Theor Appl Genet (2008) 117:927–933
DOI 10.1007/s00122-008-0832-1

ORIGINAL PAPER

Genetic association of *ETHYLENE-INSENSITIVE3*-like sequence with the sex-determining *M* locus in cucumber (*Cucumis sativus* L.)

Shiqiang Liu · Liang Xu · Zhiqi Jia · Yong Xu · Qing Yang · Zhangjun Fei · Xiangyang Lu · Huiming Chen · Sanwen Huang

Received: 3 November 2007 / Accepted: 21 June 2008 / Published online: 16 July 2008

Abstract Cucumber (*Cucumis sativus* L.) has served as the model system for sex expression in flowering plants and its sex type is predominantly controlled by two genetic loci, *F* and *M*. Ethylene is the major plant hormone that regulates sex expression in cucumber. The current model predicts that ethylene serves as both a promoter of femaleness via the *F* locus and an inhibitor of the male sex via the *M* locus. In support of this model, genetic, genomic, and transcript analyses indicate that the *F* gene encodes a key enzyme in ethylene biosynthesis. In this study, we discovered that the *M* locus co-segregates with an *ETHYLENE-INSENSITIVE3* (*EIN3*)-like genomic sequence in an F_2 population of 96 individuals. This genetic association agrees with the prediction that the *M* locus is involved in ethylene signaling, thus providing another line of evidence in favor of the model. In addition, we generated an amplified fragment length polymorphism (AFLP®) map of the *M* locus, which was delimited into a genetic interval of 2.5 cM. The genetic association and the local map will assist the molecular isolation of the *M* gene using the combination of positional cloning and candidate gene approach.

Communicated by I. L. Goldman.

S. Liu · Q. Yang
Department of Biochemistry and Molecular Biology,
College of Life Sciences, Nanjing Agricultural University,
210095 Nanjing, China

S. Liu · Z. Jia · S. Huang (✉)
Institute of Vegetables and Flowers,
Chinese Academy of Agricultural Sciences,
100081 Beijing, China
e-mail: huangsanwen@caas.net.cn

L. Xu · X. Lu · H. Chen
Lab of Biochemistry and Fermentation,
Hunan Agricultural University,
410128 Changsha, China

L. Xu · X. Lu · H. Chen (✉)
Hunan Vegetable Research Institute,
Hunan Academy of Agricultural Sciences,
410125 Changsha, China
e-mail: huiminghm@163.com

Y. Xu
Beijing Vegetable Research Centre, 100089 Beijing, China

Z. Fei
Boyce Thompson Institute and USDA Plant, Soil,
and Nutrition Laboratory, Cornell University,
14853 Ithaca, NY, USA

Introduction

Sex determination in plants is a fundamental developmental process of economic importance since the sexual phenotypes of crops determine the processes of breeding and cultivation (Tanurdzic and Banks 2004). The diverse sex types of cucumber (*Cucumis sativus* L.) allow this organism to serve as a model system for studying the genetic, physiological and molecular basis of sex expression in flowering plants. The cucumber has three types of flowers: male, female, and bisexual. Morphologically, all cucumber floral buds are initially hermaphroditic with both male and female reproductive organs. Pistil development is then arrested in floral buds that develop into male flowers, whereas stamen development is arrested in floral buds that develop into female flowers. Bisexual flowers form from the buds in which neither pistil nor stamen development is arrested (Kubicki 1969a, 1969d). The distribution of these three flower types on the plant results in seven sex types of cucumber plants (Shifriss 1961): androecious (only male flowers), gynoecious (only female flowers), monoecious

(male flowers at the base and female flowers at the top of the main stem), hermaphroditic (only bisexual flowers), andromonoecious (male and bisexual flowers), gynomonoecious (female and bisexual flowers), and trimonoecious (male, female, and bisexual flowers). Several genes that influence sex expression in cucumber have been described including *A*, *F*, *gy*, *In-F*, *M*, *M-2,* and *Tr* (Kubicki 1969a, b, c, d; Malepszy and Niemirowicz-Szczytt 1991; Perl-Treves 1999; Pierce and Wehner 1990; Shifriss 1961). In addition, sex expression is also influenced by environmental conditions and plant hormones. Long days, high temperatures, and gibberellic acid promote the formation of male flowers, whereas short days, low temperatures, auxins, and ethylene enhance the development of female flowers (Galun 1961; Kubicki 1969d; Takahashi et al. 1983; Yamasaki et al. 2003; Yin and Quinn 1995).

