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SEX EXPRESSION IN CUCURBITS: THE ROLE OF ETHYLENE SYNTHESIS AND PERCEPTION, AND SEX DETERMINATION GENES.

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ABSTRACT

SEX EXPRESSION IN CUCURBITS: THE ROLE OF ETHYLENE SYNTHESIS AND PERCEPTION, AND SEX DETERMINATION GENES.

By

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Cucurbitaceae species display a range of heritable sex patterns that are subject to hormonal control. Ethylene, which is able to promote pistillate flowers and suppress male flower formation, appears to be the main hormone influencing sex determination. Application or inhibition of ethylene increases or decreases femaleness (pistil development) respectively; higher levels of endogenous ethylene and expression of genes for ethylene biosynthesis or perception, have been correlated with female flower production. I sought to investigate the effect of modified endogenous ethylene production and perception on sex expression by producing transgenic melons constitutively expressing the *ACS* (*1-aminocyclopropane-1-carboxylic acid synthase*) gene, encoding the enzyme that catalyzes the first step in ethylene biosynthesis, or the mutant *Arabidopsis etr1-1* (*ethylene resistant*) gene that causes ethylene insensitivity. The *ACS* melons showed increased ethylene evolution by leaves and flower buds and also exhibited increased femaleness as measured by early appearance of the first hermaphroditic flower bud, increased number of total hermaphroditic buds, and increased number of hermaphroditic buds that reached anthesis. The heterologous *etr1-1* gene was able to confer ethylene insensitivity as evidenced by higher ethylene production by male buds, decreased rooting, and decreased rate of flower pedicel abscission, even in presence

of ethylene. Expression of the *etr1-1* gene also prevented hermaphroditic flower formation in both andromonoecious and gynoecious melon lines. The phenotypic observations indicate that the ability to perceive ethylene is required for promotion of femaleness at the time of sex determination. I also investigated the role of the maize sex determination gene *TASSELSEED2* (*TS2*) and the effect of exogenous brassinosteroids on sex expression pattern of andromonoecious melons. Presence of the *TS2* gene did not affect either earliness or the number of hermaphrodite buds formed. Application of the hormone brassinosteroid increased femaleness in cucumber by inducing early formation and higher number of female flowers, likely via induction of increased ethylene production. These results presented in this dissertation provide direct demonstration of the importance of endogenous ethylene synthesis and perception in pistillate flower formation in melon.

To my mother, sister, brother and the memory of my father

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INTRODUCTION

The majority of flowering plants produce hermaphrodite (bisexual or perfect) flowers, bearing both male (stamens) and female (pistil) reproductive organs on the same individual flower. However, many angiosperm species produce unisexual flowers, carrying only one reproductive organ (Dellaporta and Calderon-Urrea, 1993). Flowering habit in cucumber (*Cucumis sativus* L.) is mainly monoecious, with individual plants bearing both male and female flowers, although in different locations along the main stem. During plant development, flowering begins with production of male flowers followed by a phase, of predominantly female flowers (Perl-Treves, 1999). In melon (*Cucumis melo* L.), andromonoecious cultivars prevail, where an individual plant bears male flowers followed by hermaphrodite flowers (Kenigsbuch and Cohen, 1989).

Genetic, hormonal and environmental factors contribute to the flower sex determination (Roy and Saran, 1990). Three major loci *F*, *M*, and *A* determine the different sex patterns observed in cucumber genotypes (Perl-Treves, 1999). The degree of femaleness is determined by the *F* (*Female*) locus, which can be influenced by modifying genes [*Intensifier of female expression* (*In-F*) and *gynoecious* (*gy*)] and environmental factors (Perl-Treves, 1999). Sexual patterns in muskmelon (*Cucumis melo*, L.), are influenced by at least three genes *A*, *G* and *M* (Kenigsbuch and Cohen, 1989; Roy and Saran, 1990). Gene *A* functions to suppress anther development in pistillate flowers while the *G* gene inhibits gynoecium development in male flowers. Genetic studies of the gynoecy inheritance showed that the *M* (maleness) locus in the recessive form *mm* is required to complete and stabilize the gynoecious phenotype (Kenigsbuch and Cohen, 1989).

Sex expression patterns in cucurbits can be modified by hormonal application, a feature that is used by breeders to facilitate production of the appropriate sex type, if it is not normally expressed (Tolla and Peterson, 1979). Environmental factors also can influence sex expression, most likely via hormonal effects. GAs, auxins and ethylene, all have been demonstrated to modify sex expression (Roy and Saran, 1990; Rudich, 1990). However, ethylene, which promotes femaleness, appears to be the dominant factor (Yin and Quinn, 1995).

Elucidation of molecular mechanisms involved in sex determination will result in scientific and practical benefits. For example gynoecious (only female flowers) lines provide a valuable tool as seed parents in hybrid production, eliminating hand-pollination and chemical treatments, which are common practices when monoecious, andromonoecious or gynomonoecious lines are used (Kenigsbuch and Cohen, 1989). These lines also show early and concentrated fruit set compared to monoecious lines. Knowledge of the regulatory mechanisms and the use of gene technology will provide new ways of manipulating sex expression in cucurbits and possibly in other species. (Dellaporta and Calderon-Urrea, 1993).

The present work will focus on the hormonal factors involved in sex determination, and the effect of the heterologous sex determination gene *TASSELSEED2* (*TS2*). In the following chapters, the transformation of melon plants with genes related to ethylene production and perception and the heterologous sex determination gene *TS2* will be discussed. Furthermore, the potential influence of the new hormonal group brassinosteroids, on sex differentiation of cucumber will be examined.

REFERENCES

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

Kenigsbuch D, Cohen Y (1989) The inheritance of gynoecy in muskmelon. Genome **33:** 317-320

Perl-Treves R (1999) Male to female conversion along the cucumber shoot: approaches to studying sex genes and floral development in *Cucumis sativus*. *In* C Ainsworth, ed, Sex determination in plants. Bios Scientific Publishers, Oxford, UK, pp 189-215

Roy RP, Saran S (1990) Sex expression in the Cucurbitaceae. *In* DM Bates, RW Robinson, C Jeffry, eds, Biology and utilization of the Cucurbitaceae. Cornell Univ. Press, Ithaca, NY, pp 251-268

Rudich J (1990) Biochemical aspects of hormonal regulation of sex expression in Cucurbits. *In* DM Bates, RW Robinson, C Jeffry, eds, Biology and utilization of the Cucurbitaceae. Cornell Univ. Press, Ithaca, NY, pp 269-280

Tolla GE, Peterson CE (1979) Comparison of gibberellin A4/A7 and silver nitrate for induction of staminate flowers in a gynoecious cucumber line. HortScience **14:** 542-544

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

LITERATURE REVIEW

Introduction

The majority of flowering plants form bisexual or hermaphroditic flowers, carrying both male and female reproductive organs. The stamens, which produce the male gametes (pollen) in the anthers, are considered the male organs of the flowers. The carpels are considered the female organs, since they produce the embryo sac containing the egg cell. Hermaphroditic flowers consist of four groups of floral organs, sepals, petals, stamens and carpels, arranged in four distinct whorls (Ng and Yanofsky, 2000; Jack, 2001). Flower development studies in *Arabidopsis* have shown that organ identity is specified by the function of three classes of homeotic genes. Class A genes, *APETALA1* (*AP1*) and *APETALA2* (*AP2*), specify sepal development at the outer most whorl. The combined function of class A and B, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), genes regulate development of the second whorl organs, the petals. Genes of classes B and C (*AGAMOUS*, *AG*) together specify stamen identity, at the third whorl, and finally function of class C genes controls carpel development in the fourth whorl. Mutations in these genes, which belong to the group of MADS-box DNA-binding transcription factors, cause transformations of one organ type to another.

