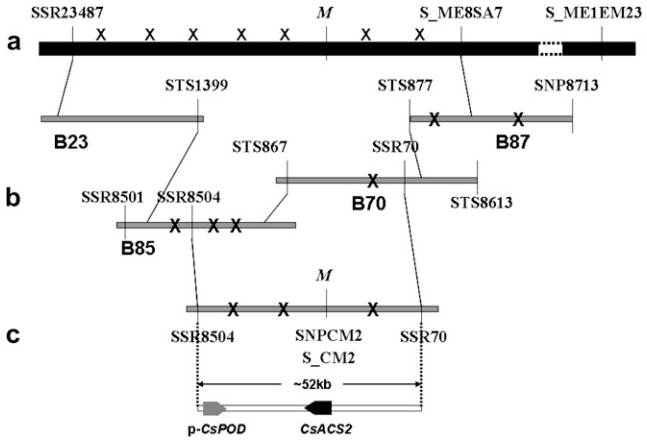

FIGURE 1.—*M* map-based cloning. (a) *M* genetic map. "X" indicates recombination events identified in population 5234 (the "right side" of the *M* locus) and population 1983 (the "left side" of the *M* locus), respectively. (b) Fine mapping *M*. New marker SNP8713, derived from the B87 end sequence, was mapped beyond the *M* locus with an additional recombination event. SSR70 was developed from the B70 inner sequence, which was mapped at one recombination event from *M* between S_ME8SA7 and SSR23487. SSR8501 and SSR8504, derived from the B85, had three and two recombination events, respectively, from the *M* locus at the other side beyond the SSR70 marker. The final contig was confirmed by an overlap test from STS877, STS867, and STS1399. BAC clones were not drawn to scale. (c) The final contig and the genomic organization for this region. A contig encompassing the *M* locus, which included three recombination events, was identified by the two markers, SSR8504 and SSR70. Broad arrows indicate the two predicted genes (*p-CsPOD* and *CsACS2*) and the predicted transcriptional orientations. Two new markers, S_CM2 and SNPCM2, were developed from the candidate gene *CsACS2* and cosegregated with the *M* locus. The primer sequences are listed in Table S2.

genetic interval. The sequence of the entire contig covered an ~52-kb chromosome section, which had two complete candidate genes (Figure 1). One gene sequence showed limited similarity (68%) to a bacterium-induced peroxidase precursor (GenBank accession no. AF155124) found in *Gossypium hirsutum*. However, the other gene, which was predicted to encode a 445-amino-acid protein, showed 100% sequence identity to the previously characterized *CsACS2* gene, which encodes a putative 1-aminocyclopropane-1-carboxylate synthase in *C. sativus* (KAMACHI *et al.* 1997).

The sequences of the entire genomic region of the putative peroxidase gene (*p-CsPOD*) and an ~2.0-kb 5′ upstream region and 1.0-kb 3′ downstream region were identical among the four parent lines. Therefore, we concluded that this putative peroxidase gene in cucumber was not the *M* gene.

We sequenced the entire genomic section of *CsACS2* along with a 1.9-kb 5′ upstream region and a 620-bp 3′ downstream region from all four parental plants. These sequences revealed two types of polymorphism in the four lines used for mapping (Figure 2). First, a 5-bp insertion/deletion difference was found in the second intron between parent plants S52 and H34. A sequence-characterized amplified region marker, S_CM2, was developed to cover this region (Figure 1c). We did not detect any recombination events after mapping in population 5234 (2700 individuals). The second type of polymorphism, a G97T conversion, was identified in the first exon of this gene in all four parent lines (WI1983G and S52 had G, while WI1983H and H34 had T). On the basis of this polymorphism, we developed the SNP marker SNPCM2, which expectedly cosegregated with the *M/m* locus in the final population of 5530 individuals (Figure 1c). Then we used this SNP marker to analyze a germplasm collection of 65 *C. sativus* cultivars obtained from different parts of the world (Table S1). All unisexual cultivars (21 monoecious cultivars and 41 gynoecious cultivars) were found to contain G, which was the dominant polymorph. However, a well-known andromonoecious line, *Lemon*, and the hermaphrodite inbred lines WI1983H and H34 were found to contain the recessive T. The quantitative reverse transcriptase–polymerase chain reaction analysis for *CsACS2* indicated significant differences (over ninefold) in the expression levels between the near-

