Date of Submission of the report: 15 January 2005

BARD Project Number: IS 3139-99

Project Title:

Ethylene mediated regulation of sex expression in *Cucumis*

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Cucumber – melon – transgenic – sex determination – ethylene receptor

Abbreviations commonly used in the report, in alphabetical order:

Budget: IS: \$

165,000

US: \$

165,000

Total: \$

330,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)		3	1	4
Submitted, in review, in preparation		3	1	4
Invited review papers				
Book chapters		1	1	2
Books				
Master theses				
Ph.D. theses		1	1	2
Abstracts		5	2	7
Not refereed (Proceedings Chapters)		7	1	8

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	2	1	3	6
Longer Visits (Sabbaticals)				

Description of Cooperation:

Grumet and Staub visited Israel to attend the Eucarpia meeting on 2000 and exchange results and ideas with the Israeli investigators. In 2002, Katzir, Staub and Grumet met at the Cucurbitaceae conference in Florida, whereas in 2004, Staub, Katzir and Perl-Treves similarly met at the Eucarpia Meeting in the Czech Republic. In between, investigators discussed their results and planned their collaboration by the electronic mail; the two Israeli and the two US investigators interacted on regular basis.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Abstract

Monoecious species such as melon and cucumber develop separate male and female (or bisexual) flowers on the same plant individual. They display complex genetic and hormonal regulation of sex patterns along the plant. Ethylene is known to play an important role in promoting femaleness and inhibiting male development, but many questions regarding critical sites of ethylene production versus perception, the relationship between ethylene and the sex determining loci, and the possible differences between melon and cucumber in this respect are still open.

The general goal of the project was to elucidate the role of ethylene in determining flower sex in *Cucumis* species, melon and cucumber. The specific Objectives were: **1.** Clone and characterize expression patterns of cucumber genes involved in ethylene biosynthesis and perception. **2.** Genetic mapping of cloned genes and markers with respect to sex loci in melon and cucumber. **3.** Produce and analyze transgenic melons altered in ethylene production or perception. In the course of the project, some modifications/adjustments were made: under Objective 2 (genetic mapping) a set of new mapping populations had to be developed, to allow better detection of polymorphism. Under Objective 3, cucumber transformation systems became available to us and we included this second model species in our plan.

The main findings of our study support the pivotal role of ethylene in cucumber and melon sex determination and later stages of reproductive development. Modifying ethylene production resulted in profound alteration of sex patterns in melon: femaleness increased, and also flower maturation and fruit set were enhanced, resulting in earlier, more concentrated fruit yield in the field. Such effect was previously unknown and could have agronomic value. Our results also demonstrate the great importance of ethylene sensitivity in sex expression. Ethylene perception genes are expressed in sex-related patterns, e.g., gynoeceious lines express higher levels of receptor-transcripts, and copper treatments that activate the receptor can increase femaleness. Transgenic cucumbers with increased expression of an ethylene receptor showed enhanced femaleness. Melons that expressed a defective receptor produced fewer hermaphrodite flowers and were insensitive to exogenous ethylene. When the expression of defective receptor was restricted to specific floral whorls, we saw that pistils were not inhibited by the blocked perception at the fourth whorl. Such unexpected findings suggest an indirect effect of ethylene on the affected whorl; it also points at interesting differences between melon and cucumber regarding the mode of action of ethylene. Such effects will require further study.

Finally, our project also generated and tested a set of novel genetic tools for finer identification of sex determining genes in the two species and for efficient breeding for these characters. Populations that will allow easier linkage analysis of candidate genes with each sex locus were developed. Moreover, effects of modifier genes on the major femaleness trait were resolved. QTL analysis of femaleness and related developmental traits was conducted, and a comprehensive set of Near Isogenic Lines that differ in specific QTLs were prepared and made available for the private and public research. Marker assisted selection (MAS) of femaleness and fruit yield components was directly compared with phenotypic selection in field trials, and the relative efficiency of MAS was demonstrated. Such level of genetic resolution and such advanced tools were not used before to study these traits, that act as primary yield components to determine economic yields of cucurbits. In addition, this project resulted in the establishment of workable transformation procedures in our laboratories and these can be further utilized to study the function of sex-related genes in detail.

Ethylene mediated regulation of sex expression in *Cucumis*

A. Achievements and their significance

(1) Cloning of cucumber genes involved in the biosynthesis and response to ethylene and characterization of their expression patterns. A collection of genomic and cDNA clones encoding cucumber and melon proteins was assembled. Northern and semi-quantitative RT-PCR analyses were used to relate gene expression patterns to sex expression in a few cucumber genotypes. For example, we observed ethylene, but not gibberellin or brassinolide induction, of *ERS* and *ETR1,2*, *ACS2*, *ACO1,3* transcripts, as well as higher *ERS1*, *ETR2* levels in gynoecious, compared to androecious /monoecious apices and buds. The autocatalytic effect of ethylene on both its synthesis and perception, and the different expression patterns among genotypes, stress the importance of both ethylene perception and synthesis in sex determination, and provide a deeper insight on the system. The cloned sequences are being tested as candidate for the identification of sex loci by linkage analysis.

(2) Map ethylene-related genes with respect to cucumber and melon sex loci. In the Staub laboratory, markers and genetic stocks were developed, to examine genetic interactions associated with sex expression in cucumber and melon. SSR markers were developed and used to map cucumber QTL (quantitative trait loci) associated with sex expression in an advanced RIL (recombinant inbred line) population, derived from gynoecious (GY-7) and monoecious (H-19) parents. To characterize epistatic genes that modify action of the major *F* gene, 54 BC₂S₄ nearly-isogenic lines were constructed with differing arrays of alleles at sex linked modifying genes. These tools allowed the assessment of variable reproductive traits and their interaction with sex loci in a detailed, quantitative manner that was previously unavailable. The loci targeted by this approach are of primary economic importance. In cucumber selection for yield has reached a plateau, and factors such as fruit-set inhibition, source-sink relationships, a narrow genetic base, and negative correlations among yield-related traits must be overcome to increase yields. Our study set the stage to explore such correlations, e.g., among multiple lateral branches, gynoecious sex expression, earliness, and fruit length-to-diameter ratio, which are four important yield-related traits. A controlled comparison of two selection strategies, marker assisted and phenotype-based, was conducted (a random-mating scheme was added as a control) on four populations, and the value of marker-aided selection was demonstrated for part of the traits. At Bar-Ilan, populations that segregate for sex determining loci of melon and cucumber were developed. Compared with

those available at the onset of this project, the new set exhibits higher polymorphism; in melon, a population segregating for gynoecy was unavailable to us. Mapping of ethylene related genes and other markers using these populations is under way.

(3) Produce and study transgenic melons/ cucumbers engineered for altered ethylene production or perception. Transgenic melons were engineered to increase ethylene production (over-expression of the ethylene biosynthetic gene, ACS), or inhibit ethylene perception (expression of the *Arabidopsis* dominant mutant, *etr1-1*). *35S:etr1-1* melons exhibited phenotypes associated with ethylene insensitivity. The majority of these plants failed to produce pistillate buds, demonstrating the critical role of ethylene perception in pistillate bud formation. Constitutively-expressing ACS plants had increased ACC levels and ethylene production, and enhanced femaleness, reflected by earlier and increased pistillate flower production, demonstrating importance of endogenous ethylene production for femaleness. Enhanced femaleness was observed also in transgenic cucumbers engineered to over-express the ERS1 receptor, showing that femaleness can be modulated also by varying ethylene sensitivity, and that receptor levels could be physiologically limiting this developmental program. ACS melons had increased, earlier occurrence of hermaphrodite buds that matured to anthesis, both in the greenhouse and field, and earlier, more concentrated fruit set. Such results provide a basis for engineering sex expression and affecting yield components in cucurbit crops. They also provide experimental tools for further research on floral differentiation and sex determination. In a more advanced set of constructs, expression was driven by organ-specific promoters; these will be used to reveal the critical sites for ethylene production or perception.

B. Agricultural and/or economic impacts of the research findings

The study provides: 1. Linkage data that, once completed, will aid cucurbit breeders to diagnose sex loci and yield-associated QTLs that relate to plant sex. 2. Start-points for positional cloning of sex loci. 3. It indicates and tests possible avenues to modify sex patterns in cucurbits by genetic engineering. For example, the impressive results obtained with the *35S:ACS* construct show that this is feasible, and breeding lines could be developed following our findings. 4. The study developed genetic stocks that, for the first time, have become available to public and private researchers. The use of these stocks in plant improvement programs will increase gynoecy in populations in preparation for the release of commercial cultivars. 5. Cucumber is an important vegetable crop whose yield has reached a

plateau in the last 15 years. Our QTL approach to map yield components (of which femaleness which is a major one) and identify physiologically important correlations among them, can help to overcome this limitation.

C. Details of cooperation

Each of the four teams pursued a distinct set of activities that, brought together, generated a more complete and deep picture of the system. The results of our groups were highly complementary, and exchanging them interactively between us stimulated and enhanced our research. The US and Israeli teams met several times during the research period at cucurbit genetics meetings, and had ample opportunity to discuss results and coordinate their research.

D. List of Publications that resulted from this Grant

Research Articles

Fazio, G, J. E. Staub, and S. M. Chung. 2002. Development and characterization of PCR markers in cucumber (*Cucumis sativus* L.). J. Am. Soc. Hort. Sci. 127:545-557.