Ethylene is highly correlated with femaleness in cucumber (Rudich et al. 1972). Yin and Quinn (1995) proposed a model of sex determination in cucumber in which ethylene serves as both a promoter of the female sex and an inhibitor of the male sex. The model predicts that the *F* gene encodes a molecule that influences the range and gradient of ethylene production along the shoot, thereby acting to promote femaleness. In addition, the model predicts that the *M* gene encodes a molecule that detects this ethylene signal and inhibits stamen development when ethylene levels reach a threshold. Recent studies have provided molecular evidence in favor of the ethylene model of sex determination in cucumber. Trebitsh et al. (1997) found the monoecious (*MMff*) cucumber genome had only one copy of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene (*Cs-ACS1*), while in gynoecious (*MMFF*) lines an additional ACC synthase (*Cs-ACS1G*) was discovered and subsequently mapped to the *F* locus. The expression of *Cs-ACS1G* as well as *Cs-ACS1* correlates with sexual phenotype, with gynoecious plants accumulating more transcript than monoecious or andromonoecious (*mmff*) plants (Kamachi et al. 1997; Yamasaki et al. 2001). In addition, Yamasaki et al. (2001) suggested that the product of the *M* locus mediates the inhibition of stamen development by ethylene. Thus, the *F* locus likely controls ethylene concentration, and the *M* locus controls the differential sensitivity of males and females to ethylene. Both of these processes are important in regulating sexual phenotype in cucumber. Molecular cloning of the *M* and *F* genes will allow the model to be tested directly. While genetic, genomic, and transcript analyses strongly suggest that the *F* gene encodes an ACC synthase (Kamachi et al. 1997; Knopf and Trebitsh 2006; Mibus and Tatlioglu 2004; Trebitsh et al. 1997; Yamasaki et al. 2001), the identity of the *M* gene remains unknown despite markers that have been identified in linkage with the gene (Deng et al. 2006; Witkowicz et al. 2003).

The ethylene signaling pathway has been well characterized in the model plant Arabidopsis (Stepanova and Alonso 2005). A central component of this pathway is a group of plant-specific transcription factors encoded by the *ETHYLENE-INSENSITIVE3* (*EIN3*) gene family. In this paper, we report the identification of an *EIN3*-like genomic sequence in cucumber that co-segregates with the *M* locus. In addition, we delimited the *M* locus to a genetic interval of 2.5 cM with AFLP markers.

Materials and methods

Plant material

Seeds of gynoecious (*Cucumis sativus* L. var *sativus* cv WI 1983G; *MMFF*) and hermaphrodite (*Cucumis sativus* L. var *sativus* cv WI 1983H; *mmFF*) nearly isogenic cucumber lines were kindly provided by Dr J. E. Staub (Agricultural Research Service, U.S. Department of Agriculture, Horticulture Department, University of Wisconsin, Madison, USA.). WI 1983G originated from a cross between inbred WI 5821 and WI 5822 (Peterson et al. 1986). An andromonoecious near-isogenic line WI 1983A (*mmff*) was developed using a hermaphrodite line as the donor parent. Five direct backcrosses to WI 1983G were made followed by three subsequent generations of self-pollination. The hermaphrodite WI 1983H line was selected from a cross between WI 1983G and WI 1983A (J.E. Staub, personal communication). The mapping population consisted of 96 individual F_2 plants derived from a cross between the WI 1983G and WI 1983H lines. Seeds were germinated and grown in trays containing a soil mixture (peat: sand: pumice, 1:1:1, v/v/v). Plants were adequately watered and grown at day/night temperatures of 24/18°C with a 16-h photoperiod. Sex type for each individual F_2 plant was determined by recording the flower type along the first 20 main-stem nodes as well as the morphology of the fruits.