Sexual reproduction is the major mechanism by which genetic variation is redistributed and passed on to the progeny. In particular, cross-pollination avoids inbreeding depression, promotes heterozygosity, genetic variability and exchange that can serve the long-term survival and adaptation of the species. Flowering plants have evolved several mechanisms to promote cross-pollination and fertilization that fall in three main categories (Ainsworth et al., 1998). One mechanism is self-incompatibility, where self-fertilization is inhibited due to genetic factors of the parent plant (sporophytic) or the pollen (gametophytic). Another mechanism is dichogamy, which refers to the temporal separation of maturation of the reproductive organs, and includes protandry when the stamens shed pollen before the stigma of the female organ is receptive, and protogyny when the stigma is receptive before the pollen is released. The final mechanism is unisexuality, the spatial separation of the reproductive organs. Unisexual flowers carrying one type of reproductive organ can be found on the same plant (monoecy), or on separate individuals (dioecy). Several reviews of plant sex determination leading to unisexual flowers have been written recently including those by (Dellaporta and Calderon-Urrea, 1993; Ainsworth et al., 1998; Grant, 1999).

Even though plants bearing unisexual flowers occur rarely in nature, they are predominantly found in three families, the Cucurbitaceae, Caricaceae and Salicaceae, and they include several agronomically important species. Monoecious species include maize, cucumber, melon, hazelnut, fig, walnut and castor bean. Dioecious species include asparagus, date palm, kiwi, papaya, hemp, hop, yam, pistachio, willow, spinach, poplar and mistletoe. In other families unisexual plants are rare and scattered, suggesting that they evolved independently several times. This literature review will focus on the mechanisms that control unisexuality. In the majority of cases, even when genetic factors controlling sex expression have been identified, the function of these genes is not known. A key area of research interest is to understand the genetic base of sexual determination.

A. Sex Determination in Dioecious Plants

Dioecy occurs in about 7% of angiosperm species (Dellaporta and Calderon-Urrea, 1993). Individuals within a population carry either male of female unisexual flowers. Sex determination may occur late in development at flower organogenesis or reproductive organ differentiation, or as early as floral organ initiation or specification (Dellaporta and Calderon-Urrea, 1993). Sex determination in dioecious species is based on an interplay between environmental factors, hormonal regulation and genetic determinants. Many species are characterized by distinct sex chromosomes, such that sex is determined by segregation of heteromorphic X and Y chromosomes, X to autosomic (X/A) chromosome ratios, or the multiple chromosome system (Dellaporta and Calderon-Urrea, 1993). In numerous other dioecious species there appear to be major loci determining gender dimorphism that segregate in meiosis. Although one sex type produces heterozygous gametes and the other homozygous gametes, it is still not clear whether the sexual dimorphism is due to allele segregation at a single locus or of a pair of chromosomes that are cytologically indistinguishable (Grant, 1999). Several different examples of dioecy will be described below.

1. Sex Chromosome Systems

In certain dioecious species sex is determined by differentiated chromosomes that can be easily identified, because they differ in size and morphology, and in meiosis are unable to pair over a large portion of their length (Ainsworth et al., 1998). These differentiated chromosomes are found in five families, Cannabidaceae, Caryophyllaceae, Cucurbitaceae, Lavanthaceae (Loranthaceae) and Polygonaceae, and usually only in few species within each family (Grant, 1999). For example, sex chromosomes are found only in white campion, *Silene latifolia* and *S. dioica* (Caryophyllaceae), two species in a genus of 500, and a family of about 2000 species. One exception is the Cannabidaceae family, where all the three species carry sex chromosomes, *Humulus lupulus*, *H. japonicus*, and *Cannabis sativa*.

In contrast to the animal sex chromosomes where Y is the smallest, and contains degenerate genes, in plants sex chromosomes are usually the largest (except in *H. lupulus*), due to accumulation of repetitive DNA (Grant, 1999). In white campion they contain close to 10% of total genomic DNA. Male sorrel (*Rumex acetosa*) plants have two chromosomes Y1 and Y2, both smaller than the X, but together they make up the largest chromosome. The sorrel Y chromosomes contain condensed heterochromatin, while in most other species chromosomes contain uncondensed euchromatin, indicating that many genes might be encoded by the Y chromosomes (Grant, 1999). The lack of condensation and the large size indicate that sex chromosomes of dioecious plants have probably originated from autosomic pairs, more recently than the animal chromosomes.

a. Heteromorphic Sex Chromosomes

i. Active-Y Chromosome System

Gender Dimorphism in *Silene latifolia*

In the dioecious species *S. latifolia* (white campion) (Caryophyllaceae), sex determination relies on an active-Y chromosome system, which carries dominant male promoting and female suppressing factors (Dellaporta and Calderon-Urrea, 1993)). In male or female flowers organ development is similar until petal and stamen primordia arise, at which time differences become apparent, e.g. the carpel whorl is larger in the female buds. In female flowers, stamen development is arrested at the stage of tapetal cell initiation, while in male flowers the gynoecium is arrested soon after its initiation, indicating that timing of female organ development arrest in male flowers is independent of stamen developmental arrest in female flowers (Ainsworth et al., 1998).

Male plants are heterogametic carrying XY chromosomes, while female plants carry XX (Ainsworth et al., 1998). The X and Y chromosomes are homologous over a part of their length, where they pair in meiosis. Even though the X chromosome is essential for both male and female plants, presence of a single Y can suppress female development even in presence of three X chromosomes, e.g. 2X: 1Y gave 89% males and 4X: 1Y gave 35% males.

Based on genetic analysis of Y chromosome deletion mutants that led to production of hermaphroditic and asexual plants, three different regions of the Y chromosome have been identified to affect sex determination (Lardon et al., 1999). One arm (p) contains factors to suppress carpel development, the gynoecium-suppressing function (GSF), since loss of that arm part resulted in hermaphroditic flowers, due to failure of gynoecium suppression (Lardon et al., 1999). The central domain of the same arm contains the stamen-promoting function (SPF) responsible for anther initiation. Mutants show very early arrest in stamen development, and asexual phenotypes result from carpel suppression and failure to initiate stamens (Farbos et al., 1999). Finally, asexual deletion mutants indicate that the opposite arm includes male fertility factors; carpel formation was suppressed and stamen development failed before maturity and led to degenerate anthers.

Female sex suppression in white campion appears to involve methylation of specific DNA sequences (Janousek et al., 1996). Treatment with the hypomethylating agent 5-azacytidine induced androhermaphroditism (gynoecium development) in 21% of male plants, while female plant sex determination was not affected. The 10% decreased hypomethylation was mainly found in CG doublets, and the effect was transmitted in two successive generations. It was postulated that the modification occurred either in the female-suppressor complex located on the Y chromosome or in a few regulatory female-promoting gene(s) located on the autosomes (Janousek et al., 1996).