Fazio, G, S.M. Chung and J. E. Staub. 2003b. Comparative analysis of response to phenotypic and marker-assisted selection for multiple lateral branching in cucumber (*Cucumis sativus* L.). Theor. Appl. Genet. 107: 875 - 883.

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Papadopoulou E, Little HA, Hammar SA, Grumet R. 2004. Effect of modified endogenous ethylene production on hermaphrodite flower development and fruit production in melon (*Cucumis melo* L.). Plant Biotechnology Journal. Submitted.

Rajagopalan P, Perl-Treves R. 2004. Improved protocol for Cucumber (*Cucumis sativus* L.) transformation by modified explant dissection and selection. HortScience: in press

Staub, J.E., L. K. Crubaugh, and G. Fazio. 2002. Cucumber recombinant inbred lines. Cucurbit Genetics Cooperative Rpt. 25:1-2.

Articles in preparation

Little HA, Papadopoulou E, Hammar SA, Grumet R. Effect of inhibited ethylene perception on flower sex organ development in melon.

Papadopoulou E, Grumet R. Brassinosteroid induced femaleness in cucumber and relationship to ethylene production.

Rajagopalan P, Saraf-Levy T, Lizhe A, Wolff D, Zelcer A, Perl-Treves R. Expression of the ERS1 gene determines the female phase in transgenic cucumber plants.

Book Chapters

Papadopoulou E, Grumet R. 2002. Production of transgenic melon. In: Khachatourins GG, McHughen A, Scorza R, Nip Y-K, Hui YH (eds). Transgenic Plants and Crops. Marcel Dekker Inc. New York, NY. p. 371-381.

Perl-Treves R. Close, yet separate: patterns of male and female floral development in monoecious species. In: Flowering and its Manipulation. Editor: C.C. Ainsworth. Blackwell, Oxford. Accepted.

Proceedings Chapters

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Staub, J. E., J. E. Zalapa, M. K. Paris and J. D. McCreight. 2004. Selection for lateral branch number in melon (*Cucumis melo* L.). Proceedings the 8th Eucarpia Conference, Cucurbitaceae 2004: Progress in cucurbit genetics and breeding research. Olomouc, The Czech Republic. July 12-17, p.381-388.

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Staub, J.E., M.D. Robbins, and A. I. López-Sesé. 2002. Molecular methodologies for improved genetic diversity assessment in cucumber and melon. Proceedings XXVI IRC,. Horticulture: Art and science for life- Advances in vegetable Breeding. Toronto, Canada. August 11-17, 2002. Acta Horticulturae 642:41-47.

Robbins, M. D., J. E. Staub and G. Fazio, 2002. Deployment of molecular markers for multi-trait selection in cucumber. Proceeding Cucurbitaceae 2002, December 8-12, Naples, Florida. Amer. Soc. Hort. Press, p. 41-47.

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Ph.D. dissertations

Papadopoulou E. 2002. Sex expression in cucurbits: the role of ethylene synthesis and perception and sex determination genes. Ph.D. Dissertation. Michigan State University.

Saraf-Levy T. Ethylene related genes and sex determination in cucumber. Doctoral dissertation. Bar-Ilan University, 2003.

Conference Abstracts

Saraf-Levy T, Kahana A, Kessler N, Silberstein L, Wang Y, Gal-On A, Perl-Treves R (2000) Genes specifying ethylene synthesis and perception in cucumber sex genotypes. The 6th International Congress of Plant Molecular Biology, Quebec, Canada, June 18-24, 2000.

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E. RESEARCH FINDINGS ("Appendix")

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1. Cloning and characterization of ethylene related genes

1.1. Cloning of F-locus ACC synthase genomic sequence – Michigan State University

An earlier study (Trebitsch et al., 1997) suggested that the *F*-locus conferring gynoecious sex expression in cucumber may represent an extra copy of the ACC synthase (1-amino-cyclopropane carboxylic acid synthase) gene coding for a key enzyme in ethylene biosynthesis. The female-specific copy was designated *Cs-ACS1G*, whereas *Cs-ACS1* is present in both monoecious and gynoecious plants. We were interested in obtaining genomic copies of these genes to examine upstream regulatory regions, and eventually directly test the hypothesis that the *F* locus is an ACS gene. We performed inverse PCR of *MspI*-digested genomic cucumber DNA from both monoecious (Straight 8) and gynoecious (GY14) genotypes. Subsequent cloning, sequencing, and BLAST analysis revealed perfect homology to *Cs-ACS3* cDNA sequence (Shiomi et al. 1998; Trebitsch et al. 1997). We evaluated ca. 2 kb of the 5' regulatory region of *Cs-ACS1/Cs-ACS3* and looked for putative auxin and ethylene response elements (details in our First Annual Report). Auxin, but not ethylene response elements, were recognized. Partial cloning of the ACS1-G copy was recently reported by Mibus and Tatlioglu (2004).

1.2. Sets of *Cucumis* clones to study ethylene synthesis and perception – Bar-Ilan University

We have prepared a set of clones for this study using different sources: PCR amplification and cloning into plasmids, based on published cucumber sequences; other probes were cloned on our lab by applying heterologous probes to cucumber cDNA libraries, or using degenerate primer-PCR based on heterologous sequences. A few were provided by colleagues. Table 1 lists all the ethylene related genes available at the conclusion of the project. Other markers are not related to ethylene, but were included because of known linkage to *Cucumis* sex loci, or sex-related expression.

Gene	Type	Species	Source	Reference
ACC oxidase1,2,3	cDNA	cucumber	Perl-Treves	Kahana et al. 1999
ACC synthase1	genomic	cucumber	Perl-Treves	Trebitsh et al. 1997
ACC synthase1,2#	genomic	cucumber	Perl-Treves	Kamachi et al. 1997
ACC synthase 4,5	genomic	cucumber	Tatlioglu, Hannover	Unpublished
ERS	cDNA	cucumber	Perl-Treves	Saraf-Levy et al. 2000
ETR1	genomic	cucumber	Perl-Treves	Yamasaki et al. 2000
ETR2	genomic	cucumber	Perl-Treves	Yamasaki et al. 2000
CTR1	genomic?	melon	Lelievre, Toulouse	Sato-Nara et al. 1999
CTR-N	genomic	cucumber	Tatlioglu, Hannover	Unpublished
EIN3	genomic?	melon	Lelievre, Toulouse	Sato-Nara et al. 1999
RAN1	genomic	cucumber	Tatlioglu, Hannover	Unpublished
CS-dh27, A-linked	cDNA	cucumber	Perl-Treves	AF104397
Copalyl diphosphate synthase 1	genomic	cucumber	Perl-Treves	Unpublished
ent-kaurene acid oxidase	genomic	cucumber	Perl-Treves	Unpublished
GAI - gibberellin insensitive	genomic	cucumber	Perl-Treves	Unpublished
GRG- unknown GA responsive clone	genomic	cucumber	Perl-Treves	Chono et al. 1996
BRI (brassinosteroid insensitive)	genomic	cucumber	Perl-Treves	Unpublished
OP-1 ovary-expressed	genomic	cucumber	Perl-Treves	AF245222
CFL -leafy like	genomic	cucumber	Perl-Treves	AF059320
CAG1,2,3 - <i>agamous</i> like	cDNA	cucumber	Perl-Treves	Perl-Treves et al.1998
CSD – <i>tasselseed</i> homolog	genomic	cucumber	Perl-Treves	AF188188

Table 1. Cucumber and melon clones, related to hormone metabolism/response or to flower development. # ACC synthases 1,2 of Yamasaki et al. (1997) differ from ACS-1 of Trebitsch et al. (1997), which is identical to ACS-3 of Yamasaki et al. (1997).

1.3. Expression analysis using RT-PCR and Northern Analysis – Bar-Ilan University

In this Objective, we tried related the expression of ethylene related genes with the developmental patterns of sex expression in cucumber.

A preliminary microarray experiment described in the Second Year Report failed to yield consistent results between biological replicates (except one ethylene-regulated ACC oxidase gene) even for genes whose expression patterns we already knew from Northern analyses. We therefore individually studied a few selected genes using RT-PCR or Northern analysis.