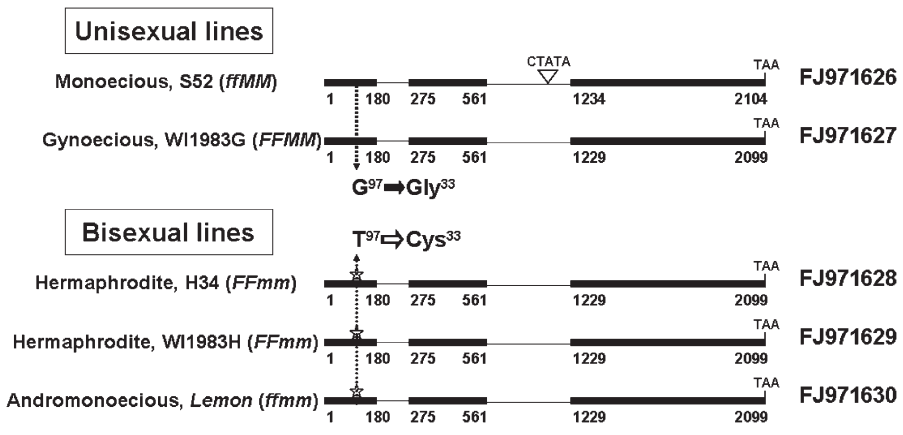

FIGURE 2.—Genomic structures of the *M/m* alleles. The monoecious plant line S52 had a unique 5-bp (CTATA) insertion/deletion in the second intron. Both of the unisexual parental lines, S52 and WI1983G, had the conserved "G." The hermaphrodite lines, H34 and WI1983H, with an additional andromonoecious line, *Lemon*, shared the same framework with WI1983G, except for the nucleotide transition from G to T, which led to the missense mutation of Gly33Cys in the deduced amino acid sequence. Solid boxes represent the full-length mRNA sequence, and thin lines indicate introns. Dashed lines showed the conserved nucleotides in the dominant and recessive (marked with asterisk) alleles.

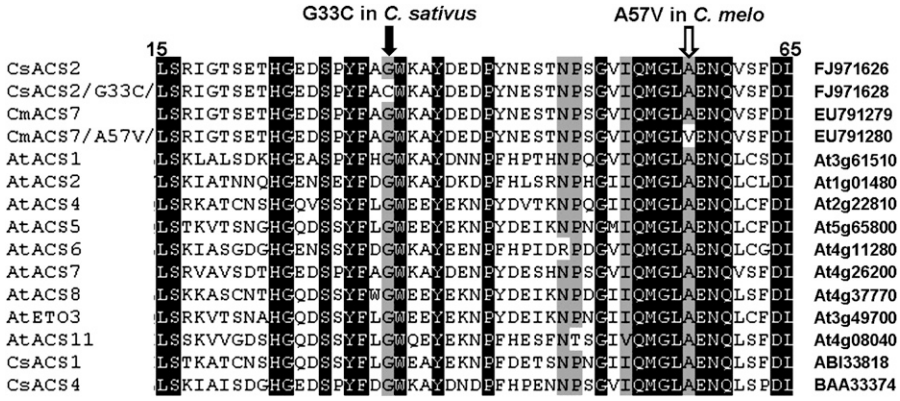

FIGURE 3.—Alignment of the deduced amino acid sequence of ACS isozymes from cucumber, melon, and *Arabidopsis thaliana*. The conserved-residue conversions, G33C in *C. sativus* for the *M/m* gene and A57V in *C. melo* for the *A/a* gene, are shown above the alignment. Identical amino acids are against a solid background, and amino acid similarity levels >75% are against a shaded background.

isogenic lines (NILs) for the *M* locus, H34 (*FFmm*) and M34 (*FFMM*) (Figure S2). These evidences strongly suggested that *CsACS2* was the candidate gene for *M*.

The G97T conversion in the coding region of *CsACS2* caused a substitution at position 33 in the protein, thereby producing differences among the proteins produced by the unisexual (gynoecious and monoecious) *M* (Gly at position 33) and the bisexual *m* (Cys at position 33) alleles. As shown in Figure 3, Gly was conserved in nearly all the functional 1-aminocyclopropane-1-carboxylic acid (ACC) synthases in plants, while the Gly33Cys isoform may show changed or reduced enzymatic activity.