Interestingly, sex determination in white campion can be modified by infection with the anther smut fungus *Ustilago violacea*, which causes stamen development in genetically female plants, such that the anthers contain fungal spores and not pollen grains (Scutt et al., 1997). Stamen development suggests that the fungus can substitute one or more functions of the Y chromosome. Conversely, infection of male plants allows in gynoecium development to proceed to further stage than in normal male flowers, indicating that the fungus is able to reduce the function of female suppressors of Y chromosome, or to activate autosomic female promoting factors (Ainsworth et al., 1998). Screening for male specific genes that are expressed in response to Y chromosome sex determination genes led to isolation of eight cDNA clones *Men-1* to *Men-8*, which show a diverse range of proteins mainly of unknown function (Scutt et al., 1997). Three of these genes showed induced expression in stamens of smut-infected female flowers.

ii. Dosage Sex Chromosome System. X to Autosomes (X/A) Balance System

Gender Dimorphism in *Humulus lupulus*

Sexual differentiation in *H. lupulus* (Cannabidaceae), the cultivated hop, occurs early in flower development; rudiments of the inappropriate organ are not apparent, suggesting that flowers do not pass through a bisexual phase (Ainsworth et al., 1998). Y chromosomes of this species are unique in that they are smaller than the X chromosome. Female plants are homogametic carrying XX, while the males have X and Y chromosomes. However, a X to autosome ratio also operates: where maleness is associated with an X/A ratio of 0.5 or less, while females have a ratio of 1, and intermediate ratios give intersexual plants. Even though Y chromosomes are not essential for the development of male phenotype, pollen does not mature in Y deletion mutants (Parker and Clark, 1991). Hormonal factors can also affect the sex determination process, with auxins and ethylene increasing femaleness, whereas cytokinins and GA increase male flowers (Ainsworth et al., 1998).

Gender Dimorphism in *Rumex acetosa*

R. acetosa (sorrel) belongs to the Polygonaceae family and is a strictly dioecious species. Male and female buds pass through a bisexual phase, but the differences are shown soon after primordia initiation, so that there is little development of the inappropriate set of organs (Ainsworth et al., 1998). Sex determination is controlled by a multiple chromosome system, with female plants carrying two X chromosomes and male plants one X and two Y chromosomes (XY1Y2). Moreover, diploid plants with XXY and XXY1Y2 produce fertile females. In polyploid plants, X/A ratio of 1 or higher is female, ratios of 0.5 or lower are males, while plants with intermediate ratios are hermaphroditic or intersexual. Furthermore, the size and number of autosomic chromosomes influence sex determination, as male or female promoting factors are also located on the autosomes that interact with the sex chromosomes (Dellaporta and Calderon-Urrea, 1993). In this case also, the Y chromosome is not the determining factor for male flower production or stamen development (e.g. near tetraploid plants, 2n=24+XXX, with a ratio of 0.75 are hermaphroditic) (Ainsworth et al., 1998), but Y is necessary for normal progression of microspore mother cells through meiosis, for pollen maturation (Negrutiu et al., 2001). In the case of sorrel, the Y chromosome does not carry gynoecium inhibitory factors, since hermaphroditic plants arise even in presence of Y, thus inhibition of gynoecium depends on X/A ratio (Parker and Clark, 1991). Y chromosomes have accumulated repetitive DNA, they display depletion of H4 histone acetylation and maintain their condensed state even in interphase, suggesting a case of constitutive heterochromatin (Negrutiu et al., 2001). However, they have a small euchromatic region, which is the X/Y pairing region. Individually, the Y chromosomes are shorter than X, but combined are 60% longer (Parker and Clark, 1991).

b. Homomorphic Sex Chromosomes

Gender Dimorphism in *Asparagus officinalis*

Morphological evidence shows that sex determination in the dioecious species *Asparagus officinalis* (Liliaceae) relies in selective abortion of the gynoecium or the androecium in initially hermaphroditic floral primordia, in male and female individuals, respectively (Bracale et al., 1991). Abortion occurs late in development, in pollen-mother cells and anthers in females and in megaspore-mother cells, but not in the ovary, in males. Higher levels of cytokinins, auxins (IAA) and abscisic acid (ABA) have been associated with male plants, with ABA regulating the balance of hormones (Bracale et al., 1991). In cultivated populations male and female individuals occur in a 1:1 ratio, but occasionally individuals carry a few perfect flowers. Genetic studies based on these individuals showed that the male plants are heterogametic, maleness is dominant and the factors involved in sex differentiation are associated with the chromosome pair L5. In addition, spontaneous hermaphroditic and asexual individuals appear, indicating that sex chromosomes carry carpel suppressing and stamen promoting factors. Hermaphroditic plants arise from mutations in the carpel suppressors, while mutations in stamen promoting factors cause an asexual phenotype (Bracale et al., 1991; Ainsworth et al., 1998; Grant, 1999).

2. Genic Sexual Determination

Gender Dimorphism in *Mercurialis annua*

In *M. annua* (Euphorbiaceae), where sex determination occurs early enough in development that the flowers do not show any rudiments of the inappropriate set of reproductive organs, sex is controlled by three unlinked loci, *A, B1* and *B2* (Durand and Durand, 1991; Ainsworth et al., 1998; Grant, 1999). In general, male plants have one dominant *A* allele and a dominant allele at the *B* loci, while female plants carry a dominant *A* allele and recessive alleles at both *B* loci, or a recessive *A* and any allele of the *B* loci. Hormones can influence sex expression; the response though to exogenous hormone treatment depends on the presence of dominant alleles at *B1* or *B2* loci. Strong males showing resistance to the feminizing effect of cytokinins have dominant alleles at both *B* loci. Intermediate males that can be partially feminized by cytokinin carry one or two dominant alleles at *B1* and one or two dominant at *B2*, while weak males carry a recessive allele at *B1* and one or two dominant alleles at *B2*. Male and female plants show qualitative and quantitative differences in endogenous cytokinins, so that female plants contain trans-zeatin and the male plants trans-zeatin mononucleotide (riboside). Strong male plants contain high levels, weak males lower levels, and female plants the lowest. The dominant *A* allele controls synthesis of trans-zeatin riboside levels, while the

B loci prevent its conversion to another form. Thus, female plants either produce low levels of trans-zeatin mononucleotide if carry the recessive *A* allele, or if they carry the dominant allele, it is metabolized quickly due to presence of dominant alleles at the *B* loci. In addition, high zeatin induces auxin oxidases that decrease auxin concentration. High levels of auxin oxidases are found in strong males, with dominant *B1* and *B2* alleles and minimum levels in female plants. In conclusion, in this case where function of the sex genes has been determined, the sex controlling gene products interact to control the levels of growth substances in the plants.

B. Sex Determination in Monoecious Plants

Chromosome differences are not associated with sexual dimorphism in monoecious species as both male and female flowers are produced on the same plant. Various genes have been identified, however, that can modify sex expression and in several cases, as described for *M. annua* above, they appear to be associated with plant hormone levels (Kamachi et al., 1997; Trebitsh et al., 1997). Two of the best-studied monoecious species are maize and cucumber.

<u>Gender Dimorphism in *Zea mays*</u>

Maize or corn (*Zea mays* L.) carries a terminal male inflorescence, the tassel, and a lateral female inflorescence, the ear. The ear florets are arranged in spikelets, with an upper and a lower floret. At early stages flower buds are hermaphroditic; later in development, pistil primordia abort in male florets and only stamens develop. The reverse occurs in female florets. In the tassel both florets develop, while in the ear, only the upper floret of the spikelet matures to female and the lower aborts (Dellaporta and Calderon-Urrea, 1993).