Molecular markers

DNA was isolated from fresh leaves using the modified CTAB method (Murray and Thompson 1980). The cleaved amplified polymorphic sequence marker *CsEIL1* (Table 1) was developed according to the *CmEIL1* gene sequence (accession: AB063191) in melon (*Cucumis melo* L.). For PCR analysis, 15 µl reaction mixtures contained 20 ng DNA template, 7.5 ng of each primer, 0.1 mM of each dNTP, 0.3 units *Taq* polymerase (Tiangen Biotech Co. Ltd, Beijing China), 10 mM Tris-HCl pH 9, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, and 0.01% (w/v) gelatin. PCR-amplification protocol was as following: an initial DNA denaturation for 5 min at 94°C, followed by 35 cycles

Table 1 CAPS and AFLP markers linked to *M* locus. AFLP markers are listed according to their position on the genetic map (Fig. 4)

Markers	Primer combinations	Type	Size (bp) and linkage with *M*/*m* alleles
CsEIL1	Sense: 5′-GGA AGT CTG TAA TGC TCA AGG TT-3′ Antisense: 5′-GAC CTT GGT CCT TCG GCA-3′	Codominant	380/327(*M*/*m*)[a]
PGGMCCC_450/453	*Pst*I primer: 5′-GAC TGC GTA CAT GCA GGG-3′ *Mse*I primer: 5′-GAT GAG TCC TGA GTA ACC C-3′	Codominant	450(*m*)/453(*M*)
PAGMCTC_122	*Pst*I primer: 5′-GAC TGC GTA CAT GCA GAG-3’ *Mse*I primer: 5′-GAT GAG TCC TGA GTA ACT C-3′	Dominant	122(*M*)
EACAMCAT_202/203	*Eco*RI primer: 5′-GAC TGC GTA CCA ATT CAC A-3′ *Mse*I primer: 5′-GAT GAG TCC TGA GTA ACA T-3′	Codominant	202(*M*)/203(*m*)
EATGMCAA_380	*Eco*RI primer: 5′-GAC TGC GTA CCA ATT CAT G-3′ *Mse*I primer: 5′-GAT GAG TCC TGA GTA ACA A-3′	Dominant	380(*M*)
PGTMCTA_185	*Pst*I primer: 5′-GAC TGC GTA CAT GCA GGT-3′ *Mse*I primer: 5′-GAT GAG TCC TGA GTA ACT A-3′	Dominant	185(*M*)

The primer combinations, type (dominant or codominant), size of polymorphism bands, and linkage to the *M* or *m* alleles are indicated

[a] The association between the 380 and 327-bp bands with the *M* and *m* alleles refers to the results

of 30 s DNA denaturation at 94°C, 30 s annealing at 61°C, and 45 s elongation at 72°C, then a final seven minute elongation at 72°C. The amplification reactions were performed using a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, California, USA.). The PCR products were digested with appropriate restriction enzymes and subsequently analysed by electrophoresis in agarose gels.

AFLP analysis (Vos et al. 1995) was performed on a Licor sequencer (LI-COR, Lincoln Nebraska) using fluorescently labeled *Eco*RI or *Pst*I primers. Experiments were performed following a published protocol (Myburg et al. 2001). Bands that were polymorphic (Table 1) in the parental lines were scored for all F_2 progeny.

Linkage and statistical analyses

A X^2 test was used to test the goodness-of-fit of the Mendelian segregation of the *M* gene classes. Marker orders and map distances for the F_2 population were calculated using the software JoinMap Ver. 3.0 with a LOD threshold of 3.0 and the mapping function of Kosambi (Kosambi 1944; Stam 1993).

Results

The phenotypes of flowers and fruits

The mother plant WI 1983G bears only pistillate flowers and white spined, slightly tapered fruits, whereas the mother plant WI 1983H bears bisexual flowers that typically produce nearly round fruit (Fig. 1). All F_1 progeny that were derived from the cross between WI 1983G and WI 1983H lines bore pistillate flowers and slightly tapered fruits. This finding confirmed the complete dominance of the maternal sex type over the paternal sex type. Hereafter, we designate the genotype of WI 1983G as *MMFF* (gynoecious) and of WI 1983 H as *mmFF* (hermaphroditic). In the 96 F_2 individuals, 71 had only pistillate flowers and were scored as gynoecious (*M_FF*). In addition, 25 F_2 individuals had only bisexual flowers and were, thus, scored as hermaphroditic (*mmFF*). These results fit the Mendelian 3:1 ratio ($\chi^2 = 0.056$; $p > 0.8$), indicating the single *M* locus controlled the segregation of the sex expression in the F_2 progeny. Therefore this population is ideal for genetic mapping of the *M* locus.