Because of the technical difficulties in cucumber transformation, we used the *Escherichia coli* system to analyze the activity of the putative ACC synthases encoded by the *M* and *m* alleles. Various reports have shown that plant ACC synthase can serve the same function in *E. coli* (VAN DER STRAETEN *et al.* 1990; TARUN *et al.* 1998; YAMAGAMI *et al.* 2003). TARUN *et al.* (1998) integrated the ACC deaminase gene into the genome of the mutant strain JHM544, which is an Ile auxotroph, and developed the *E. coli* strain JAde 6, which is effective for testing putative ACC synthases. To test the potential loss-of-function mutation attributed to the Gly33Cys conversion in CsACS2, we subcloned two full-length coding sequence fragments derived from the cucumber lines M34 (*FFMM*) and H34 (*FFmm*) into the expression vector *pQE-30*. Additionally, we induced artificial mutagenesis at position 33 of the original CsACS2 to produce two mutant proteins (Gly33Ser and Gly33Ala), which were used to study the putative conserved active site of ACC synthase. Furthermore, CsACS1G, encoded by the well-characterized *F* gene (KNOPF and TREBITSH 2006), was used as a positive control in the cucumber plant to test the biochemical activity of the enzyme. After a 3-day incubation at 37° on basic M9 media, CsACS1G and CsACS2 demonstrated ACC synthase activity, while the other three CsACS2 isoforms, namely, Gly33Cys, Gly33Ser, and Gly33Ala, did not show any activity (Figure 4). Therefore, the Gly33Cys conversion may cause a functional alteration resulting in a new phenotype in cucumber plants.

DISCUSSION

The conserved ACC synthase gene *CsACS2* was first cloned by KAMACHI *et al.* (1997) using a homology-based cloning strategy. This gene was subsequently reported in many studies (MATHOOKO *et al.* 1999; KAMACHI *et al.* 2000; YAMASAKI *et al.* 2000, 2001, 2003a,b; SAITO *et al.* 2007). Since the *mm* genotype was associated with significant expression, *CsACS2* was first defined as a downstream product regulated by the *M* gene. However, despite this aberration, the information from these studies was very useful in clarifying the regulated expression and functional pattern of this gene. KAMACHI *et al.* (1997) reported that the *CsACS2* mRNA was detected at the apexes, and the results of the Southern blot analysis suggested that a single copy of the gene was present in the examined cucumber genomes. Furthermore, KAMACHI *et al.* (2000) noted that the expression of *CsACS2* at the apexes was localized to the floral buds

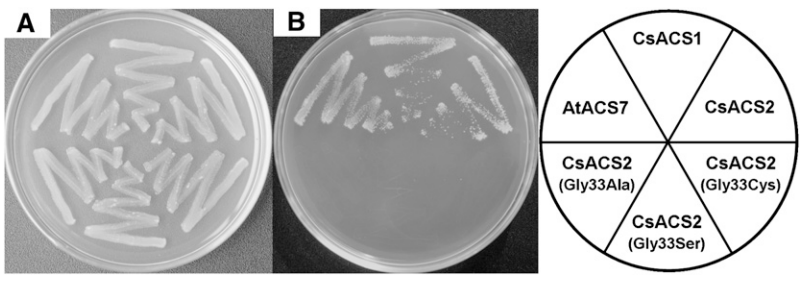

FIGURE 4.—Complementation of the *E. coli* integrative strain JAde 6 with various *CsACS* and *AtACS7* cDNAs. Six plasmids were used to transform *E. coli* JAde 6 as described in File S1. The streaked sections in A and B correspond to the cDNAs in the pie chart. All the plates were grown at 37° overnight (on LB media) or for 3 days (on minimal media) and then photographed. (A) Growth of all six strains of JAde 6 on LB media for positive control. (B) Growth of all six strains of JAde 6 on minimal media.

that developed into female flowers. Yamasaki *et al.* (2000) found a positive correlation between *CsACS2* expression and ethylene evolution in shoot apexes. Later, Yamasaki *et al.* (2001) showed that ethylene caused substantial increases in the accumulation of *CsACS2* mRNA and inhibited stamen development.

Saito *et al.* (2007) performed comprehensive *in situ* hybridizations to study the expression pattern of *CsACS2*. The results revealed that the *CsACS2* transcript began to accumulate just beneath the pistil primordia of flower buds at the bisexual stage, and this accumulation coincided with the sex-determination stage in cucumbers (Bai *et al.* 2004). During the later developmental stages, the accumulation of *CsACS2* was correlated with the establishment of female flowers. These results may imply a relationship between the expression of *CsACS2* and the continuous arrestment of stamen development.