Gibberellins (GAs) are involved in sex determination of maize by inhibiting maleness, a role that is supported by the higher GA content in the ears than the tassel and the sex phenotypes of GA-deficient (*dwarf, d*) (Rood and Pharis, 1980) and GA-responsive mutants (*anther ear1, an1*) (Bensen et al., 1995). The phenotypes of *d* and *an1* include reduced internode length, delayed flowering, and stamen development in ear florets, resulting in hermaphroditic florets (Rood and Pharis, 1980; Bensen et al., 1995). Pistil abortion in tassel florets is unaffected in *d* and *an1* mutants (Rood and Pharis, 1980; Bensen et al., 1995). In contrast, mutation in the *TASSELSEED 2* (*TS2*) gene leads to the formation of female flowers in the normally male tassel and in the ears the lower floret does not abort (DeLong et al., 1993). Thus, the wild type *TS2* gene product inhibits femaleness. The proposed function of *TS2* is to trigger a programmed organ death that results in gynoecium abortion in the tassel and the suppression of the lower floret development in the ear (Calderon-Urrea and Dellaporta, 1999). The *TS2* gene has been cloned and the predicted protein shows similarity to bacterial short chain steroid alcohol dehydrogenases (DeLong et al., 1993). GAs have been suggested as potential substrates of the proposed steroid alcohol dehydrogenase activity of TS2 (DeLong et al., 1993). In this case, *TS2* would act antagonistically to the feminizing effect of GAs by direct inactivation, or indirectly by inhibiting pistil development. However, the phenotype of the double mutant *ts2/d1* is additive; hermaphrodite flowers are formed both in tassel and ear, suggesting the possibility that *TS2* works independently from GAs (Dellaporta and Calderon-Urrea, 1993). Brassinosteroids, also have been suggested as a possible substrate for the TS2 steroid alcohol dehydrogenase (Lebel-Hardenack et al., 1997).

Recently, it has been shown that pistil abortion in the tassel is caused by DNA degradation in the sub-epidermal cells, in the central region of the pistil primordia (Calderon-Urrea and Dellaporta, 1999). As the spikelets progressively mature the cell area becomes greater, but the epidermal cells keep their integrity during the whole process, until the pistil collapses (Calderon-Urrea and Dellaporta, 1999). Two more genes are involved in the proposed model, *TS1*, which is required for *TS2* expression, and *silkless1* (*sk1*), which is partially epistatic to *TS2* and promotes pistil development by protecting pistil primordia in the ear upper floret from the *TS2* function.

Gender Dimorphism in *Cucumis sativus* and *Cucumis melo*

Plant species of the *Cucurbitaceae* family, show numerous patterns of sex expression regulated by genetic and hormonal factors, and so have been used to study mechanisms of sex differentiation (Roy and Saran, 1990; Perl-Treves, 1999). Monoecy and andromonoecy are the most common patterns in cucurbits, where male flowers are produced first, followed by a predominantly female or hermaphroditic phase, along the main stem. Genetic variants form a spectrum of patterns including hermaphroditic plants that produce bisexual flowers, gynoecious plants producing only pistillate flowers, and androecious plants producing only staminate flowers (Roy and Saran, 1990).

In developing flower buds both sets of sex organs are initiated, subsequently the inappropriate organ development arrests. Several studies have examined the possible role of MADS-box genes in differential sex organ development. Based on the ABC model, loss of an A, B, or C gene, affects two adjacent whorls, rather than a single organ (Coen and Meyerowitz, 1991). For example mutations in B-function genes cause transformation of petals into sepals and stamens into carpels. These homeotic changes are not observed in unisexual flowers, where only one whorl is affected. Study of homeotic mutants, however, has added to our understanding of the process leading to unisexual development (Kater et al., 2001). In the spontaneous B-function cucumber mutant *green petals* (*gp*) the female buds show a typical B-function homeotic transformation in the second whorl, but in third whorl the predicted carpels do not develop. Thus this phenotype indicates that developmental arrest of the inappropriate sex organ depends on its position rather than its sexual identity, e.g. in a *gp* homeotic mutant, whorl three is eliminated even though it would produce carpels. Similarly, in the node positions that would normally produce male buds, only the predicted first three whorls developed, even though the third whorl produced carpels instead of stamens or became indeterminate. Other MADS-box related genes, however, might play a role in sex-specific development.

Three cucumber (*Cucumis sativus* L.) homologues (*CAG1, CAG2,* and *CAG3*) of the *Arabidopsis AGAMOUS* (*AG*) C-function gene were isolated by screening c-DNA libraries of flower buds at the bisexual stage (Perl-Treves et al., 1998a). Transcripts of *CAG1* and *CAG3* continued to be present at a later stage, in both male and female buds, showing a classic C-function gene expression, however the *CAG2* clone was expressed only in the carpels of pistillate buds. Expression levels of *CAG* genes were not affected by application of hormones that influence sex expression (GA or ethylene) suggesting that although *CAG2* may be expressed during pistil development, it is not likely to be the regulatory factor causing pistillate vs. staminate flowers.

Last year, a new class of ABC-model MADS-box genes, *ERAF17*, was identified in apices of ethephon-treated monoecious and gynoecious cucumber genotypes (Ando et al., 2001). Timing of expression was correlated to induction of female flowers, leading the authors to suggest that induction of female bud formation may be regulated by expression of a novel MADS-box gene. Like *CAG2*, but unlike standard C-function genes that promote both stamens and pistils, *ERAF17* appears to be associated with development of pistils, but not development of stamens. Thus, even though the MADS-box genes play important roles in flower development, it appears that the canonical A, B, C type genes are not likely to be responsible for development of unisexuality. Whether suppression of stamens results from other aspects of the ethylene treatment or normal development and not from *ERAF17* expression remains to be determined.

Several genes influencing sex expression have been described in cucumber (Perl-Treves, 1999). The primary genes appear to be *F* and *M*. The partially dominant *F* locus regulates the degree of femaleness, while the *M* locus is responsible for the monoecious vs. andromonoecious pattern. The proposed genetic profile of the monoecious sexual type, which is the most common, is *M-ff*. As will be discussed later, *F* is thought to be associated with ethylene production and *M* is thought to be associated with ethylene perception (Yin and Quinn, 1995; Perl-Treves, 1999; Yamasaki et al., 2000). Additional genes have been identified that modify the effect of the major genes. The recessive *A* locus intensifies maleness in homozygotes for the recessive allele at the *F* locus. *Tr* (*trimonoecious*), a co-dominant gene, removes the inhibition of carpel development in unisexual male buds and converts them to hermaphrodites. Finally, the *In-F* (*intensifier-female*) gene intensifies femaleness in monoecious genotypes and when crossed with a gynoecious genotype abolishes responsiveness to GA treatment (Perl-Treves, 1999).

Sexual patterns in muskmelon (*Cucumis melo*, L.), are influenced by at least three genes *A*, *G* and *M*. Gene *A* functions to suppress anther development in pistillate flowers while the *G* gene inhibits gynoecium development in male flowers (Roy and Saran, 1990). Genetic studies of the gyneocy inheritance showed that the *M* (maleness) locus in the recessive form *mm* is required to complete and stabilize the gynoecious phenotype (Kenigsbuch and Cohen, 1989). The most prevalent type of sex pattern is andromonoecy, where hermaphroditic buds follow the male bud phase. The hermaphrodite plants have a *aagg* genetic profile, andromonoecious *aaG---*, monoecious *A-G---*, gynomoecious *A-gg* and gynoecious *A-ggmm* (Poole and Grimball, 1939; Kenigsbuch and Cohen, 1989).

Sex expression patterns in cucurbits can be modified by hormonal application, a feature that is used by breeders to facilitate production of the appropriate sex type, if it is not normally expressed (Tolla and Peterson, 1979). Environmental factors also can influence sex expression, most likely via hormonal effects (Roy and Saran, 1990). GAs, auxins and ethylene, all have been demonstrated to modify sex expression. However, as will be discussed below, ethylene, which promotes femaleness, appears to be the dominant factor (Yin and Quinn, 1995).