A pair of cucumber-specific primers was constructed for *ACO2*, *ACS1*, *ERS1*, *ETR1* and *ETR2* as well as an actin control. The RT step and the PCR step were calibrated for each of the probes, in terms of the annealing temperatures at both steps, and the amount of total RNA used as template. We compared transcript levels of floral buds of different developmental

stages from three genotypes - hermaphrodite (bisexual buds), gynoecious (female buds) and androecious (male buds). *ERS1* and *ETR1* transcript levels were higher in female buds than in male and perfect buds, mainly in the medium-larger size buds. *ETR2* levels did not differ among bud types. In a second set of experiments, we checked the expression in whole shoot apices following ethrel and gibberellin treatments. *ERS1* levels rose in response to ethrel in the gynoecious, hermaphrodite and – to a lesser extent - in the androecious line (Fig. 1). This sex-differential response may indicate a role for ethylene sensitivity in determining the sex of the young shoot. *ETR1* showed a similar but less pronounced effect in all three genotypes, while results with *ETR2* were inconclusive. In general, it appears that in our system, a primary ethylene signal may increase sensitivity to ethylene, causing an "autocatalytic effect". Gibberellin treatment had no effect on these three ethylene response genes. In the third year, a series of Northern analyses was completed, in which the response of whole apices from an androecious and a gynoecious line to ethylene and gibberellin was examined; a response of a monoecious line to brassinolide was tested too (Figure 2). GA and BR did not affect transcript levels, showing that these hormones could act independent of ethylene action – judging from these selected genes. It is possible however that other ethylene synthesis /perception genes are affected by gibberellin.

	x3	x1	:3								
<i>ERS</i>											
treatment	<hr/>			GA	C	E	GA	C	E	GA	C
Genotype	G			A			H			C	
	x3	x1	:3								
<i>ETR1</i>											
treatment	<hr/>			GA	C	E	GA	C	E	GA	C
Genotype	G			A			H			C	
Total RNA											
treatment	<hr/>	GA	C	<hr/>	G	A	C	<hr/>	GA	C	
genotype	G			A			H			C	

Figure 1. Semi-quantitative RT-PCR analysis ERS and ETR1 transcripts in shoot apices of a gynoeious (G) an androeious (A) and a hermaphrodite line (H). E, ethrel treatment, 16 hrs, GA – gibberellin GA3 treatment, C-control. Lower panel shows ethidium bromide stain to demonstrate equal loading; in each RT-PCR series, one sample was reacted in a dilution series to show semi-linear correlation of RT-PCR product with the amount of RNA template in the samples.

Figure 2. Ethylene-synthesis genes ACO2,3 were induced in ethrel-treated apices after 16 hrs in the gynoeocious and androeocious lines. Ethylene receptor genes ETR1,2 and ERS have higher basal levels in the gynoeocious line and are further induced by ethrel in both genotypes. No induction by gibberellin, or by brassinolide applied to a monoecious line, was observed in this experiment.

Preliminary results of Northern analysis with other genes (see Table 1 for gene list; results should be repeated): CTR1 – higher in gynoeocious line, probably ethrel inducible; BRI – higher in gyno- and monoecious, response to gibberellin; DH27 – decreased by gibberellin, increased by brassinolide; CSD – induced by ethrel and brassinolide (Saraf-Levy 2003).

1.4. Do copper treatments affect sex expression? - Bar-Ilan University

The possible role of ethylene receptors in female sex expression was studied also at the physiological level, by performing copper and copper-chelator treatments, on cucumber young plant apices. Copper was shown to be the active co-factor in ethylene receptor proteins. After two weekly treatments, sex expression was monitored along the shoot. We observed no effect of the chelator, 15 mM bathocuproinedisulfonic acid, on sex expression of three genotypes, gynoeocious monoecious and hermaphrodite. Copper treatment (15 mM CuSO₄) only affected the monoecious line (Table 2), causing an increase in the number of

female flowers and earlier onset of the mixed-female phase. Similar results were reported by Engelke et al. (2000).

	Copper treatment	Control	F	p
Total nodes	17.7 ±0.1	20±0.06	14.08	0.0005
First female node	7.5±0.13	10.4±0.12	10.45	0.002
% female nodes	17.7±0.2	14.6±0.2	5.09	0.03
%female nodes in first 18 nodes	17.0±0.2	13.0±0.1	8.4	0.005

Table 2. Effect of copper treatment on sex expression along monoecious cucumber shoots. F values larger than the F_{α} values are significantly different from the control at a 0.95 threshold-probability.

2. Linkage analysis of sex loci

2.1. Mapping sex loci with random markers – Bar-Ilan and Neue Yaar

Using bulked segregant analysis on an initial set of populations, we looked for sex-linked RAPD and AFLP. This activity was described in the first annual report and did not yield tightly linked markers to sex genes; several distantly linked markers to locus *M* in cucumber and *A* in melon were recovered. The screen was resumed in the last year using the new populations that we developed.

2.2. Developing a new set of mapping populations - Bar-Ilan University

At the onset of this project we used the populations in Table 3A, each scored for a different sex- determining gene, to perform linkage analysis.

	Population	Segregating gene	Crossing	Genotypes crossed
Cucumber	F2-1022	<i>A</i>	(W1 x W3)- self	(<i>MMffAA</i> x <i>MMffaa</i>)-self
	BC-1035	<i>M</i>	(W2 x W4)xW4	(<i>MMFFAA</i> x <i>mmFFAA</i>) x <i>mmFFAA</i>
	F2-1053	<i>F</i>	(W2 x W1)- self	(<i>MMFFAA</i> x <i>MMffAA</i>)-self
Melon	F2-BIU	<i>A</i>	(PI414723 x Topmark)-self	(<i>AAMMGG</i> x <i>aaMMGG</i>)- self
	RIL-BIU	<i>A</i>	(PI414723 x Topmark)-RIL	(<i>AAMMGG</i> x <i>aaMMGG</i>)-repeated self
	RIL2 INRA	<i>A</i>	(PI414723 x Védraçais)-RIL	(<i>AAMMGG</i> x <i>aaMMGG</i>)-repeated self

Table 3A. Cucumber populations segregating for sex determining genes. The first four populations were produced, scored and sampled at BIU. DNA samples and hybridization filters of RIL2 INRA were kindly provided by Drs. Dogimont and Pitrat, INRA, Avignon.

In view of the difficulty to detect RFLP or SNP in our present populations, we followed, during years 2-4 of the project, a crossing scheme based on a wide cross with *C.sativus* var. *hardwickii*. An accession from Oman, displaying a monoecious phenotype, with a very

delayed female phase, was used as the "exotic" parent; its presumed genotype is *MMffAA*. In the past year we completed the preparation of the four populations (Table 3B), that should allow an easier detection of polymorphism and more efficient mapping of sex determining loci. The populations were grown in the greenhouse during summer 2003 and scored phenotypically. Leaves and shoot tips sampled and stored, and DNA prepared for marker analysis. In *Cucumis melo*, a mapping population that segregates for gynoecy was prepared by crossing AGY, a gynoecious line kindly donated by Mr. J. Fuchsenbaum, Hazera Genetics. The inheritance of gynoecy in melon is different from cucumber (Kenigsbuch and Cohen 1989) and the complete set of populations will allow us to map sex-linked markers on both species and assess the evolutionary relatedness of sex determination between them. The BC-AGY population was grown in summer 2004, scored phenotypically and sampled for DNA preparation.

	Population	Segregating gene	Crossing	Genotypes crossed
Cucumber	F2-A9	<i>A</i>	(Oman x W3)- self	(<i>MMffAA</i> x <i>MMffaa</i>)-self
	BC-A7	<i>A</i>	(Oman x W3) x W3	(<i>MMFFAA</i> x <i>mmFFAA</i>) x <i>mmFFAA</i>
	F2-1123	<i>F</i>	(W2 x Oman)- self	(<i>MMFFAA</i> x <i>MMffAA</i>)-self
	BC-1121	<i>F</i>	(W2 x Oman) x Oman	(<i>MMFFAA</i> x <i>MMffAA</i>) x <i>MMffAA</i>
Melon	BC-1108	<i>F</i> and <i>M</i>	(Oman x W4) x W4	(<i>MMffAA</i> x <i>mmFFAA</i>) x <i>mmFFAA</i>
	BC-AGY	<i>G</i> and <i>M</i>	(AGY x PI414723)x AGY	(<i>Aammgg</i> x <i>aaMMGG</i>) x <i>Aammgg</i>

Table 3B. New mapping populations prepared during this project.

Populations BC-A7 and F2-A9 segregated for the *A* locus. In the F2-A9 population, the first 40 individuals analyzed segregated 28 monoecious (first female node around 20, earlier female flowers on side branches) and 12 androecious plants. In BC-A7, 55 plants segregated into 28 monoecious, 27 androecious. This fits the expected model for a single gene for androecy in this cross.

Populations BC-1121 and F2-1123 segregate for the *F* locus (Fig. 3). It is apparent that, compared to our previous populations (Table 3A) where a cultivated monoecious parent (W1) was used, Oman enhances maleness; it may be due to a strongly-male *f* allele, or by additional genes segregating in the populations. In addition, a gene(s) that contribute a long phase of sterile nodes segregates as well (Fig. 4); these phenotypic phenomena and their possible relatedness to sex expression will require closer inspection. In BC-1121, a sample of 51 plants showed a rather continuous variation between monoecious plants with very long male phases (over 30 male or sterile nodes on main shoot preceding first female flower) and plants with more moderate monoecious patterns (node numbers up to 30). In F2-1123, 57

plants segregated between 17 gynoeious plants (30%), and monoecious plants with varying level of maleness (Fig. 3). Quantitative trait analysis could help in mapping the femaleness trait in this background, although the *F* genotype assignment is possible, at least in the F2, where *FF* plants are recognized, while *Ff* and *ff* ones are less readily separated.

Figure 3. Disribution of sex patterns in population F2-1123, segregating for gene *F*. Percentage of female nodes, out of non-sterile nodes in main shoot.

Figure 4. Distribution of number of sterile nodes on main shoot (22 nodes scored) in Population BC-1121.