Development of the *CsEIL1* markers co-segregating with the *M* locus

As the *M* locus is presumed to be involved in perceiving the ethylene signal and the *EHTYLENE-INSENSTIVE3* (*EIN3*) and *EIN3-LIKE* (*EIL*) gene families are key components in the ethylene signaling pathway, we investigated whether the *M* locus and the *EIN3/EIL* genes are genetically associated in cucumber. A pair of primers was designed from the sequence of the *CmEIL1* gene (AB063191) from melon (*C. melo*) to amplify a single 380-bp fragment (Table 1). The PCR products of WI 1983G and WI 1983H were cloned and sequenced. Two amplicons from WI 1983H were found and they differ by a single nucleotide polymorphism (SNP) (Fig. 2), while only one amplicon from WI 1983G was found and it is identical to one of the amplicon from WI 1983H. This SNP, when cleaved with the restriction enzyme *Sfc*I, resulted in a cleaved amplified polymorphic sequence (CAPS) marker, *CsEIL1* (Fig. 3a). This marker

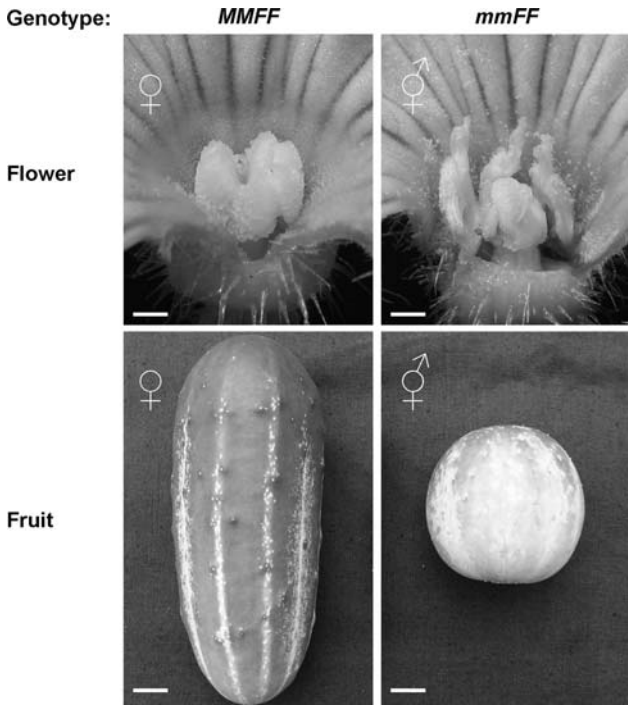

Fig. 1 The effects of the *M* gene on the sexual type and fruit shape of cucumber. Gynoecious plants (*MMFF*), bearing the dominant *M* allele, develop only female flowers and slightly tapered fruits, while hermaphroditic plants (*mmFF*), bearing the homozygous *m* recessive alleles, develop hermaphrodite flowers and round fruits. *Scale* represents 0.2 cm in flower plates and 1 cm in fruit plates

```
1   GGAAGTCTGTAATGCTCAAGGTTTTGTATACGGAATAATTCCTGAGAAGGGAAAACCAGT CsEIL1-G
1   GGAAGTCTGTAATGCTCAAGGTTTTGTATACGGAATAATTCCTGAGAAGGGAAAACCAGT CsEIL1-H1
1   GGAAGTCTGTAATGCTCAAGGTTTTGTATACGGAATAATTCCTGAGAAGGGAAAACCAGT CsEIL1-H2

61  AACCGGGGCATCGGATAATCTGCGAGAGTGGTGGAAAGACAAAGTCAGATTTGATAGAAA CsEIL1-G
61  AACCGGGGCATCGGATAATCTGCGAGAGTGGTGGAAAGACAAAGTCAGATTTGATAGAAA CsEIL1-H1
61  AACCGGGGCATCGGATAATCTGCGAGAGTGGTGGAAAGACAAAGTCAGATTTGATAGAAA CsEIL1-H2

121 CGGACCAGCTGCCATAGCCAAGTACCAGGCAGACAATGCAATTCCTGGACGAAATGATGG CsEIL1-G
121 CGGACCAGCTGCCATAGCCAAGTACCAGGCAGACAATGCAATTCCTGGACGAAATGATGG CsEIL1-H1
121 CGGACCAGCTGCCATAGCCAAGTACCAGGCAGACAATGCAATTCCTGGACGAAATGATGG CsEIL1-H2

181 CTGTAATTCAATCGGTCCAACCCCTCACACCTTGCAGGAACTTCAGGATACCACCTTAGG CsEIL1-G
181 CTGTAATTCAATCGGTCCAACCCCTCACACCTTGCAGGAACTTCAGGATACCACCTTAGG CsEIL1-H1
181 CTGTAATTCAATCGGTCCAACCCCTCACACCTTGCAGGAACTTCAGGATACCACCTTAGG CsEIL1-H2

241 TTCTCTTTTATCAGCTCTGATGCAGCACTGTGACCCTCCTCAAAGAAGATTTCCATTGGA CsEIL1-G
241 TTCTCTTTTATCAGCTCTGATGCAGCACTGTGACCCTCCTCAAAGAAGATTTCCATTGGA CsEIL1-H1
241 TTCTCTTTTATCAGCTCTGATGCAGCACTGTGACCCTCCTCAAAGAAGATTTCCATTGGA CsEIL1-H2
                                  ↓
301 GAAAGGAGTTCCTCCGCCATGGTGGCCTACTGGAGTCGAGGAATGGTGGCCTCAGCTTGG CsEIL1-G
301 GAAAGGAGTTCCTCCGCCATGGTGGCCTACTGGAGTCGAGGAATGGTGGCCTCAGCTTGG CsEIL1-H1
301 GAAAGGAGTTCCTCCGCCATGGTGGCCTACAGGAGTCGAGGAATGGTGGCCTCAGCTTGG CsEIL1-H2

361 ATTGCCGAAGGACCAAGGTC                                          CsEIL1-G
361 ATTGCCGAAGGACCAAGGTC                                          CsEIL1-H1
361 ATTGCCGAAGGACCAAGGTC                                          CsEIL1-H2
```