As in the case of maize, GA influences sex expression in cucurbits, but in the opposite direction. Application of GA enhances the male tendency of the plant by increasing initiation of male buds or delaying the appearance of the continuous female phase, either by inhibiting pistillate buds from full development or causing abortion of pistillate buds (Atsmon et al., 1968; Freidlander et al., 1977; Tolla and Peterson, 1979; Perl-Treves, 1999). Endogenous GA levels are usually higher in monoecious and andromonoecious genotypes compared to gynoecious, and higher in long-day conditions rather than in short days.

In contrast, exogenous auxins enhance female flower production. The effect is likely to be indirect, however, as endogenous auxin levels were found to be lower in gynoecious than monoecious lines and auxin inhibitors did not modify sexual pattern of gynoecious plants (Trebitsh et al., 1987). Application of auxins induced ethylene production and the ethylene biosynthesis enzyme, 1-aminocyclopropane-1-carboxylic acid (ACC), synthesis in cucumber, suggesting that the effect of exogenous auxins is likely to be mediated via ethylene.

Ethylene, which increases femaleness, is able to promote pistillate flower formation, and in some species suppresses male organ development (Rudich, 1990; Yin and Quinn, 1995). Application of ethylene or ethylene releasing agents increased female flower production in monoecious cucumber and squash, and androecious cucumber (Augustine et al., 1973), and hermaphroditic flower production in andromonoecious muskmelon plants (Rudich et al., 1969). In addition, higher levels of endogenous ethylene (usually 2-3 fold) have been associated with gynoecious sex pattern in cucumber and muskmelon (Byers et al., 1972; Rudich et al., 1972; Rudich et al., 1976). Inhibitors of ethylene synthesis (aminoethoxyvinyl glycine, AVG and α-aminoxyacetic acid, AOA,) or action (silver ions: AgNO3, silver thiosulfate, STS) induced the development of male and hermaphroditic flowers in gynoecious cucumber and muskmelon plants, respectively (Byers et al., 1972).

Two hypotheses have been proposed to explain the role of hormones in sex determination; the two-hormone system, where each hormone promotes one type of sexual organ and the one-hormone system, where a single hormone can promote one sex and suppress the other. In the one-hormone case, two receptors would be required, one to promote development of one reproductive organ and the other to inhibit development of the opposite organ (Yin and Quinn, 1995). The female receptor would perceive ethylene at a threshold concentration and send a promoting signal to carpel primordia, while the male receptor would suppress stamen development when activated (Yin and Quinn, 1995). Yin and Quinn (1995) used a range of sex phenotypes and hormone levels to show that ethylene alone can explain the observed sex patterns. This model also proposes that the *F* gene controls endogenous ethylene concentration and the *M* gene controls male organ sensitivity to ethylene. In agreement with this hypothesis, application of ethylene was not able to inhibit stamen development in the andromonoecious (*mm*) genotype and led to hermaphroditic flowers, indicating that stamen primordia of andromonoecious cucumber are less sensitive to ethylene. More over, it has been suggested that the *M* locus is epistatic to, and functions downstream of the *F* locus, regulating responsiveness to ethylene. Subsequent molecular studies are providing support for this model (Yamasaki et al., 2001).

Regulation of endogenous ethylene levels has been associated with sex determination by the isolation of cucumber genes, involved in ethylene production (Kamachi et al., 1997; Trebitsh et al., 1997). Ethylene is synthesized in plant tissue via the Yang cycle, where S-adenosyl methionine (AdoMet) is converted to ACC by the function of ACC synthase (ACS), which is then oxidized to ethylene by ACC oxidase (ACO) (Johnson and Ecker, 1998). Comparative studies between near-isogenic monoecious and gynoecious genotypes indicated that the gynoecious genotype contained a second copy of the cucumber *ACS* gene, gene, *CS-ACS1G* (Trebitsh et al., 1997). Genetic studies detected a tight linkage between the *F* locus and the *CS-ACS1G* gene, indicating that the *F* locus might regulate endogenous ethylene availability (Trebitsh et al., 1997). Kamachi et al., (1997) identified another cucumber *ACS* gene, *CS-ACS2*. Time and level of expression of the *CS-ACS2* at the apices of monoecious and gynoecious cucumbers, coincided with the development of pistillate flowers and with the action of ethylene in the induction of the first pistillate bud (Kamachi et al., 1997; Kamachi et al., 2000). Apical expression of *CS-ACS1/1G*, however, did not correlate with development of pistillate flowers (Kamachi et al., 2000). This evidence led to the suggestion that *CS-ACS2* was critical for female flower development, and that the role of *CS-ACS1* is to accelerate the timing and increase the levels of *CS-ACS2* expression via higher ethylene production, in gynoecious vs. monoecious cucumbers (Kamachi et al., 2000).

The enzyme ACC oxidase (ACO) catalyzes the final step in ethylene biosynthesis (Johnson and Ecker, 1998). Studies to compare expression patterns and relation of ACOs with sex determination in cucumber, resulted in characterization of three *ACO* genes (*CS-ACO1*, *CS-ACO2* and *CS-ACO3*) (Kahana et al., 1999). The genes have distinct expression patterns during cucumber development and among the different genotypes studied, showing higher levels in *ff* (androecious and monoecious) genotypes compared to *FF* (gynoecious and hermaphrodite). At later stages of development, *CS-ACO2* levels were higher in leaves of female genotypes, but expression in the apices was inversely correlated to female tendency, being lower in *ff* genotypes. These results might indicate that these *ACO* genes are not involved in sex determination or that expression in the apices is not critical (Kahana et al., 1999).

The role of ethylene perception in cucumber sex determination also was recently investigated (Yamasaki et al., 2000; Yamasaki et al., 2001). Higher expression levels of the cucumber putative ethylene receptors *CS-ETR1*, *CS-ETR2* and *CS-ERS* were observed in shoot apices of a gynoecious rather than monoecious genotype, and transcripts of these genes increased in gynoecious cucumber, at the 4-5 leaf stage, when the first female buds are initiated (Augustine et al., 1973; Yamasaki et al., 2000). Ethylene application increased expression of *CS-ETR1*, CS-*ETR2* and *CS-ERS* in apices of both monoecious (*M-ff*) and gynoecious (*M-F-*) cucumber genotypes but not in the andromonoecious genotype (*mmff*), indicating that the *M* locus product regulates accumulation of *CS-ETR1*, CS-*ETR2* and *CS-ERS* (Yamasaki et al., 2000; Yamasaki et al., 2001). In contrast, application of AVG (aminoethoxyvinyl glycine), an ethylene synthesis inhibitor, decreased expression of *CS-ETR1*, *CS-ETR2* and *CS-ERS* in the gynoecious genotype, suggesting regulation of expression by endogenous ethylene (Yamasaki et al., 2000). As was described by Yin and Quinn (1995), ethylene treatment sufficient to induce female flowers in monoecious cucumber, could promote pistil development, but not inhibit stamen development, in andromonoecious cucumber, leading to production of hermaphroditic flowers (Yamasaki et al., 2001). These observations indicate that stamen primordia in andromonoecious cucumber are insensitive to ethylene, but pistil primordia are still sensitive (Yamasaki et al., 2001). Based on the molecular evidence and phenotypic evaluation of sex pattern modification by ethylene, it was suggested that the *M* locus inhibits stamen development via ethylene perception.