In BC-1108, the two genes, *F* and *M*, segregated into four neatly-separated categories: 46 plants were mono- or gynoeious (*Mm* genotype), while 33 were hermaphrodite /andromonoecious (*mm*). Each of the two classes segregated bi-modally between *FF* and *Ff* genotypes. In the *mm* class (Fig. 5A), 16 andromonoecious plants with a long male phase segregated from 13 near-hermaphrodite plants (*FFmm*), where bisexual flowers developed (along with later-appearing male flowers) from earlier nodes; we could not assign an *F* genotype to the remaining plants. The 25 plants that had the *Mm,FF* genotype (Fig. 5B)

were gynoecious, while 20 *Mm,Ff* plants were monoecious, with a rather long male phase (unlike *Ff* plants in cultivated background, where femaleness is stronger). This population can be used to study the possible interaction between the *F* and *M* genes.

Figure 5. Distribution of Node number among plants of BC-1108. A. Plants of the *mm* genotype, segregating between near-hermaphrodite (*FF*) plants and andromonoecious ones with long male phase (*Ff*). B. Plants of the *Mm* class segregating between gynoecious (*FF*) and monoecious (*Ff*) types.

2.3. Linkage analysis of known genes using the mapping populations - Bar-Ilan University

In the first part of the project we searched for RFLP and SNP polymorphism in the older set of mapping populations, and reported linkage and mapping data in the first and second annual reports. Table 4 summarizes the data obtained at that stage. In the last year we

searched for SNP polymorphism between parents of the new populations (Table 3B). We performed PCR reactions that amplify these genes from parental genotypes and search for single nucleotide polymorphism between the whole set of parental genotypes. Detection and scoring of such SNPs was performed either by the CAPS or the dCAPS methods that involve digestion of the amplicons using restriction enzymes. The clones for which an SNP was detected are reported in Table 4B. Work under this Objective is in progress.

A	Cucumber populations scored			Melon populations scored	
clone	F2-1022 gene <i>A</i>	BC-1035 gene <i>M</i>	F2-1053 gene <i>F</i>	RIL INRA gene <i>A</i>	F2 BIU gene <i>A</i>
<i>ERS1</i>		+ unlinked			
<i>ETR1</i>					+ unlinked
<i>CTR1</i>		+ unlinked			
<i>EIN3</i>		+ unlinked			
<i>ACO2</i>			+ linked	+ unlinked	
<i>DH27</i>					+ linked
<i>CAG1</i>				+ unlinked	+ unlinked
<i>CAG2</i>				+ unlinked	+ unlinked
<i>CAG3</i>				+ unlinked	+ unlinked

B	Cucumber populations			Melon populations	
clone	F2-A9, BC A7 gene <i>A</i>	F2-1123, BC-1121 gene <i>F</i>	BC-1108 genes <i>F</i> , <i>M</i>	RIL BIU gene <i>A</i>	BC-AGY genes <i>G</i> , <i>M</i>
<i>ACS1</i>	p	p	p		p
<i>ACS2</i>			p, s		p
<i>ACS3</i>		p			
<i>CFL</i>	p	p	p	p	
<i>OPI</i>	p, s	p, s	p	p	p
<i>CTR1</i>	p	p	p		

Table 4. A: Linkage data obtained between known genes and sex loci using original set of populations. + marker developed for specific parental combination and scored in the respective population. Linkage to sex locus indicated. B: Search for SNP and scoring progress in the new populations. P – parental polymorphism uncovered; s - SNP scored in mapping population.

2.4. Developing mapping populations to study sex modifying genes – University of Wisconsin

The unique sub-project addressed by the Wisconsin laboratory is the elucidation of epistatic interactions among the *F* locus conditioning gynoecy in cucumber and loci which modify the expression of *F*. This involved the identification of markers that are closely linked to *F* and the genes that modify its expression, the creation of nearly isogenic lines with an array of modifying genes with differing allelic constitutions associated with the *F* locus, and the evaluation of the interaction of these genes modifying genes.

2.4.1. Identification of quantitative genes that modify sex expression – University of Wisconsin

Two quantitative trait loci (QTL) for number of pistillate nodes on the main stem, *sex 1.1* and *sex 1.2*, have been detected on linkage Group I of a map constructed using the gynoeceious (*FF*), determinate (*de*) line G421 possessing standard-sized leaves (*LL*) and the monoecious (*Ff*), indeterminate (*De*) line H-19 possessing little leaves (*ll*). These QTL are associated with the *F* and *de* loci and explain 16.4% and 9.4% of the observed phenotypic variation, respectively. A third QTL maps to linkage Group 6 (*sex 6.1*) and explains 5.5% of the observed variation. The H-19 alleles of all three QTL had a negative effect on femaleness.

Four QTL, each explaining about 5% of the observed phenotypic variation for the number of pistillate nodes on primary lateral branches are associated with linkage Group 1. Two of these QTL (*fnl 1.1* and *fnl 1.3*) are associated with either the *F* locus or the *de* locus. In each case the H-19 allele had a negative effect on the trait. Another QTL (*fnl 1.4*) is associated with the H-19 allele *ll*, resulting in a positive effect (more pistillate flowers).

2.4.2. Development of nearly-isogenic lines – University of Wisconsin

Near-isogenic lines have been developed by backcrossing procedures that resulted in either BC₁S₄ or BC₂S₄ individuals with differing arrays of alleles at sex linked modifying genes. This required the development of BC₁S₁ and BC₂S₁ individuals, which were self-pollinated after their sex expression and allelic constitution at marker loci were determined. In each successive generation, progeny were selected to develop unique near-isogenic lines with contrasting sex expressions and associated alleles.

In 2000/2001, BC₁S₁ and BC₂S₁ families (Table 5) were developed and evaluated for sex expression in an open-field nursery (Hancock, WI, summer 2001). These were self-pollinated for further evaluation of their sex expression and marker constitution. This involved planting 580 individual plants in 116 plots (4 plots /family, 5 plants /plot). Of these, 193 plants reached maturity such that 131 selections were self-pollinated (Table 6). In addition to sex expression scores, the plants were also evaluated for leaf size and multiple lateral branching characteristics. This allowed for the production of nearly-isogenic genotypes with differing plant habits that are directly associated with sex expression in cucumber, but have been poorly studied.

Of these 131 plants, 23 were strategically selected, based on the number and distribution of female flowers on the first 15 nodes of the main stem (Table 7), to represent

an array of phenotypes ranging from gynoeious to monoecious with low female-tendency. Twenty-four seedlings from each selfed selection were genotyped with markers linked to the QTL described above (Table 8). At the same time, additional backcross material produced from the same G421 and H19 parents during concurrent studies in the USDA cucumber breeding program (Table 9) was genotyped with the same markers. All the material was divided by leaf type into two groups (little leaf and normal leaf). From the genotypic data, strategic crosses were designed between selected plants to create progeny that were heterozygous at as many QTL as possible. Although 25 crosses were designed for the little leaf population, only 11 gave a sufficient amount of seed. A total of 15 crosses were designed for normal leaf selections and nine gave sufficient seed. Ten to 20 seeds of these crosses were planted in the field to determine if the sex expression of the progeny matched that predicted by the genotype of the parents. In some cases, sex expression was as predicted, and not in others. From these phenotypic data and the genotypic data of the parents, we identified the 10 (five little leaf and five normal leaf) most promising crosses. The progeny from these crosses were genotyped to select plants that were heterozygous at as many QTL as possible, then selfed for three generations. Heterozygotes were selected at each selfing generation based on marker genotype to produce an array of lines that differ in allelic constitutions of QTL associated with sex expression (Table 10). Unfortunately, we were unable to recover an array of NIL in a little leaf background with the *F* locus in combination with modifying genes by the third generation of selfing (BC₂S₄). The normal leaf NIL have only recently been developed and seed is currently being collected to complete the testing of our nearly-isogenic lines. This portion of the project will be completed in 2005. Once sufficient seed has been collected, the NIL created in part II will be tested in replicated trials to determine the effect of individual QTL alleles and if interactions exist between QTL alleles associated with sex expression.

3. Produce and analyze transgenic melons and cucumbers altered in ethylene production or perception

Our primary objective was to further understand the roles of, and critical sites for, endogenous ethylene production and perception in sex expression in *Cucumis*.

3.1 Constructs for modulation of ethylene biosynthesis and perception and transformation into melon – Michigan State University

The ACS gene for ethylene production from petunia and the dominant negative *etr1-1* ethylene perception mutant gene from *Arabidopsis* were provided by R. Woodson and C. Chang, respectively. These genes were engineered into binary *Agrobacterium*-mediated transformation vectors in combination with three promoters: CaMV 35S for strong constitutive expression; the *Arabidopsis* floral specific *Apetala3* (*AP3*) promoter (provided by V. Irish, Yale); and the *Arabidopsis* *Crabscraw* (*CRC*) promoter for carpel expression (provided by J. Bowman). Details of the constructs and cloning methods were described in the year 1 and year 2 reports. Transgenic melons and progeny were produced with the *35S:ACS*, *35S:etr1-1*; *AP3:etr1-1* and *CRC:etr1-1* constructs. Analyses are described below.