Fig. 2 Sequence alignment of the *CsEIL1* fragments from WI 1983G (CsEIL1-G, accession: EU814481) and WI 1983H (CsEIL1-H1, accession: EU814482 and CsEIL1-H2, accession: EU814483). CsEIL1-G is identical to CsEIL1-H1, both differ from CsEIL1-H2 by an SNP that is denoted with an arrow. The SNP creates an *Sfc*I restriction site in CsEIL1-H2

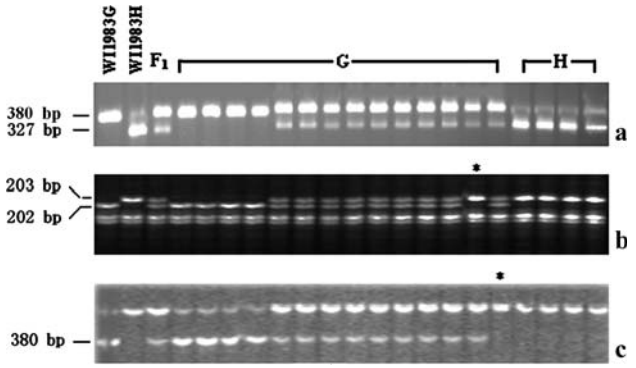

Fig. 3 Molecular markers linked to the *M* locus. **a** *CsEIL1*, **b** EACAMCAT_202/203, and **c** EATGMCAA_380. Analyzed plants included the parental WI 1983G and WI 1983H lines, F_1, and 18 F_2 progenies (14 gynoecious and 4 hermaphrodite). The sizes of the diagnostic bands are indicated on the left. *Asterisks* (*) show recombinant events between the *M* locus and the markers

can be co-dominantly scored. After digestion with *Sfc*I, the cleaved 327-bp fragment was present in WI 1983H and F_1 individuals but absent in WI 1983G individuals. The intensity of the 327-bp fragment in WI 1983H, however, was more than two times greater than that in F_1 individuals.

Due to the near isogenic nature of WI 1983G and WI 1983H, the presence of the SNP suggested the association of the *CsEIL1* marker and the *M* locus. To confirm this association, we analyzed the 96 F_2 progeny with *CsEIL1* and discovered that the marker and the *M* locus co-segregated.