Although we could not perform the complementation test in cucumber plants, the analysis of the physical location of the gene, the enzymology results obtained from this study, and the results obtained from previous studies indicate that the *CsACS2* gene is the *M* gene in cucumbers.

To our knowledge, the presence of a gene for controlling unisexual expression is unique to the Cucurbitaceae plants, particularly cucumber and melon (Tanurdzic and Banks 2004). Therefore, the unisexuality-determining genes *M* (in cucumbers) and *A* (in melons) may represent a homology. Boualem *et al.* (2008) described an experiment to clone the *a* gene in melon; homology analysis showed that the two dominant alleles encoded almost identical amino acid sequences, except for eight residues, all of which were in nonconserved active sites (Figure 3). Since the BLASTX analysis did not show any other proteins with such a high degree of similarity (98%), they may be the ortholog gene in the Cucumis plants. Nevertheless, in addition to the different expression patterns of *m* and *a*, there were differences between the protein sequences of the natural mutants [Gly33Cys in cucumbers and Ala57Val (Gly19Glu for the targeting-induced local lesions in genomes analysis) in melons]. These results suggest that the evolution of the functional unisexuality-determination genes occurred prior to the differentiation of the two Cucumis species, namely, cucumber and melon. Subsequently, the two species independently evolved two different nonfunctioning recessive mutants.

In cucumber, the *F* gene and the *M* gene were shown to encode 1-aminocyclopropane-1-carboxylate synthase, the key regulatory enzyme in the ethylene biosynthetic pathway. Therefore, in cucumber plants, the genes related to the ethylene-signaling pathways might be related to the unique development of sex organs.

We thank Rentao Song (Shanghai University, Shanghai, China) for his technical assistance in the BAC library; Athanasios Theologis and Guixia Yu (University of California, Berkeley) for their helpful assistance with *E. coli* JAde 6; Lingxia Zhao (Shanghai Jiaotong University, Shanghai, China) for the vector *pQE-30*; and Qiguang Wen (Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China) for the chemical reagent 1-aminocyclopropane-1-carboxylic acid. The authors also thank Zhangjun Fei (Cornell University, Ithaca, NY) for his critical reading. This work was supported by the National Natural Science Foundation of China (no. 30871707), National "863" Project (no. 2008AA10Z150), Ministry of Agriculture "948" Program (no. 2008-Z42), Ministry of Science and Technology (no. 2006DFA32140), and the Shanghai Leading Academic Discipline Project (no. B209).

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Molecular Isolation of the *M* Gene Suggests That a Conserved-Residue Conversion Induces the Formation of Bisexual Flowers in Cucumber Plants

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FILE S1

Material and Methods

Plant materials

Four cucumber inbred lines S52 (monoecious), H34 (hermaphrodite), WI1983G (gynoecious) and WI1983H (hermaphrodite), were used to generated two segregating populations. Sex expression conditions for the four lines had been reported previously (Liu *et al.* 2008; Li *et al.* 2008). The first F_2 population, 5234, which included 2,700 individuals, was obtained from the crossing of S52 and H34 followed by subsequent self-pollination. The second population was derived from WI1983G and WI1983H, which included two sub-populations: an F_2 population of 2,080 plants and a BC_1 population (backcross to WI1983H) of 750 individuals. Plants were adequately watered and grown under natural sunlight in greenhouses. The estimation of sex types for all the plants was according to the methods described previously (Liu *et al.* 2008; Li *et al.* 2008).

A well-known andromonoecious line, *Lemon*, and a set of near-isogenic lines (NILs), M34/H34, were used for the gene structure and expression analysis. Seeds of Lemon cucumber plants were purchased from Heirloom Seeds Co. (Pennsylvania, USA). The NIL, which developed following at least six direct backcrosses, was nearly identical except for the *andromonoecious* locus (M34/H34). All seeds, which were maintained by inbreeding at our laboratory in Shanghai, were germinated and grown in trays containing a soil mixture (peat:sand:pumice, 1:1:1, v/v/v) at day/night temperatures of 25/20°C with a 16-hr photoperiod (light intensity: ~112.5μmol×m^{-2}×s^{-1}) in a light incubator (Snijders Scientific Co., economic deluxe, STEK-NO.S1084).