Objectives of the Following Research

Plants offer a unique opportunity to study the evolution of sex determination. Unisexual species, monoecious or dioecious, have evolved recently and independently from hermaphroditic progenitors multiple times, and each time a different mechanism was adopted to differentiate the gender. Evidence of physiological, biochemical and molecular genetic studies indicate that regulation of hormonal levels or the balance between hormones is a key factor controlling gender differentiation. In several cases, sex determination genes have been shown or hypothesized to be associated with hormone production or perception. The purpose of the following research is to further understand the role of hormone production and perception in sex expression, in cucumber and melon.

To this end I produced transgenic muskmelon plants modified for ethylene production or perception. Endogenous ethylene production was modified by introduction of the ethylene biosynthesis *ACS* gene, in an andromonoecious genetic background. The transgenic melons, which were expressing the *ACS* gene, both constitutively and in a tissue specific manner within floral primordia, exhibited increased ethylene production and earlier pistillate flower formation.

Similarly, modification of ability of a cucurbit plant to perceive ethylene is expected to alter the plant predisposition to produce pistillate flower buds. Introduction of the ethylene mutant receptor gene, *etr1-1* from *Arabidopsis*, rendered transgenic melon plants insensitive to ethylene and abolished the ability of the plant to form pistillate buds.

Another objective of this work was to study the effect of the maize sex determination gene *TS2*, in the heterologous melon system. It was hypothesized that overexpression of the *TS2* gene in cucurbits might affect the amount of available endogenous active GA or brassinosteroid (BR) levels and consequently influence melon sex expression. Given the range of developmental activities of BRs, and the role of several hormones in influencing sex determination in cucurbits, I was also interested in testing whether exogenous BRs also would exert an effect on sex differentiation of cucurbits. Although, *TS2* gene did not alter sex exression in melon, BR application increased femaleness in cucumber. Our results suggest that the ability of BRs to stimulate female flower production, in cucumber, was an indirect effect of BR-induced ethylene production.

References

Ainsworth C, Parker J, Buchanan-Wollaston V (1998) Sex determination in plants. *In* Curr. Topics Devel. Biol., Vol 38. Academic Press, San Diego, pp 167-223

Ando S, Sato Y, Kamachi S, Sakai S (2001) Isolation of a MADS-box gene (*ERAF17*) and correlation of its expression with the induction of formation of female flowers by ethylene in cucumber plants (*Cucumis sativus* L.). Planta **213:** 943-952

Atsmon D, Lang A, Light EN (1968) Contents and recovery of gibberellins in monoecious and gynoecious cucumber plants. Plant Physiol. **43:** 806-810

Augustine JJ, Baker LR, Sell HM (1973) Female flower induction on androecious cucumber *Cucumis sativus* L. J. Amer. Soc Hort. Sci. **98:** 197-199

Bensen RJ, Johal GS, Crane VC, Tossberg JT, Schnable PS, Meeley RB, Briggs SP (1995) Cloning and characterization of the maize *an1* gene. Plant Cell **7:** 75-84

Bracale M, Caporali E, Galli MG, Longo C, Marziani-Longo G, Rossi G, Spada A, Soave C, Falavigna A, Raffaldi F, Maestri E, Restivo FM, Tassi F (1991) Sex determination and differentiation in *Asparagus officinalis* L. Plant Sci. **80:** 67-77

Byers RE, Baker LR, Sell HM, Herner RC, Dilley DR (1972) Ethylene: A natural regulator of sex expression of *Cucumis melo* L. Proc. Natl. Acad. Sci. USA **69:** 717-720

Calderon-Urrea A, Dellaporta SL (1999) Cell death and cell protection genes determine the fate of pistils in maize. Development **126:** 435-441

Coen ES, Meyerowitz EM (1991) The war of the whorls: Genetic interactions controlling flower development. Nature **353:** 31-37

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

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

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

Farbos I, Veuskens J, Vyskot B, Oliveira M, Hinnisdaels S, Aghmir A, Mouras A, Negrutiu I (1999) Sexual Dimorphism in white campion: Deletion on the Y chromosome results in a floral asexual phenotype. Genetics **151:** 1187-1196

Freidlander M, Atsmon D, Galun E (1977) Sexual differentiation in cucumber: Abscisic acid and gibberellic acid of various sex genotypes. Plant Cell Physiol. **18:** 681-691

Grant RS (1999) Genetics of gender dimorphism in higher plants. *In* MA Geber, TE Dawson, LF Delph, eds, Gender and Sexual Dimorphism in Flowering Plants. Springer-Verlag, Berlin, Heidelberg, pp 247-274

Jack T (2001) Plant development going MADS. Plant Mol. Biol. **46:** 515-520

Janousek B, Siroky J, Vyskot B (1996) Epigenetic control of sexual phenotype in a dioecious plant, *Melandrium album*. Mol. Gen. Genet. **250:** 483-490

Johnson PR, Ecker JR (1998) The ethylene gas signal transduction pathway: A Molecular perspective. Annu. Rev. Genet. **32:** 227-254

Kahana A, Silberstein L, Kessler N, Goldstein R, Perl-Treves R (1999) Expression of ACC oxidase genes differs among sex genotypes and sex phases in cucumber. Plant Mol. Biol. **41:** 517-528

Kamachi S, Mizusawa H, Matsuura S, Sakai S (2000) Expression of two 1-aminocyclopropane-1-carboxylate synthase genes, *CS-ACS1* and *CS-ACS2*, correlated with sex phenotypes in cucumber plants (*Cucumis sativus* L.). Plant Biol. **17:** 69-74

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

Kater MM, Franken J, Carney KJ, Colombo L, Angenent GC (2001) Sex determination in the monoecious species cucumber is confined to specific floral whorls. Plant Cell **13:** 481-493

Kenigsbuch D, Cohen Y (1989) The inheritance of gynoecy in muskmelon. Genome **33:** 317-320

Lardon A, Georgiev S, Aghmir A, Le Merrer G, Negrutiu I (1999) Sexual dimorphism in white campion: complex control of carpel number is revealed by y chromosome deletions. Genetics **151:** 1173-1185.

Lebel-Hardenack S, Ye D, Koutnikova H, Saedler H, Grant SR (1997) Conserved expression of a TASSELSEED2 homolog in the tapetum of the dioecious *Silene latifolia* and *Arabidopsis thaliana*. Plant J **12:** 515-526

Negrutiu I, Vyskot B, Barbacar N, Sevdalin G, Moneger F (2001) Dioecious Plants: A key to the early events of sex chromosome evolution. Plant Physiol. **127:** 1418-1424

Ng M, Yanofsky MF (2000) Three ways to learn the ABCs. Curr. Opin. Plant Biol. **3:** 47-52

Parker JS, Clark MS (1991) Dosage sex-chromosome systems in plants. Plant Sci. **80:** 79-92

Perl-Treves R (1999) Male to female conversion along the cucumber shoot: approaches to studying sex genes and floral development in *Cucumis sativus*. *In* C Ainsworth, ed, Sex determination in plants. Bios Scientific Publishers, Oxford, UK, pp 189-215

Perl-Treves R, Kahana A, Rosenman N, Xiang Y, Silberstein L (1998a) Expression of multiple AGAMOUS-like genes in male and female flowers of cucumber (*Cucumis sativus* L.). Plant Cell Physiol. **39:** 701-710.