Construction of an AFLP map of the *M* locus

To develop more markers for fine mapping, we generated a local map for the *M* locus using high-throughput AFLP technology. We screened 256 primer combinations (128 *Eco*RI/*Mse*I plus 128 *Pst*I/*Mse*I, three selective bases for *Eco*RI and *Mse*I primers and two selective bases for *Pst*I primers) for polymorphisms using WI 1983G, WI 1983H, and the F_1 plants. These primer combinations resulted in approximately 15,000 bands with an average of about 58 bands per primer combination. Only ten bands (<0.1% of the total number of bands) from eight primer combinations revealed polymorphisms between the parental lines in confirmation of their near isogenic nature. Further analysis was conducted with the eight primer combinations on an F_2 population subset that consisted of 30 gynoecious and 10 hermaphroditic plants. Three of the ten bands, however, were not linked to the locus. This result indicated that additional genomic fragments from the donor parent remained in the genome of WI 1983H. The remaining seven bands that were derived from five primer combinations were significantly associated with the sex phenotype in the subset. Thus, three dominant and two codominant AFLP markers were linked to the *M* locus (Table 1).

Subsequently, five AFLP markers were scored on the remaining 56 plants of the F_2 population to generate a linkage map for the *M* locus (Fig. 4). The local map spanned a

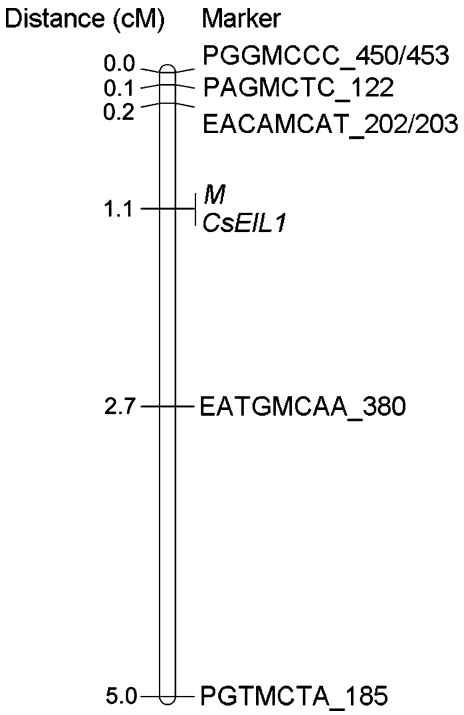

Fig. 4 Genetic map of the *M* locus. Genetic distances in cM on the left were estimated based on the linkage analysis of 96 F_2 plants derived from the cross between WI 1983G and WI 1983H

genetic interval of 5.0 centimorgans (cM), which was defined by the AFLP markers PGGMCCC_450/453 and PAGMCTC_122 on one side and PGTMCTA_185 on the other side. The *M* locus co-segregated with the *CsEIL1* marker and was flanked by the AFLP markers EACAMCAT_202/203 and EATGMCAA_380 that defined a 2.5-cM interval (Figs. 3b, c and 4). The linkage map provides a solid basis for high-resolution mapping and ultimately for molecular isolation of the *M* gene.

Discussion

In this report, we describe the finding that the sex-determining *M* locus in cucumber genetically associates with an *EIN3/EIL* genomic sequence and construct an AFLP map of the locus. The association provides another line of evidence to support the ethylene model of sex determination, which predicts that the *M* locus mediates the ethylene signal and inhibits stamen development in cucumber. Together with the local map, this finding will assist the molecular isolation of the *M* gene via a combined strategy of positional cloning and the candidate gene approach.

Information gained in model species will be an indispensable tool for deciphering the genetic and molecular basis of agronomical traits in 'orphan' crops such as cucurbits. Ethylene is a plant hormone involved in the regulation of various stress responses and developmental adaptations. Ethylene biosynthetic and signaling pathways have been well characterized, particularly in the model species Arabidopsis (Guo and Ecker 2004; Kende 1993). The direct relationship between sex expression and ethylene has long been established in cucurbits. Therefore, application of comparative genetics to uncover the mechanism of sex expression in cucumber and other cucurbits is valid using the large body of knowledge on ethylene biosynthesis and signaling available for Arabidopsis and other model species. Using these methods, the *Female* (*F*) locus of cucumber was found to correspond to an ACC synthase, a key enzyme in the ethylene biosynthetic pathway (Trebitsh et al. 1997). Here, our results suggest that the *M* locus, another sex-determining locus of cucumber, may correspond to the *EIN3*-like transcription factors. Clearly, comparative genomics are a powerful tool for unveiling the sex-determining pathways.