Molecular markers

All plant genomic DNA was extracted according to the previous studies (Liu *et al.* 2008; Li *et al.* 2008). The PCR conditions for the three SCAR markers (S_ME1EM26, S_ME1EM23, and S_ME8SA7) were reported previously (Li *et al.* 2008). Based on the draft genome sequence of the cucumber plant derived from the Cucumber Genome Initiative (CuGI; Huang *et al.* 2008), 2,000 SSR markers were identified with RepeatMasker (http://www.repeatmasker.org/). After amplification, the products were separated on a 6% denatured polyacrylamide gel with 1× TBE buffer and 8 M urea. After electrophoresis, the gel was stained with $AgNO_3$ solution (Bassam *et al.* 1991). All the primers (shown in Supplemental Table 2) used in this study were synthesized by Sangon Biological Engineering Technology & Service Co. (Shanghai).

BAC library screening, end sequencing, and shotgun sequencing

The cucumber genomic BAC library was constructed by a previously reported procedure (Guan *et al.* 2008). After screening with the PCR method, positive clones, B70 (100.8-kb) and B85 (80.6-kb), were sequenced by the Beijing Genomics Institute (Beijing). The sequences, derived from two ends or the whole BAC inserts, utilized to design PCR primers to develop simple sequence repeat (SSR), single nucleotide polymorphism (SNP), or sequence tagged sites (STS) markers for fine mapping and chromosome walking to *M*.

Linkage analysis

MAPMAKER/EXP3.0 (Lander *et al.* 1987) was used for linkage analysis with a LOD threshold of 3.0 or more. The recombination rates were converted to genetic distance by using the Kosambi mapping function (Kosambi 1944).

Allele sequencing of the candidate genes

Specific primers (Supplemental Table 2) were designed to amplify the corresponding fragments from different alleles. The resulting PCR products were then cloned and sequenced from both ends as previously described (Li *et al.* 2008).

To identify putative genes in the final physical interval, the contig sequences were analyzed with the programs GENSCAN (http://genes.mit.edu/GENSCAN.html) and FGENESH gene prediction software (http://www.softberry.com) using the *Arabidopsis* setting, and then manually edited. The putative gene sequences were then searched against the public non-redundant protein using the BLASTX program (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi).

Prokaryotic expression system

The integrating bacterial strain, *E. coli* JAde 6, used for functional analysis was provided by Prof. Athanasios Theologis (University of California, Berkeley). Detailed information for this strain was provided by Tarun *et al.* (1998). The *pQE-30* vector used for expression of *ACSs* was supplied by Dr. Lingxia Zhao (Shanghai Jiaotong University, Shanghai). 1-aminocyclopropane-1-carboxylate (ACC) used to validate the ability to grow on minimal media (M9) for the original JAde 6 was provided by Prof. Qiguang Wen (Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai).

The well-characterized ACC synthase gene *AtACS7*, which shared the highest similarity with *CsACS2* in *Arabidopsis*, was used as the positive control. The cDNA of *AtACS7* was isolated using the method described by Yamagami *et al.* (2003). As a positive control in the cucumber plants, CsACS1G (Knopf and Trebitsh 2006), encoded by the well-characterized *F* gene, was also used to test its biochemical activity. cDNA fragments for all the *ACS* gene in cucumber plants were obtained by the method described in the next part. All the natural gene fragments tested in this study were amplified with the corresponding ORFs primers, bAtACS7-F/sAtACS7-R (for *AtACS7*), bCsACS1-F/sCsACS1-R (for *CsACS1G*), and bCsACS2-F/sCsACS2-R (for *CsACS2*).

To obtain mutated isozymes of CsACS2, two pairs of complementary primers, mCsACS2_A-U/mCsACS2_A-D and mCsACS2_S-U/mCsACS2_S-D, were used to mutate Gly to Ala and Ser, respectively. The PCR productions derived from the corresponding primer combinations (for Ala: bCsACS2-F+mCsACS2_A-U and sCsACS2-R+mCsACS2_A-D; for Ser: bCsACS2-F+mCsACS2_S-U and sCsACS2-R+mCsACS2_S-D) were purified using the AxyPrep™ DNA Gel Extraction Kit (AXYGEN, China). Then, mixed the two relevant reclaimed DNA fragments, and performed a second cycle PCR with the ORFs primers.