Poole CF, Grimball PC (1939) Inheritance of new sex forms in *Cucumis melo* L. J. Heredity: 21-25

Rood SB, Pharis RP (1980) Changes of endogenous gibberellin-like substances with sex reversal of the apical inflorescence of corn. Plant Physiol. **66:** 793-796

Roy RP, Saran S (1990) Sex expression in the Cucurbitaceae. *In* DM Bates, RW Robinson, C Jeffry, eds, Biology and utilization of the Cucurbitaceae. Cornell Univ. Press, Ithaca, NY, pp 251-268

Rudich J (1990) Biochemical aspects of hormonal regulation of sex expression in Cucurbits. *In* DM Bates, RW Robinson, C Jeffry, eds, Biology and utilization of the Cucurbitaceae. Cornell Univ. Press, Ithaca, NY, pp 269-280

Rudich J, Baker LR, Scott JW, Sell HM (1976) Phenotypic stability and ethylene evolution in androecious cucumber. J. Amer. Soc. Hort. Sci. **101:** 48-51

Rudich J, Halevy AH, Kedar N (1969) Increase in femaleness of three cucurbits by treatment with ethrel, an ethylene releasing compound. Planta **86:** 69-76

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

Scutt CP, Li T, Robertson SE, Willis ME, Gilmartin PM (1997) Sex determination in dioecious *Silene latifolia*. Effects of the Y chromosome and the parasitic smut fungus (*Ustilago violacea*) on gene expression during flower development. Plant Physiol. **114:** 969-979

Tolla GE, Peterson CE (1979) Comparison of gibberellin A4/A7 and silver nitrate for induction of staminate flowers in a gynoecious cucumber line. HortScience **14:** 542-544

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

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.

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 (2000) The ethylene-regulated expression of *CS-ETR2* and *CS-ERS* genes in cucumber plants and their possible involvement with sex expression in flowers. Plant Cell Physiol. **41:** 608-616.

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

CHAPTER 1

Influence of the introduction of heterologous *ACC synthase* gene on sex expression of andromonoecious muskmelon.

Abstract

The plant hormone ethylene regulates many physiological and developmental plant processes, including sex expression in cucurbits. Application of ethylene or ethylene-releasing agents induces formation of hermaphroditic or perfect flower buds on melon (*Cucumis melo* L.) plants. The effect of increased endogenous ethylene production on sex determination was investigated on andromonoecious melon plants transformed with a petunia *ACC synthase* gene (*ACS*) encoding 1-aminocyclopropane-1-carboxylate synthase, the first committed step in ethylene biosynthesis. Constitutive expression of the *ACS* gene increased ethylene evolution of leaves, male buds and hermaphroditic bus. Plants carrying the *ACS* gene showed one or more of the following measures of femaleness; the first hermaphroditic flower bud and first mature hermaphroditic flower on the main stem appeared earlier, increased number of the hermaphroditic buds formed and increased the number of hermaphroditic buds that reached anthesis, relative to wild type plants. The phenotypic observations indicate that the heterologous *ACS* gene influenced sex expression of melon plants.

Introduction

Plant species of the *Cucurbitaceae* family, show numerous heritable patterns of sex expression, and so have been used to study mechanisms of sex differentiation. In the

Cucumis species, cucumber (*Cucumis sativus* L.) and melon (*Cucumis melo* L.), key genes have been identified that influence sex expression. The most prevalent pattern of sex expression in cucumber is monoecious, where a phase of male flower production is followed by a phase of female flower production (Perl-Treves, 1999). Other types including hermaphrodite, andromonoecious (male flowers followed by hermaphroditic), androecious (Kater et al., 2001) and gynoecious (all female flowers), are controlled by an interplay of at least three genes, *F*, *M* and *A*. The dominant/semi-dominant *F* (*Female*) locus has been of significant commercial value by enabling the production of gynoecious lines which allow for earlier and more uniform fruit production. Sex expression in melon also is controlled by an interplay of at least three major genes *A*, *G* and *M*, which can result in a range of sex types (Kenigsbuch and Cohen, 1989; Roy and Saran, 1990). Melon differs from cucumber, however, in that the most prevalent sex pattern is andromonoecious, and there does not appear to be an equivalent locus to the cucumber *F* locus conferring gynoecy.

Cucurbit sex expression patterns also can be modified by hormonal and environmental factors (Rudich, 1990). Ethylene, which is able to promote pistillate flowers and suppress male flower formation appears to be the main hormonal factor affecting sex determination in cucumber (Rudich, 1990; Yin and Quinn, 1995). Numerous studies have shown that application of ethylene or ethylene releasing agents promote female tendency by increasing pistillate flower production in monoecious cucumber and squash, androecious cucumber and hermaphroditic flower production in andromonoecious muskmelon plants (Robinson et al., 1969; Rudich et al., 1969; Karchi, 1970; Augustine et al., 1973). In addition, 2-3 fold higher levels of endogenous ethylene have been associated with gynoecious sex pattern in cucumber and muskmelon (Byers et al., 1972; Rudich et al., 1972; Rudich et al., 1976; Trebitsh et al., 1987). Conversely, inhibitors of ethylene synthesis or action induce the development of male and hermaphroditic flowers in gynoecious cucumbers and muskmelons, respectively (Byers et al., 1972; Byers et al., 1972; Tolla and Peterson, 1979; Den Nijs and Visser, 1980). Silver ions are used to facilitate crossing of gynoecious breeding lines (Tolla and Peterson, 1979; Den Nijs and Visser, 1980). In melon, increased levels of endogenous ethylene did not differ significantly among sex phenotypes, however the significance of endogenous ethylene was demonstrated by application of hypobaric atmospheric conditions to reduce internal gas concentrations (Byers et al., 1972).

Ethylene is synthesized in plant tissue via the Yang cycle, where S-adenosyl methionine (AdoMet) is converted to 1-aminocyclopropane-1-carboxylic acid (ACS) by the function of *ACC* synthase, which is then oxidized to ethylene by ACC oxidase (ACO) (Johnson and Ecker, 1998). Regulation of endogenous ethylene production has been associated with sex determination by the isolation of cucumber *ACS* genes, *CS-ACS1* (Trebitsh et al., 1997) and *CS-ACS2* (Kamachi et al., 1997). Comparative studies between near-isogenic monoecious and gynoecious genotypes indicated that the gynoecious genotype contained a second copy of the *CS-ACS1* gene, *CS-ACS1G* (Trebitsh et al., 1997). Genetic analysis indicated a complete co-segregation between the *F* locus, and the *CS-ACS1G* gene, suggesting that the *F* locus might directly regulate endogenous ethylene availability (Trebitsh et al., 1997). Time and level of expression of a second *ACS* gene, *CS-ACS2* at the apices of monoecious and gynoecious cucumbers, coincided with the development of pistillate flowers and with the action of ethylene in the induction of the first pistillate bud, suggesting that *CS-ACS2* is critical for the increase in ethylene evolution and the female flower development. The relative contribution of different *ACS* genes remains to be resolved (Kamachi et al., 1997). Similar studies have not been performed for muskmelon. The lack of an equivalent gene to the *F* locus in melon may relate to non-significant differences in ethylene levels among melon sex types observed by Byers at al., (1972). Perhaps in melon gynoecy arises from increased sensitivity to ethylene rather than different levels of endogenous ethylene production.

The goal of the proposed work was the genetic modification of sex expression pattern of muskmelon plants by modifying endogenous ethylene production. Introduction of the *ACS* gene in an andromonoecious genetic background was expected to increase ethylene production and consequently to increase pistillate flower formation. The *ACS* gene was introduced and expressed either constitutively, or in a tissue specific manner within floral primordial cells destined to generate stamens and petals.