3.2. Analysis of transgenic plants – Michigan State University

A.35S-ACS transgenic melons

Gene integration and expression. Transgenic *CaMV 35S-ACS* melons were produced from the andromonoecious cultivar Hale's Best Jumbo by *Agrobacterium*-mediated transformation using the method of Fang and Grumet (1990) and progeny produced in the greenhouse. As has been frequently observed for melons regenerated from tissue culture (Ezura et al. 1992; Yadav et al. 1996), analysis of pollen morphology indicated that the transgenic ACS lines were tetraploid. The observed segregation ratios for the T₁ progeny (Table 11) were consistent with ACS gene insertion at either one or two sites.

Table 11 - 12

Expression of the introduced *ACS* gene was verified by northern blot analysis of leaf and flower bud tissue of T₁ and T₂ plants (Figure 6). Consistent with segregation ratios implicating possible presence of two copies of the *ACS* transgenes in lines H (Hale's Best Jumbo)-ACS3 and H-ACS4, expression levels in these plants were higher than in H-ACS1 plants. Preliminary tests of leaf tissue indicated that these two lines also had higher ethylene production and so were chosen for subsequent analyses in the greenhouse and field.

Ethylene evolution. It is predicted that constitutive *ACS* expression would cause elevated ethylene production in the transgenic melon plants. Young, fully expanded leaves of H-ACS3, H-ACS4, and wild type Hale's Best Jumbo (H-WT) were sampled for ethylene production from main stem node positions 5 (at the onset of flowering), 10 (during male flowering phase), and 15 (at the onset of hermaphrodite flowering) (Figure 7A). Leaves from the transgenic 35S:*ACS* T₁ plants produced 2 - 3 fold higher ethylene levels than H-WT plants at all three node positions. The elevated ethylene production did not result from tetraploidy as ethylene production by azygous tetraploid segregant plants (H-4nAZ), 58.0 ± 18.2 pmol/g, was equivalent to the H-WT controls, which produced 72.7 ± 11.6 pmol/g (data are mean of five sample dates, 4 replicates/genotype/date; $t=1.19ns$). The 2-3 fold increase in ethylene production was comparable to the difference observed between apices of monoecious and gynoecious cucumbers, which reached a maximum of 2-3 fold at the 4-5 leaf stage, when the

first pistillate buds were visible on the excised apices of the gynoecious plants (Rudich et al., 1972, 1976; Yamasaki et al., 2000).

Figure 7. Ethylene evolution from young, fully expanded leaves and flower buds 1-2 days pre-anthesis of wild type (WT) and transgenic 35S::ACS melon plants. **(A)** Leaves from node positions 5, 10, and 15. Data are combined from two experiments with a total number of samples n=16, 18 and 5 for WT leaves 5, 10, and 15, respectively; n=18, 14, and 12 for ACS3 plants; and n=12, 13 and 9 for ACS4 plants. **(B)** Male buds. The experiment was repeated five times with n=17, 31, and 18 for the WT, ACS3 and ACS4 plants, respectively. **(C)** Hermaphrodite buds. The experiment was repeated four times with n=12, 23, and 10 for the WT, ACS3 and ACS4 plants, respectively. All data are combined means $\pm$ standard error.

Excised male and hermaphrodite buds sampled 1-2 days prior to anthesis (petals closed, but beginning to develop yellow color) also showed higher ethylene production than H-WT plants (Figure 7B, C). Ethylene evolution from H-4nAZ male buds was equivalent to H-WT

buds (H-WT: 42.7 ± 2.7 pmol/g, H-4nAZ: 68.0 ± 17 ; data are mean of three sample dates; 4 replicates/genotype/date; $t=1.31$ ns). Although there was a fair bit of variability in ethylene production by hermaphrodite buds, they generally produced more ethylene than male buds, with levels 8-10-fold higher for ACS hermaphrodites vs. males (ca. 800 pmol/g vs. 105 pmol/g for H-ACS3 and 1800 pmol/g vs. 170 pmol/g for H-ACS4) and ca. 3-fold higher for H-WT hermaphrodites vs. males (200 pmol/g vs. 60 pmol/g). This reflects what has been observed for cucumber, where ethylene evolution by female flower buds was generally higher than male buds (Rudich et al., 1976). Unlike leaves and buds, apical growing points excised from lateral branches of 35S:ACS transgenic melons did not show elevated ethylene evolution relative to controls (data not shown).

Leaves and flower buds were also tested for ACC content (Table 12). As predicted, H-ACS3 plants had higher ACC levels in leaves and hermaphrodite flower buds than did H-WT or H-4nAZ plants. Comparison of levels of ACC to ethylene evolution in the different genotypes and tissues, showed a strong correlation between ethylene production and ACC content for leaves and hermaphrodite buds (Figure 8A; $r^2 = 0.994$), suggesting conversion of ACC to ethylene. The male buds at an equivalent stage, however, accumulated considerably higher amounts of ACC that was not correlated to rate of ethylene production, possibly reflecting other steps of control of ethylene production in this tissue. ACC is converted to ethylene by the ACC oxidase (ACO) enzyme. Expression patterns of *ACO* genes relative to sex expression has been less well studied than *ACS*, but Kahana et al. (1999) observed weak expression of one of the *ACO* genes, *CS-ACO2* in mature, male cucumber buds, just prior to anthesis, whereas *CS-ACO2* expression increased in mature hermaphrodite buds.

Figure 8. Comparison of ACC content and ethylene production by wild type (WT), azygous segregant (AZ), and transgenic 35S::ACS melon (ACS3) leaves, male buds and hermaphrodite buds. ACC and ethylene data are as described for Table 2 and Figure 2.

Sex expression and floral development. The normal sex expression pattern along the main stem of andromonoecious melon plants is 4 - 5 vegetative nodes followed by several nodes that carry only male buds, and then a hermaphrodite phase which includes a mixture of hermaphrodite and male buds, including both sex types at a single node. In our conditions in the greenhouse, the wild type control plants formed the first hermaphrodite bud at approximately node 11; H-4nAZ plants were not significantly different than H-WT controls (Figure 9A,B). The transgenic H-ACS3 and H-ACS4 plants showed earlier appearance of the first hermaphrodite bud by 2 - 4 nodes (node 7 - 9). The time of appearance of the first male bud was not affected in the transgenic plants (data not shown) so that the male only phase was reduced from an average of 4.2 nodes for the wild type to an average of 2.4 nodes for the H-ACS3 plants and 0.3 for the H-ACS4 plants. The H-ACS3 and H-ACS4 plants also produced 3-6 more hermaphrodite buds on the first 30 nodes of the main stem than did the H-WT and H-4nAZ controls (Figure 9E,F).

Thus, modification in endogenous ethylene production can alter floral development in transgenic melons and is consistent with previously reported effects of exogenous ethylene treatments in promoting pistil development (e.g., Augustine et al., 1973; Galun et al., 1963; Karchi, 1970; McMurray and Miller, 1968; Pike and Peterson, 1969; Tolla and Peterson, 1979; Yin and Quinn, 1995).

An interesting and unexpected effect of the ACS gene was on flower bud maturity. The first several hermaphrodite buds often abort before developing fully to anthesis. Thus, the first hermaphrodite flower to reach anthesis generally occurred 10-15 nodes later than did appearance of the first hermaphrodite bud, at node 22 - 27 for the H-WT and H-4nAZ plants (Figure 9C,D). This pattern of flowering was modified in the transgenic ACS lines, where the first mature hermaphrodite flowers occurred at nodes 10 - 17, with only a delay of 2 - 8 nodes relative to appearance of first the hermaphrodite bud. The ACS lines also had an increased total number of mature hermaphrodite flowers on the first 30 nodes of the main stem (Figure 9G,H).

Figure 9. Sex expression pattern of wild type (WT), azygous segregant (AZ) and transgenic 35S::ACS melon plants (ACS3 and ACS4) in the greenhouse. **(A, B)** Node position on the main stem of the first hermaphrodite bud. **(C, D)** Total hermaphrodite buds on the first thirty nodes of the main stem. **(E, F)** Node position of the first hermaphrodite flower to reach anthesis. **(G, H)** Total number of hermaphrodite flowers that reached anthesis on the first thirty nodes of the main stem. The experiments were repeated three times with total number of samples $n=22$, $n=22$, $n=12$ for WT, ACS3 and ACS4, respectively (left panels); and $n=16$, $n=16$, $n=8$ for WT, AZ, and ACS3, respectively (right panels).

The transgenic ACS3 and ACS4 lines were also tested for sex expression under field conditions. In general, the first hermaphrodite flowers occurred earlier in the field (node 5-7) than in the greenhouse. Although there was no significant difference in time of appearance of the first hermaphrodite bud for the ACS transgenic lines (Figure 10A), there was significantly earlier appearance of the first mature hermaphrodite flower in the H-ACS 3 and H-ACS 4 lines than for the H-WT plants and H-4nAZ plants (Figure 10B; analysis of variance, $P \leq 0.05$). The transgenic ACS lines also produced significantly more mature hermaphrodite flowers in the first 25 nodes of the main stem (Figures 10D, 11) and a higher percentage of hermaphrodite flowers reached maturity (Table 13). Although the non-transgenic tetraploids also showed an increased number of mature hermaphrodite flowers, the effect was more pronounced in the ACS transgenic plants. There also was a higher frequency of adjacent mature flowers on the main stem of ACS plants (Table 13).