The PCR-amplified products were digested with restriction enzymes, gel-purified, and subcloned into *pQE-30* vector as BamHI/SalI fragments using FastDigest™ Pack Kit (Fermentas, China). Plasmids containing inserts were transformed into *E. coli* DH5α by electroporation and sequenced to confirm that the reading frames and mutated nucleotides. Then, plasmids purified using AxyPrep™ Plasmid Miniprep Kit (AXYGEN, China) from the positive DH5α colonies were transformed into JAde 6 using TransformAid™ Bacterial Transformation Kit (Fermentas, China).

Transformed cells were plated on Luria-Bertani medium (LB) with 100 μg/ml amp and incubated at 37°C overnight. Colonies from the LB plates were patched onto minimal media (M9) with 25 μg/ml amp and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Negative results were identified for their inability to grow on M9 plates after 3 days of incubation at 37°C.

Quantitative RT-PCR

The developmental stage for *CsACS2* expression analysis was according with Yamasaki *et al.* (2001). Total RNA was isolated from shoot apices at the 4-leaf stage of cucumber plants (~20 days after seeding). The apical shoot, excised as the shoot apex, included immature leaves shorter than 1 cm in length. The reagents for RNA extraction was purchased from TIANGEN (China). DNaseI (TaKaRa, China) was used to remove the contaminating DNA, and first strand cDNA was synthesized with the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, China). PCR was performed in a 96-well plate using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA), with SYBR Green Realtime PCR Master Mix (TaKaRa, China). PCR was started at 94°C for 10 min, followed by 40 cycles at 94°C for 5 s, and 62°C for 34 s. The amplification specificity was tested by a dissociation curve (65°C to 90°C). To compare results from different reactions and samples, *CsACS2* was normalized to the *CsActin3* with the CT value.

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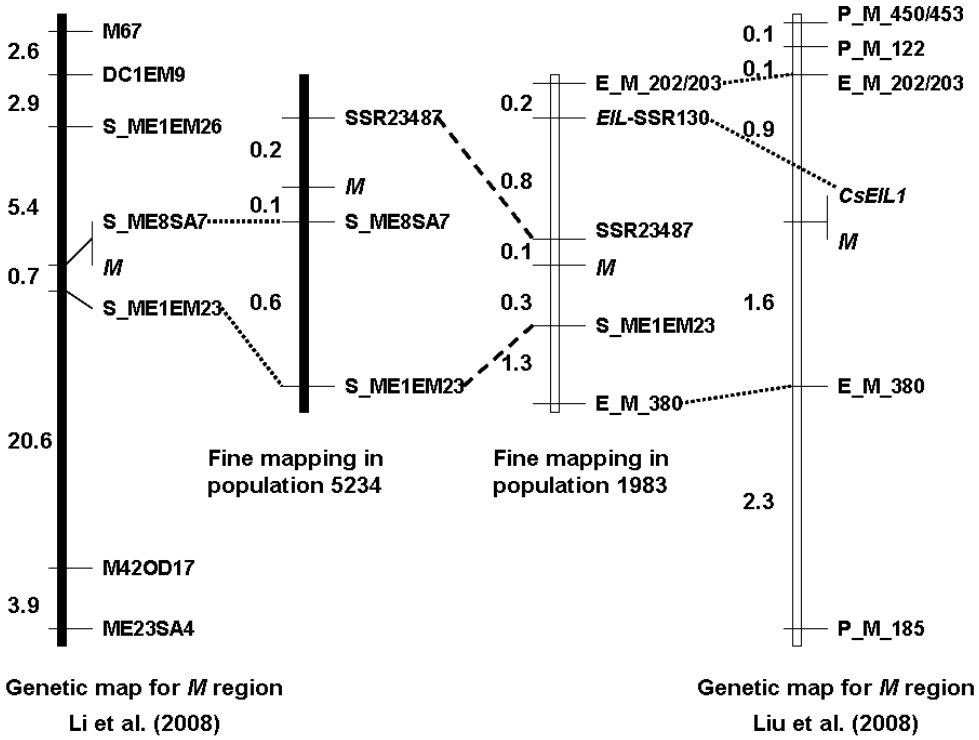

FIGURE S1.—Fine mapping for *M* locus. Two segregating populations were used to map the *M* locus. SCAR markers, S_ME1EM23 and S_ME8SA7, were derived from previously mapped markers (Li *et al.* 2008). EIL-SSR130 was developed based on the nearby sequence of the previous CsEIL1 marker (Liu *et al.* 2008). The collinear locations between the two linkage maps are shown by *dashed*. Genetic distance in centimorgans (cM) was calculated using the Kosambi function. Different scales were used in the four maps.