Two additional lines of evidence support our working hypothesis that the *M* locus of cucumber may correspond to the *EIN3/EIL* gene. Firstly, Arabidopsis *EIN3* mutants are insensitive to ethylene-mediated inhibition of hypocotyl and root elongation (Chao et al. 1997). In cucumber, the inhibitory effect of ethylene on hypocotyls elongation in andromonoecious (*mmff*) plants is less than that in monoecious (*MMff*) and gynoecious (*MMFF*) plants (Yamasaki et al. 2001), suggesting that the *m* allele confers reduced response to ethylene response. Although mutants of other genes in the signaling pathway may also lack an inhibitory effect, we detected no polymorphisms between the near-isogenic WI 1983G and WI 1983H lines in the surveyed region of other ethylene signaling genes as *ETR1*, *ETR2*, *CTR1*, *ERF*, and *ERS* (data not shown); however, we cannot exclude the possibility that polymorphisms occur beyond the regions defined by the PCR primers used. Secondly, the *EIN3*- or *EIL1*-overexpressing transgenic lines of Arabidopsis exhibited greatly reduced fertility, and the gynoecium protruded from a flower while the pollen was still immature (Chao et al. 1997). Thus, we offer a preliminary hypothesis that the stimulated gynoecium development by *EIN3* inhibits stamen development. Further experiments are critical to address this hypothesis.

However, the reduced response to ethylene response of the *m* allele is unlikely related to the lower transcriptional level of *EIN3/EIL* in cucumber. Recently we performed deep EST sequencing on flower buds of these two cultivars using the 454 pyro-sequencing technology (Margulies et al. 2005) and obtained ~195,000 and ~171,000 ESTs for the near-isogenic WI 1983G and WI 1983H, respectively (Fei et al. unpublished data). Digital expression information derived from these ESTs indicates that there is no significant difference of *EIN3/EIL* expression in the flower buds of the two cultivars as the same number of ESTs (16) were

from *EIN3/EIL* in both lines. Similarly, tomato *EIN3/EIL* gene expression is not regulated by exogenous ethylene (Tieman et al. 2001).

At least two copies of *EIN3/EIL* occur in the genome of the hermaphrodite cucumber line (Fig. 2). From the 2x genome shotgun sequences of a monoecious cucumber inbred line(*MMff*) (Huang et al. unpublished data), we discovered three *EIN3/EIL* fragments with about 90% homology to the *CmEIL1* gene (data not shown). Therefore, the cucumber genome may also contain a family of *EIN3*-like genes similar to Arabidopsis (Chao et al. 1997) and other plants (Iordachescu and Verlinden 2005; Rieu et al. 2003; Tieman et al. 2001). These *EIN3*-like genes may fine tune the ethylene response to environmental and developmental adaptation. Isolation of full-length *EIN3/EIL* genes in *MM* lines and functional complementation in *mm* lines are required to conclude whether these genes indeed involve in sex determination of cucumber.

The current map (Fig. 4) provides a basis for high-resolution mapping of the *M* locus. In future experiments, we will screen a large population using the flanking markers and identify rare recombination events that can further narrow down the genetic interval of the *M* locus. We will also screen and sequence cucumber genomic clones carrying *EIN3*-like genes. These efforts will result in the molecular cloning of the *M* locus and a model of its origin as well.

Acknowledgments The experiment was carried out in the Sino-Dutch Joint Lab of Horticultural Genomics Technology and the Opening Lab of Vegetable Genetics and Physiology of Ministry of Agriculture, both located in the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences. We thank Dr. J. E. Staub (Agricultural Research Service, U. S. Department of Agriculture, Horticulture Department, University of Wisconsin, Madison) for providing the cucumber materials and Dr. Zhonghua Zhang of our lab for bioinformatics analysis. This work was supported by grants from Ministry of Agriculture ("948" Program: 2008-Z42) and Ministry of Science and Technology (2006DFA32140) to S. H.