Figure 10. Sex expression pattern of wild type (WT), azygous segregant (AZ) and transgenic 35S::ACS melon plants (ACS3 and ACS4) in the field. **(A)** Node position on the main stem of the first hermaphrodite bud. **(B)** Node position of the first hermaphrodite flower to reach anthesis. **(C)** Total hermaphrodite buds on the first 25 nodes of the main stem. **(D)** Total number of hermaphrodite flowers that reached anthesis on the first 25 nodes of the main stem.

Thus the effects on pistillate flower maturation were observed reproducibly in both the greenhouse and field, and suggest that ethylene may be important not only for promotion of pistil development at the time of sex determination, but also to sustain further development of the female organ. Whether expression of the introduced ACS gene and ACC or ethylene levels play a specific role in melon ovary or ovule development remains to be determined.

Fruit production and development. Consistent with the earlier appearance and greater number of mature hermaphrodite flowers on the main stem, field-grown transgenic ACS plants showed earlier fruit set and a higher total number of fruit on the main stem (Figure 11, A-C). This observation indicates that the plants were developmentally ready to set fruit sooner than typically occurs, and that they had the resource capacity to develop fruit earlier. This characteristic could be horticulturally valuable, especially in regions with a short growing season.

The ACS plants also were more likely to set fruit on closely spaced nodes (Fig. 11A, Table 13); 59% of the H-ACS plants set two fruits within four nodes, vs. only 12% for the non-transgenic lines. Multiple ACS plants set four or more fruit on the main stem, and in one extreme case, five fruit were set on consecutive nodes. The percent of mature hermaphrodite buds on the main stem that formed fruit was similar for all lines (Table 13).

Despite earlier fruit set and increased fruit set on the main stem of ACS plants, total fruit set per plant did not differ significantly between the ACS and control lines. Expression of ACS did not influence fruit size, however, there were marked differences in fruit size between the tetraploid and diploid plants (Table 13) such that fruits on the tetraploid plants (H-4nAZ, H-ACS3, and H-ACS4) were smaller than the diploid fruits. Smaller fruit size resulting from tetraploidy has been observed previously (Nugent, 1994a,b).

Increased fruit set on the main stem of the ACS plants was not due to smaller fruit size, as the azygous tetraploid plants also had smaller fruit, but did not have increased fruit set. The greater number of fruit along the main stem may result from the increased number of mature hermaphrodite flowers, as the percent mature hermaphrodites that bore fruit, was similar among the genotypes. Clearly, however, the presence of a mature hermaphrodite flower was not sufficient for fruit set. Previous work in the area of plant reproductive ecology has suggested that in some monoecious plant species, sex expression and fruit set can be influenced by the presence of prior flowers or developing fruits. Developing fruit on *Cucurbita* species resulted in a reduction in pistillate flower production and increased fruit abortions, while lack of fruit or fruit removal increased pistillate flower production (Avila-Sakar et al., 2001; Krupnick et al., 1999; Stephenson et al., 1988). Similarly fruit production in the cucurbit, *Lagenaria siceraria*, was associated with a shut-down of subsequent flower production (Delesalle and Mooreside, 1995); melon fruit removal stimulated production of

male flowers and new fruit set (El-Keblawy and Lovett Doust, 1996); and deflowering of early nodes in cucumber plants increased the number of simultaneously developing cucumbers on individual plants (Janoudi and Widders, 1993). While we cannot always separate the effects that increase fruit set from those that increase pistillate flower production, these observations imply that the presence of integrated developmental processes regulate resource allocation, and that developing fruits may send hormonal or other signals that limit subsequent hermaphrodite flower production or fruit set. Given that the total number of fruits per plant was equivalent, the increased percent fruit set on the main stem of transgenics suggests a redistribution of resources, presumably mediated by ethylene signaling.

The ACS plants also had ripe fruit sooner than the non-transgenic melon plants (Figure 11D). When observed at 8 ½ weeks post-transplanting, fruits in the overripe (very soft, beginning to break open or decay) and ripe (full slip) categories came almost exclusively from the H-ACS3 and H-ACS4 lines. An average of 18% of the ACS fruits were ripe or overripe, whereas only 3% of the non-transgenic fruits were ripe or overripe. The non-transgenic plants did not reach a similar percent ripe fruit until a week and half later, when 15% were ripe or overripe.

Figure 11. Fruit set and ripening pattern of wild type (WT), azygous segregant (AZ) and transgenic 35S::ACS melon plants (ACS3 and ACS4) in the field. **(A)** Node positions of mature hermaphrodite flowers (○) and fruits (●) for melon plants in the field. Each horizontal line represents the main stem of an individual plant. The three sets of plants represent the three replicate plots. For ease of visualization, the plants are grouped by genotype in the figure, rather than in randomized complete block design as they were arranged in the field. **(B)** Average node position of the first fruit on the main stem. **(C)** Average number of total fruits on the main stem. **(D)** Stage of fruit development at 8 1/2 weeks after transplanting to the field. All fruit within each plot were scored for maturity as described in Methods.

The earlier occurrence of ripe fruit on the ACS plants may be due to a combination of factors. Earlier fruit set would be expected to result in earlier ripening, if the time to mature remained constant between the ACS and non-transgenic fruits. There may also be a confounding effect of increased ethylene production leading to accelerated ripening. In climacteric fruit, a burst in ethylene production and respiratory activity initiates the ripening process in mature fruit (review: Giovannoni, 2001), and studies with transgenic melons expressing antisense genes to ACO showed inhibited ripening processes such as pigment development and fruit softening (Ayub et al., 1996; Guis et al., 1997; Silva et al., 2004). Further studies would be required to distinguish the relative effects of earlier fruit set from possible enhanced ripening by increased ethylene production.

Conclusions from 35S:ACS studies. The above results indicate that ethylene production plays a central role sex determination and female flower organ development in melon. Constitutive expression of an additional copy of the ACS genes in transgenic andromonoecious melon plants induced earlier and increased formation of hermaphroditic flower buds, an increased number of hermaphrodite buds that reached anthesis, earlier fruit set, and increased fruit set on the main stem. These results provide a direct demonstration of the importance of endogenous ethylene production for hermaphrodite flower formation and development in melon, and may provide a route for producing melon genotypes with earlier fruit production or reduced growing season requirements. Future research will be needed to understand the role of specific timing and location of ethylene production relative to the developing flower primordia to allow for targeted ACS expression.

B. 35S-etr1-1 transgenic melons

Ethylene perception phenotypes. Transgenic 35S:*etr1-1* melon plants (cv. Hale's Best Jumbo; Chesmore Seed Company, St Joseph MO) were generated by cotyledon transformation according to the methods of Fang and Grumet (1990). Regenerated kanamycin resistant plantlets evaluated for the presence of the introduced genes by polymerase chain reaction (PCR). PCR-positive individuals were transplanted to soil, acclimated in the growth chamber, and transferred to the greenhouse to produce T₁ progeny. Expression of the *etr1-1* was verified by northern analysis (Fig. 12). The T₁ progeny of line *etr1-1* showed a segregation ratio consistent with a single gene insertion (51:14 transgenic:non-transgenic; $\chi^2=0.42$ ns).

To verify effectiveness of the *Arabidopsis etr1-1* gene in inhibiting ethylene perception in the heterologous melon system, the transgenic 35S:*etr1-1* melon plants were examined for several phenotypic effects associated with ethylene insensitivity, such as including impaired root development, increased ethylene production, delayed flower bud senescence and abscission, and reduced responsiveness to exogenous ethylene (Wilkinson et al., 1997). Vegetative cuttings from T₁ transgenic plants had reduced and delayed rooting ability as evidenced by decreased maximum root length and weight compared to WT plants and non-transgenic segregating progeny (NT) from the same family (Table 14). Reduced ability of vegetative cuttings of transgenic *etr1-1* melon plants to form adventitious roots is consistent with prior studies implicating ethylene as a positive regulator of root growth and root hair development (Clark et al., 1999; Mensuali-Sodi et al., 1995; Tanimoto et al., 1995).

Excised flower buds of transgenic plants also showed significantly higher ethylene evolution (130 pmol/g) compared to WT and NT plants (41 and 31 pmol/g). Elevated ethylene production has been observed in leaves of *etr1-1* *Arabidopsis* plants (Schaller et al. 1995) and *etr1-1* petunia flowers (Wilkinson et al., 1997), presumably due to disruption of a feedback mechanism controlling ethylene biosynthesis.

Transgenic flower buds also showed delayed abscission and senescence (Fig. 13). Pedicels of *etr6* male buds abscised an average of 1/2-1 day later than WT and NT6 buds. Furthermore, while exogenous ethylene caused WT and NT6 buds to abscise a day earlier, ethylene treated *etr6* buds abscised the same time as in air, indicating insensitivity to exogenous ethylene. Consistent with the delayed abscission of excised buds, male flower buds formed on the *etr1-1* plants were more likely to develop to anthesis, rather than abort. Nearly all of the male buds produced on *etr1-1* melon plants (98%) developed fully, while

only ca. 70% of the WT and NT6 male buds reached anthesis. These results correspond with observations with transgenic *etr1-1* tomatoes and petunias that showed delayed senescence and abscission, and failure to respond to exogenous ethylene with accelerated senescence (Wilkinson et al., 1997).