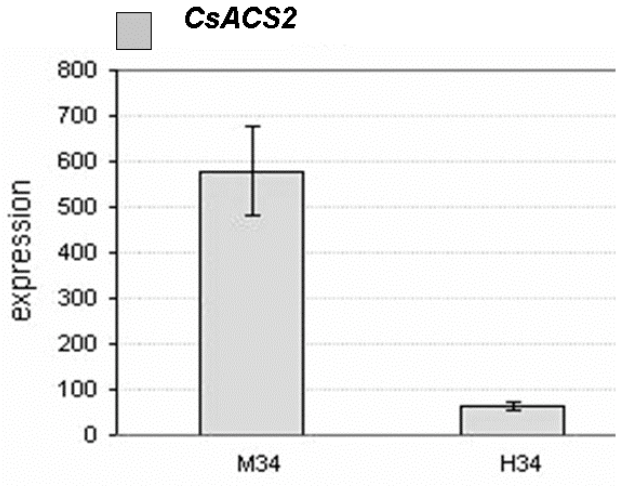

FIGURE S2.—Quantitative RT-PCR of *CsACS2* in near-isogenic lines, M34 (*FFMM*) and H34 (*FFmm*). Total RNA extracted from shoot apices of cucumber plants at the four leaf stage. Each result was the average of three independent biological replicates, and bars indicated ± SE.

TABLE S1

Different cucumber sexual lines tested with *CsACS2* specific marker SNPCM2 for *M/m* locus

Lines	Origin	Sexual phenotype	Screened with SNPCM2	Lines	Origin	Sexual phenotype	Screened with SNPCM2
S52	China	monoecious	M[a]	S45-2	Israel	gynoecious	M
H34	Europe	hermaphrodite	m	S47	Israel	gynoecious	M
1983G	America	gynoecious	M	S48-1	Israel	gynoecious	M
1983H	America	hermaphrodite	m	S48-2	Israel	gynoecious	M
Lemon	Europe	andromonoecious	m	S48-4	Israel	gynoecious	M
S106	America	gynoecious	M	S73-3	Japan	gynoecious	M
S35-2	China	gynoecious	M	S109-6	Japan	gynoecious	M
S59-3	China	gynoecious	M	S118-1	Japan	gynoecious	M
S57	China	gynoecious	M	S119-17	Japan	gynoecious	M
S82	China	gynoecious	M	S112-7	Korea	gynoecious	M
S80	China	gynoecious	M	S75	Spain	gynoecious	M
S105	China	gynoecious	M	S76	Spain	gynoecious	M
S122-2	China	gynoecious	M	S107	America	monoecious	M
S122-16	China	gynoecious	M	S50-3	China	monoecious	M
S33-1	Europe	gynoecious	M	S58	China	monoecious	M
S34	Europe	gynoecious	M	S60	China	monoecious	M
C19	France	gynoecious	M	S61-2	China	monoecious	M
C21	France	gynoecious	M	S78-2	China	monoecious	M
C17-1	Holand	gynoecious	M	S94	China	monoecious	M
C17-2	Holand	gynoecious	M	S98	China	monoecious	M
S17-2	Holand	gynoecious	M	S99	China	monoecious	M
S23	Holand	gynoecious	M	S100	China	monoecious	M
S46-1	Holand	gynoecious	M	S103	China	monoecious	M
S46-2	Holand	gynoecious	M	S110-7	China	monoecious	M
S49-1	Holand	gynoecious	M	S115-3	China	monoecious	M
S49-2	Holand	gynoecious	M	S54-2	Japan	monoecious	M
S51-2	Holand	gynoecious	M	S66	Japan	monoecious	M
S55-1	Holand	gynoecious	M	S67	Japan	monoecious	M
S05	Israel	gynoecious	M	S69-2	Japan	monoecious	M
S06	Israel	gynoecious	M	S70	Japan	monoecious	M
S36	Israel	gynoecious	M	S74	Japan	monoecious	M
S43	Israel	gynoecious	M	S53	Korea	monoecious	M
S45-1	Israel	gynoecious	M				

[a]Genotype designation: M: dominant allele of *M/m* locus; m: recessive allele of *M/m* locus