References

Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR (1997) Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein *ETHYLENE-INSENSITIVE3* and related proteins. Cell 89:1133–1144

Deng S-l, Pan J-s, He H-l, Wu A-z, R Cai (2006) SRAP molecular marker linked to *M* Gene in cucumber. J Shanghai Jiaotong Univer (Agric Sci) 24:240–244

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

Guo H, Ecker JR (2004) The ethylene signaling pathway: new insights. Curr Opin Plant Biol 7:40–49

Iordachescu M, Verlinden S (2005) Transcriptional regulation of three *EIN3*-like genes of carnation (Dianthus caryophyllus L. cv. Improved White Sim) during flower development and upon wounding, pollination, and ethylene exposure. J Exp Bot 56:2011–2018

Kamachi S, Sekimoto H, Kondo N, Sakai S (1997) Cloning of a cDNA for a 1-aminocyclopropane-1-carbo-xylate synthase that is expressed during development of female flowers at the apices of *Cucumis sativus* L. Plant Cell Physiol 38:1197–1206

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

Knopf RR, Trebitsh T (2006) The female-specific *Cs-ACS1G* gene of cucumber. A case of gene duplication and recombination between the non-sex-specific 1-aminocyclopropane-1-carboxylate synthase gene and a branched-chain amino acid transaminase gene. Plant Cell Physiol 47:1217–1228

Kosambi DD (1944) The estimation of map distance from recombination values. Ann Eugen 12:172–175

Kubicki B (1969a) Investigation of sex determination in cucumber (*Cucumis sativus* L.). Genet Pol 10:101–121

Kubicki B (1969b) Investigation of sex determination in cucumber (*Cucumis sativus* L.). Genet Pol 10:5–22

Kubicki B (1969c) Investigation of sex determination in cucumber (*Cucumis sativus* L.). Genet Pol 10:123–143

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

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

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer ML, Jarvie TP, Jirage KB, Kim JB, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu P, Begley RF, Rothberg JM (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437:376–380

Mibus H, Tatlioglu T (2004) Molecular characterization and isolation of the *F/f* gene for femaleness in cucumber (*Cucumis sativus* L.). Theor Appl Genet 109:1669–1676

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

Myburg AA, Remington DL, O' Malley DM, Sederoff RR, Whetten RW (2001) High-throughput AFLP analysis using infrared dye-labeled primers and an automated DNA sequencer. BioTechniques 30:348–357

Perl-Treves P (1999) Male to female conversion along the cucumber shoot: approaches to studying sex genes and floral development in *Cucumis sativus*. In: Ainsworth CC (ed) Sex determination in plants. BIOS Scientific Publisher, Oxford

Peterson CE, Staub JE, Williams PH, Palmer MJ (1986) Wisconsin 1983 cucumber. HortScience 21:1082–1083

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

Rieu I, Mariani C, Weterings K (2003) Expression analysis of five tobacco *EIN3* family members in relation to tissue-specific ethylene responses. J Exp Bot 54:2239–2244

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

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

Stam P (1993) Construction of integrated genetic linkage maps by means of a new computer package: Join Map. Plant J 3:739–744

Stepanova AN, Alonso JM (2005) Arabidopsis ethylene signaling pathway. Sci STKE 2005:cm4

Takahashi H, Saito T, Suge H (1983) Separation of effects of photoperiod and hormones on sex expression in cucumber. Plant Cell Physiol 24:142–154

Tanurdzic M, Banks JA (2004) Sex-determining mechanisms in land plants. Plant Cell 16(Suppl):S61–S71

Tieman DM, Ciardi JA, Taylor MG, Klee HJ (2001) Members of the tomato *LeEIL* (*EIN3*-like) gene family are functionally redundant and regulate ethylene responses throughout plant development. Plant J 26:47–58

Trebitsh T, Staub JE, O'Neill SD (1997) Identification of a 1-aminocyclopropane-1-carboxylic acid synthase gene linked to the female (*F*) locus that enhances female sex expression in cucumber. Plant Physiol 113:987–995

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M et al (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Witkowicz J, Urbanczyk-Wochniak E, Przybecki Z (2003) AFLP marker polymorphism in cucumber (*Cucumis sativus* L.) near isogenic lines differing in sex expression. Cell Mol Biol Lett 8:375–381

Yamasaki S, Fujii N, Matsuura S, Mizusawa H, Takahashi H (2001) The *M* locus and ethylene-controlled sex determination in andromonoecious cucumber plants. Plant Cell Physiol 42:608–619

Yamasaki S, Fujii N, Takahashi H (2003) Photoperiodic regulation of *CS-ACS2*, *CS-ACS4* and *CS-ERS* gene expression contributes to the femaleness of cucumber flowers through diurnal ethylene production under short-day conditions. Plant Cell Environ 26:537–546

Yin T, Quinn JA (1995) Tests of a mechanistic model of one hormone regulating both sexes in *Cucumis sativus* (Cucurbitaceae). Am J Bot 82:1537–1546