Sex expression. As was observed in the ACS experiments, initiation of the hermaphroditic flower phase on WT and NT plants in the greenhouse occurred at ca. node 10, following 5-7 male nodes (Fig 14). The majority (86%) of *etr1-1* transgenic plants, however, failed to produce any hermaphrodite flower buds during the observation period of 30 nodes of the main stem. Some were observed until the 40th node and still did not form hermaphrodites. A small number of *etr1-1* transgenic plants (7 of 51) formed 1-2 hermaphrodites at approximately the time that WT and NT6 plants formed their first hermaphrodite bud, but then converted back to only males. The occasional hermaphrodite buds formed on the *etr1-1* transgenic plants reached anthesis, whereas only 11% of the female buds on H-WT plants developed fully.

Thus inhibition of ethylene perception largely abolished formation of hermaphrodite buds in the *etr1-1* transgenic melons. These results demonstrate that ethylene perception plays a critical role in pistil formation and are consistent with the effects of exogenous inhibitors of ethylene action which cause a delay in the pistillate flower phase (Rudich, 1990). In summary, these experiments provide a direct demonstration of the importance of endogenous ethylene perception for sex determination in melon.

Figure 14. Production of vegetative and flowering nodes along the main stem of transgenic T₁ *etr1-1* plants. Each node produced multiple buds and was scored pistillate if at least on pistillate bud was formed. Data are pooled from four experiments. WT, n=43; NT6, n=14; *etr6**, transgenic plants that produced 1-2 pistillate buds, n=7; *etr6*, transgenic *etr1-1* T₁ plants line 6, n=44.

B. CRC:- and AP3:*etr1-1* transgenic melons.

Study of these lines is in preliminary stages, however, we have observed very interesting phenotypes. Preliminary observations of several melon lines expressing *etr1-1* under control of the Arabidopsis *CRABSCLAW* (CRC) promoter, gave a very surprising result. Inhibition of ethylene perception in carpels did not prevent carpel establishment, as might be predicted; more strikingly, the pistillate buds did not contain stamens (Fig. 15). Thus typically hermaphrodite flowers were converted to female flowers. Examination of the few pistillate buds that occurred on lateral branches of *35S:etr1-1* melons also revealed a lack of stamens. Conversely, very preliminary observations of *AP3:etr1-1* melons suggest an increase in maleness. These observations suggest that the sex determination mechanism for melon may differ from that for cucumber, where ethylene perception by the stamens is proposed to inhibit stamen development (Yamasaki 2001, 2003a; Yin and Quinn, 1995), and leads us to propose a model whereby melon stamen and carpel primordia reciprocally inhibit development of the alternate whorl, unless they perceive sufficient ethylene. These observations raise many questions to be addressed with further analysis of tissue specific expression of ethylene related genes.

3.3. Construct preparation and set up of cucumber transformation – Bar-Ilan University

In the first and second annual reports, preparation of a series of constructs was detailed. These included sense and antisense constructs designed to affect ethylene production: ACC oxidase-3 of cucumber, a construct for RNAi mediated silencing of ACC synthase 1, all under the control of the 35S promoter. Constructs were also made to alter ethylene sensitivity via over-expression of cucumber ERS, under the control of the 35S and the *Apetala3* promoters, respectively, and antisense silencing of ETR2.

Initial help in setting up cucumber transformation was obtained from Dr A. Zelcer of ARO. The protocol, modified from Tabei et al. (1998) worked best with gynoeocious cultivar, Eyal. In addition, post-doctoral fellow Dr. Premanand Rajagopalan optimized his protocol and concentrated on monoecious Poinsett76 cultivar. The results of such optimization are detailed in a paper appended to this report.

3.4. Transgenic cucumbers with increased sensitivity to ethylene - Bar-Ilan University

Ph.D. student Talya Saraf-Levy, helped by Dr. A. Zelcer at ARO, introduced the 35S-ERS1 construct to gynoeocious *C. sativus* cv Eyal. Seven independent T0 transgenic plants were obtained, and verified by PCR and Southern analysis; ERS transcripts were present at high levels in the floral buds, whereas in the wild type they are barely detectable (Saraf-Levy, 2003). The construct did not affect the phenotype in the gynoeocious Eyal background, and the plants were crossed with a hermaphrodite line, W4 (*FFmm*) to assess the possible effect of increased ethylene sensitivity on the *mm* genotype. Two independent progenies were analyzed but no alteration of the hermaphroditic or gynoeocious sex types, or in typical ethylene-associated traits, was observed. If such result can be repeated with more transformants, it would mean that ERS over-expression cannot complement the ethylene-sensing defect of plants that carry the *mm* alleles. It has been shown that such plants do not sense ethylene normally, but this could result from other genes being affected. A signaling component that acts downstream of ERS or in parallel to ERS would not be complemented. At present however this conclusion requires further support.

The 35S-ERS construct was then introduced into a monoecious cultivar, Poinsett76, where its effect and interaction with the *f* allele could be followed. The regeneration frequency was 9%, and average transformation frequency was about 1.5%, and a total of seven 35S-ERS plants were verified by PCR and Southern blot analysis of the primary transformants (the T0 generation) and the progeny (T1 generation; see Second Annual report). The Southern-positive plants were self crossed in the glasshouse and seeds were collected. Progeny

seedlings were once again confirmed by PCR and Southern. Five families had monoecious sex patterns similar to the non-transformed plants, but in two families we observed segregation between plants with a "normal" monoecious phenotype (Type I) and plants with an enhanced-female phenotype (Type II). Table 15 and Fig. 16 show the sex patterns of the different T1 families grown during three growing seasons. Type II plants formed their first female node at least four nodes earlier than the other plants and the proportion of female flowers was several folds higher. The enhanced-femaleness plants represented two thirds of the transgenic progeny in families 6 and 7, and displayed, at a young stage, higher levels of *ERS* transcripts, compared to control plants and to their "phenotype-negative" siblings (Fig. 17). The feminized plants also exhibited accelerated yellowing of detached leaves, suggesting that the phenotype is related to the expression of the ERS transgene (Table 16, Fig. 18). These data implicate ethylene perception in sex determination, and support the notion that receptor levels, and not only ethylene hormone levels, could be a limiting factor in female bud development. Because unoccupied receptor proteins were shown, in *Arabidopsis*, to block ethylene response, our results suggest that the extra receptor made by the transgenic plants can be activated by sufficient amounts of endogenous ethylene, otherwise the transgenic plants could have exhibited a delayed femaleness-delayed senescence response. Data were published in an appended Proceedings paper and are in preparation for a full-length paper.

		1 st Female Node $\pm$ SE			Total Female Nodes $\pm$ SE (first 19 nodes)		
Family	No. plants	Exp. 1 Winter ²	Exp. 2 Spring ³	Exp. 3 Summer ⁴	Exp. 1 Winter	Exp. 2 Spring	Exp. 3 Summer
Wild Type	16	13.0	13.9 $\pm$ 0.6	15.6 $\pm$ 0.8	3.0	2.1 $\pm$ 0.3	1.3 $\pm$ 0.2
Fam. 2	18	11.6 $\pm$ 1.0	13.1 $\pm$ 0.9	14.8 $\pm$ 0.8	1.8 $\pm$ 0.2	1.4 $\pm$ 0.2	1.2 $\pm$ 0.2
Fam. 3	12	12.6 $\pm$ 0.5	12.1 $\pm$ 1.2	--	2.2 $\pm$ 0.2	1.6 $\pm$ 0.2	--
Fam. 4	18	11.0 $\pm$ 0.7	13.5 $\pm$ 1.1	13.8 $\pm$ 1.8	2 $\pm$ 0.0	1.4 $\pm$ 0.2	1.4 $\pm$ 0.2
Fam. 5	5	11.6 $\pm$ 1.0	--	--	1.8 $\pm$ 0.2	--	--
Fam. 6 –group I	9	9.8 $\pm$ 0.8	12.0	13.8 $\pm$ 0.2	2.8 $\pm$ 0.3	1.0	1.0 $\pm$ 0.0
Fam. 6 –group II	16	6.3 $\pm$ 0.9	8.1 $\pm$ 1.4	5.3 $\pm$ 0.9	12.6 $\pm$ 0.3	5.6 $\pm$ 0.7	8.8 $\pm$ 1.2
Fam. 7 –group I	9	12.0	12.3 $\pm$ 0.6	15.3 $\pm$ 0.8	1.0	1.5 $\pm$ 0.5	1.5 $\pm$ 0.5
Fam. 7 –group II	15	5.3 $\pm$ 0.7	6.5 $\pm$ 0.3	5.3 $\pm$ 0.6	9.0 $\pm$ 0.6	9.0 $\pm$ 0.7	8.0 $\pm$ 1.2

Table 15. Sex phenotype of transgenic T1 plants and Poinsett76 non-transformed plants.

¹ – Total number of plants examined in three experiments. ² – Winter season: November 02 to February 2003. ³ – Spring season: March-June 2003. ⁴ – Summer-fall season, August-November 2003.

Table 16. Chlorophyll content of detached leaves. No. of plants assayed and chlorophyll content after 4 days detachment; discs from additional plants were inspected visually for yellowing.