Table S2

Primers used in this study

Analysis	Primer name	Sequence (5′ to 3′)	Size (bp)
Fine mapping	S_ME8SA7-F	CGATAACAAGCCGCTGAGGAGAT	149/165 (*M/m*)
	S_ME8SA7-R	GTGGCCCGTTACCACTAATAC	
	S_ME1EM23-F	GAAACCACAAGATTCAACCACAC	227/223 (*M/m*)
	S_ME1EM23-R	GCATGAGGTTCCATCTCAAAGC	
	SSR23487-F	TGTTTCAAGGTGCTGACCTG	139/147 (*M/m*)
	SSR23487-R	CCACAACAACAAAAGAATGTGAA	
Physical interval	SNP8713-F	TGCATTATATATCTCGAGGTGGC	802
	SNP8713-R	TGGGGTTCATCAAATTCAGAGTC	
	STS877-F	CGCGCATGCCAATGATTGAGT	827
	STS877-R	AAAGTATTTGGTCCACTCTGCTC	
	STS8613-F	GCTTGACTGACTAACTCATTTG	273
	STS8613-R	TAAGTCCCTGGAAGAAATGAAG	
	SSR70-F	GACATGATGGAGAGGGAAAGTG	223/221 (*M/m*)
	SSR70-R	TTCTTCCTCATCTCATGTACCTA	
	STS867-F	TGGAAAAAGAAAAGTTTGCTGA	151
	STS867-R	TCGAAACAATGGTTATTCATTCC	
	SSR8501-F	AGGAAAGGAATATTAACCTCC	155/157 (*M/m*)
	SSR8501-R	TCATAACAACATAGGTATAGAT	
	SSR8504-F	ATGCTCAGGTTCATGGATTG	108/110 (*M/m*)
	SSR8504-R	TATTTTTTTGAGGGAGCTGG	
	STS1399-F	GGAAATGTAAATGGAAGTGAGGAG	1762
	STS1399-R	GATATTGCCACCACCATAGTCC	
Nucleotide diversity	SNPCM2-F	TTGGCTCTCAAGAAGGGAAA	654
	SNPCM2-R	AGGAGTGGGGACAAGCAAAG	
	S_CM2-F	AAATCCTGGTGATGCTTTGC	845/840 (*M/m*)

	S_CM2-R	TTTTCATTTCCGTTGCTGAA	
	p-CsACS2-F	AAATTATATATGCATGGGTTGGTGG	1972
	p-CsACS2-R	GATGTTTGTTCTTTTTTCCTTGAGC	
	p-CsPOD-F	ATAGAGTTGTTTCCTTTGGTTGTGG	2224
	p-CsPOD-R	GGCTAAAAGGAAAAGAACAATGAAA	
	CsACS2_full-F	TTGGCTCTCAAGAAGGGAAA	2828
	CsACS2_full-R	TAAATGCCACGTCAGATGGA	
	CsPOD_full-F	AACCCTCACGCTCTCTAAGTTTCAT	1754
	CsPOD_full-R	GCAACAACACACATAGAAACCAACAA	
	bAtACS7-F	AAGCCGGATCCAAAAAAATGGGTCTTCCTCTAATG	1434
	sAtACS7-R	TCTTTGTCGACCCCCTCCCCCATATATTTAACC	
	bCsACS1-F	AGCCGGGATCCATTAGCAGCACAACCGAAGAA	1496
	sCsACS1-R	CAACTGTCGACCAGGCTATCGTTCATAATGGAG	
Prokaryotic	bCsACS2-F	ACGGCGGATCCGAACAAACATCAATGGCGA	1372
expression	sCsACS2-R	TGGTCGTCGACTTAATTAGCTTCGTTTTCCTTC	
	mCsACS2 _A-U	ATCATACGCTTTCCAGGCAGCAAAATACGG	
	mCsACS2 _A-D	CGCCGTATTTTGCTGCCTGGAAAGCGTATG	
	mCsACS2 _S-U	ATCATACGCTTTCCAGCTAGCAAAATACGG	
	mCsACS2 _S-D	CGCCGTATTTTGCTAGCTGGAAAGCGTATG	
	qCsACS2-F	TGGGCTTAGCTGAGAATCAAGTGTC	171
Quantitative	qCsACS2-R	TTTCTTCCATAAAACTTCCCATTGC	
RT-PCR	CsActin3-F	TCGTGCTGGATTCTGGTG	161
	CsActin3-R	GGCAGTGGTGGTGAACAT	