Family	No. plants	Chlorophyll, mg/gfw
Wild Type	4	2.4 ± 0.6
Fam. 2	3	2.0 ± 0.2
Fam. 4	4	2.0 ± 0.1
Fam. 6 –group I	4	2.3 ± 0.4
Fam. 6 –group II	6	0.9 ± 0.3
Fam. 7 –group I	4	1.9 ± 0.5
Fam. 7 –group II	4	1.0 ± 0.1

Figure 16. Poinsett 76 cucumber plants transformed with the 35S:ERS gene. Left: example of Type I and Type II transgenic plants: in Type I a single female node in the middle of several male nodes, in Type II, seven consecutive female nodes are apparent. On the right, schematic representation of non-transformed, Type I and Type II plants.

Figure 17. Northern analysis of ERS transcripts in non-transformed and 35S:ERS transgenic plants of the T1 generation. All plants harbored the trans-gene. Bottom panel – ethidium bromide stain of the total RNA samples. Plants No. 7, 8, 11, 15 in Family 6, and plants 2, 7, 8 and 14 in Family 7 displayed Type II phenotype with increased femaleness, all others were designated Type I, i.e., similar to non-transformed Poinsett 76 plants.

Figure 18. Delayed senescence of detached leaves and leaf disks of feminized 35S:ERS plants. WT – untransformed Poinsett76; 4-1, 4-8 – transgenic plants with no phenotypic alteration; 6-7, 7-3 – transgenic plants with enhanced femaleness.

3.5. Other transgenic lines – Bar-Ilan University and Newe Yaar, ARO

In addition, transgenic plants harboring an *ACS1*-silencing construct were produced and selfed during Spring 2003. Their phenotypic evaluation during 2004 did not reveal visible alterations. A few plants that expressed ERS under the AP3 promoter failed to produce progeny. Tobacco AP3:GUS plants were made to demonstrate the functioning and specificity of the Arabidopsis AP3 promoter that we cloned in an heterologous species (Second Annual Report). In Newe-Yaar, melon transformation experiments were carried out with 35S:ACO3 constructs in the sense and antisense conformation. Seeds of muskmelon (*Cucumis melo* L. cv. 'Cezan') were used in all the experiments. Regeneration and transformation were performed using modifications to Fang and Grumet (1990). Sterilized seeds were germinated on agar medium and 48-72 hr. later transformed with *Agrobacterium tumefaciens* containing either the sense or antisense sequences encoding the ACC-oxidase 3 gene. Freshly dissected cotyledon pieces were soaked for 15 min in *Agrobacterium* suspension, blotted dry and transferred to regeneration medium. About two weeks later, leaf explants were transferred to an elongation medium for two more weeks. For rooting - the regenerated plants were transferred to a rooting medium and the resulting plants were consequently transferred to a hardening medium (Jiffy) for additional three weeks. Once the plants were established they were moved to the greenhouse.

A total of 80 muskmelon seeds were germinated. Approximately 80 cotyledons were transformed with the sense construct, and 80 with the antisense. In ca. 70 of the ~80

transformed cotyledons with each construct, callus formation was observed, and each of those developed 1 to 5 leaf explants. Altogether, an average of 210 leaf explants were formed in both treatments (sense/antisense) and transferred to the elongation medium. About one quarter (50) of the sense leaf explants developed roots and were then placed on hardening medium. Half of them (~25) survived. With the antisense gene, roots development was less successful (~25) and none of them survived on the hardening medium. The presence of the construct could be detected in six out of the 25 sense-plants tested using PCR with specific primer pair (35S forward and ACC-oxidase reverse) (Figure 1). Plantlets are being hardened and transferred to greenhouse condition for phenotypic and molecular analysis.

Figure 19: PCR products of the ACC-oxidase gene on 1.2% agarose gel.
SA-SD: leaf explants from the elongation medium, S20, S30, S37 & S48: greenhouse plants.
M: DNA marker.

Figure 20: *Agrobacterium*-mediated transformation steps.
Plants in (A) callus induction medium, (B) elongation medium, (C) hardening medium, (D) greenhouse.

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Line	Ploidy	Segregation	Expected ratios	χ^2
H-ACS1	4n	21:12	3:1	1.71 ns
H-ACS3	4n	30:3	3:1 / 15:1 / 35:1*	3.64 ns / 0.13 ns / 2.92 ns
H-ACS4	4n	12:0	3:1 / 15:1 / 35:1	2.77 ns / 0.09 ns / 0.14 ns

Table 11. Segregation analysis of T₁ progeny of the transgenic 35S-ACS Hales' Best Jumbo (H) melons.

Segregating progeny were assayed for presence of the introduced gene by PCR amplification of the *ACS* gene or by ELISA assay for expression of the *NPTII* gene
ns - χ^2 value not significant at $P < 0.05$

χ^2 values were calculated based on one or two insertions. If chromosome doubling occurs before insertion, 3:1 transgenic:non-transgenic ratios are expected for a single site of integration, and 15:1 for two sites of integration. If doubling occurs after insertion, a ratio of 35:1 is expected for a single insertion site.

ACC content (nmol evolved ethylene/g)			
Genotype	Leaf	Male bud	Hermaphrodite
H-WT	18.5 $\pm$ 2.9 a	100.6 $\pm$ 15.4 a	42.7 $\pm$ 11.3 a
Azygous (4n)	14.5 $\pm$ 2.6 a	257.5 $\pm$ 41.4 c	56.3 $\pm$ 9.8 a
H-ACS3	36.7 $\pm$ 5.6 b	177.8 $\pm$ 27.2 b	134.3 $\pm$ 25.5 b

Table 12. ACC content of Hales' Best Jumbo wild type, azygous tetraploid, and transgenic H-ACS3 leaves, male buds, and hermaphrodite buds.

Values are the mean of combined data from three experiments $\pm$ S.E. The total replicates were: n=5, n=12, and n=7 for H-WT, H-4nAZ, and H-ACS3 leaves, respectively; n=6, n=12, and n=7 for H-WT, H-4nAZ, and H-ACS3 male buds, respectively; and n=4, n=10, and n=6 for H-WT, H-4nAZ, and H-ACS3 hermaphrodite buds, respectively. The trends were the same in all three experiments. Significant differences in ACC content were observed among genotypes for all three tissues (ANOVA, $P < 0.01$). Values within a column followed by different letters are significantly different by $LSD_{0.05}$.

Trait	H-WT	H-4nAZ	H-ACS3	H-ACS4
% mature hermaphrodite flowers (main stem)	24.0 $\pm$ 2.0 a	39.1 $\pm$ 1.1 b	57.4 $\pm$ 1.1 c	52.0 $\pm$ 3.4 c
Number of instances of adjacent mature hermaphrodite flowers (main stem)	0.9 $\pm$ 0.3 a	3.2 $\pm$ 0.7 b	6.2 $\pm$ 0.6 c	5.7 $\pm$ 0.6 c
% mature hermaphrodite flowers that set fruit (main stem)	15.0 $\pm$ 4.0 a	20.8 $\pm$ 8.1 a	27.8 $\pm$ 1.8 a	22.2 $\pm$ 1.6 a
% plants with 2 fruit within 4 nodes (main stem)	6.7 $\pm$ 1.7 a	16.7 $\pm$ 17 a	65.0 $\pm$ 5.0 b	53.3 $\pm$ 13 b
Average fruit size (kg)	0.91 $\pm$.07 a	0.47 $\pm$.01 b	0.45 $\pm$.02 b	0.43 $\pm$.01 b
Total number of fruits set per plant	9.6 $\pm$ 1.0 a	6.8 $\pm$ 2.7 a	11.6 $\pm$ 1.0 a	9.9 $\pm$ 1.5 a

Table 13. Reproductive characteristics of ACS and control melons in the field.

Data are the mean values $\pm$ S.E. of three replicate plots/genotype. Numbers within a row followed by different letters are significantly different by LSD, P=0.05

Genotype (n)	Length	Fr. Weight	Dry Weight
WT	15	12.6 $\pm$ 2 cm	0.62 $\pm$.25 g
NT6	7	12.8 $\pm$ 4 cm	0.81 $\pm$.2 g
etr6	17	3.4 $\pm$ 3 cm	0.13 $\pm$.1 g

Table 14. Root growth on lateral cuttings from transgenic and control melon plants.

Data are pooled from three experiments.

WT: control Hale's Best Jumbo; NT6: non-transgenic segregant T₁ progeny from etr line 6;

etr6: transgenic *etr1-1* T₁ plants from line 6.

Line	Leaf	F locus	QTL locus				
			SE1	SE2	SE3	SE4	SE5
SE-21	N	FF	a1a1	a1a1	a1a1	a1a1	a1a1
SE-22	N	FF	a1a1	a1a1	a1a1	a1a1	a2a2
SE-29	N	FF	a1a1	a1a1	a2a2	a1a1	a1a1
SE-34	N	FF	a2a2	a1a1	a2a2	a1a1	a2a2
SE-37	N	FF	a2a2	a1a1	a2a2	a1a1	a1a1
SE-38	N	FF	a1a1	a1a1	a2a2	a1a1	a2a2
SE-39	N	FF	a1a1	a1a1	a2a2	a2a2	a2a2

Table 10:
 Nearly Isogenic Lines (NIL) and their genotype at
 QTL associated with sex expression
 Notes:
 Leaf: as in previous tables
 F locus: genotype at the F locus. All lines are FF.
 SE1-SE5: QTL loci associated with sex
 expression
 a1: allele associated with pistillate flowers
 a2: allele associated with staminate flowers