Materials and Methods

Plasmid Construction

The *ACS* cDNA from petunia flower petals (kindly provided by Dr. R. Woodson, Purdue Univ.) was transferred into the pGA643 vector (An et al., 1988), as a *XbaI-Kpn/BglII* fragment, for constitutive expression downstream of the cauliflower mosaic virus *35S* promoter. The clone was also transferred into the pCIB10 (CIBA-GEIGY, Research Triangle Park, NC) (Rothstein et al., 1987) vector under the control of the *APETALA3* (Jack et al., 1994) gene promoter (kindly provided by Dr. V. Irish, Yale Univ.) from *Arabidopsis thaliana*, for tissue specific expression in petals and stamens (Jack et al., 1994). The ATG codon from the 3' end of the *AP3* promoter was removed by polymerase chain reaction (PCR) and a *PstI* site was introduced. In addition a *BamHI* restriction site was introduced at the 5'end, using gene specific primers 5'**GGATCC**AAGCTTCTTAAGAATTATAGTAGC3' (RG74) and for the 5' end 5'**CTGCAG**ATTCTTCTCTCTTTGTTTAATCTT3' (RG75) and 3' end, respectively. The *AP3* fragment was introduced as a *BamH1-Pst1* into the pBS-*ACS* plasmid upstream of *ACS*. Subsequently the promoter and the gene were introduced as a *XbaI-KpnI* fragment into the pCIB10 vector. Both constructs were introduced in the *Agrobacterium tumefasciens* strain EHA105 (Hood et al., 1993) and used for melon transformation.

Plant Transformation

Transgenic muskmelon plants of the andromonoecious cv. Hale's Best Jumbo (Chesmore Seed Company, St Joseph, MO) were generated by cotyledon transformation according to the method of Fang and Grumet (1990). The regenerated plantlets (T_0) were selected on kanamycin (200mg/L), and evaluated for presence of the kanamycin resistance gene *neomycin phosphotransferase* (*NPTII*) by polymerase chain reaction (PCR) (as described below). The positive plants were transferred to the growth chamber and then in the greenhouse to be self-pollinated for progeny (T_1) production.

PCR and Northern analysis

Genomic DNA from leaf tissue was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Amplification by Taq DNA polymerase (GIBCO Invitrogen Co, Carlsbad, CA) was performed in 1.5mM $MgCl_2$, for 5 min at 95°C, 2 min at 62°C and 1 min at 72°C for 1.5min cycle, followed by 1min at 95°C, 1min at 62°C and 1.5 min at 72°C for 35 cycles and for the last cycle 5min at 72°C. Total RNA from leaf and flower bud tissue was isolated with the RNeasy Plant Mini Kit (Qiagen).

Northern analysis was performed with 10μg per lane total RNA. Following electrophoresis and blotting to Hybond N+ membrane (Amersham Pharmacia) using standard procedures (Sambrook and Russell, 2001), RNA was hybridized with radiolabeled cDNA probe encoding the *ACS* gene. Hybridization was done in PerfectHyb™ Plus (SIGMA) at 65°C overnight, and washed in 0.5X SSC, 0.1% SDS and 0.01% sodium pyrophosphate at 65°C for 30 min and with 0.2XSSC, 0.1% SDS and 0.01% sodium pyrophosphate at 65°C for 30 min.

Ethylene Experiments

Young, fully expanded leaves with 1cm long petioles were excised from node positions 5, 10 and 15, with a razor blade, weighed and placed individually in 250ml plastic containers (Magenta boxes) with a Whatman filter paper #2 moistened with 2ml of distilled water (dH_2O). The containers were tightly closed with covers fitted with a hole sealed with a piece of electric tape and silicon and then sealed with parafilm. Headspace gas samples were taken at 3 and 6 hours with an airtight syringe (1cc) and analyzed in a gas chromatographer (HACH CARLE series 100AGC, Linear 1200 recorder), with an activated alumina column and flame ionization detector. The experiments were performed twice using a completely randomized design.

Similarly, lateral apices, male buds and hermaphroditic buds (1-2 days before anthesis) were excised with a razor blade, weighed and placed individually in 5ml glass vials, containing 2 discs (6mm) of Whatman filter paper #2 and 30μl dH_2O. The vials were tightly closed with rubber lids; headspace gas samples were taken and analyzed as above. The experiments were performed twice for the apices, five times for the male buds, and four times for the hermaphroditic buds using a completely randomized design.

ACC Content

ACC was extracted according to (Sitrit et al., 1988) and assayed according to Lizada and Yang (1979) modified as follows. One g of leaf, male bud and hermaphroditic bud ground tissue was extracted with 2 ml of 80% ethanol, centrifuged for 10 minutes at 10000rpm (Sorvall RC-5B). The supernatant was evaporated *in vacuo*, resuspended in water and transferred to a 16X100mm test tube. Briefly, 1μM $HgCl_2$ was added to the assay sample and the volume was brought to 0.9ml with water. The tube was sealed with a rubber stopper and kept in ice and 0.1ml of a cold mixture of 5.25% NaOCl-saturated NaOH (2:1, v/v) was injected with a syringe into the test tube. The tube was mixed on a vortex, incubated for 30 minutes, mixed again and 1ml gas sample was removed for ethylene analysis by gas chromatography, as described above.

Sex Expression

Seeds of T_0 plants were imbibed overnight at 30°C and planted in 23cm diameter plastic pots containing commercial soil mix (sphagnum peat 70-80%, pH 5,5-6,5) (Baccto, Michigan Peat Company, Houston, TX) in the greenhouse, under natural photoperiod, at average temperature 25°C. The plants were fertilized twice per week with 300ppm of the commercial fertilizing mix Peter's Special 20-20-20. The T_1 plants were evaluated for the time of appearance of the first hermaphroditic flower bud, the total number of hermaphroditic buds formed, the node position of the first mature hermaphroditic bud and the total number of mature hermaphroditic buds, along 30 nodes of the main stem. The experiments repeated three times, with total number of scored plants n=22 for the WT plants and the ACS3 transgenic plants, and n=12 for the ACS4 transgenic plants.

Results

Gene introgression and expression

Agrobacterium-mediated transformation of cotyledon explants with the *35S-ACS* construct produced three T_0 plants that were self-pollinated to produce progeny. As has frequently been observed for melons regenerated from tissue culture (Ezura et al., 1992; Yadav et al., 1996), the ACS lines were tetraploid as verified by pollen morphology (data not shown). The observed segregation ratios for the T_1 progeny (Table 1) were consistent with *ACS* gene insertion at either one or two sites, depending on whether chromosome doubling occurs before or after insertion. If doubling occurs before insertion, 3:1 transgenic:non-transgenic ratios are expected for a single insertion site and 15:1 for two insertion sites. If doubling occurs after insertion, a ratio of 35:1 is expected for a single insertion site. The ratio for ACS1 was consistent with a single copy (3:1), while ratios for ACS3 and ACS4 were consistent with either 15:1 or 35:1 predictions. Southern analyses are needed to differentiate between these possibilities. Northern analysis verified expression of the introduced *ACS* gene in transgenic Hale's ACS3 plants (Figure 1). The *ACS* message was not detected in wild type or non-transgenic Hale's Best Jumbo tetraploid controls derived from tissue culture. Transformation with the *AP3-ACS* construct also produced a transgenic plant and progeny (AP3-ACS1). However, the *AP3-ACS* line was not characterized as seed was not available in time; experiments are currently being performed in the greenhouse.

Table 1. Segregation analysis of the T_1 progeny of transgenic *35S-ACS* melons.

Line	PCR-Positive:Negative	Expected ratios	χ^2
35S-ACS1	21:12	3:1	1.71ns
35S-ACS3	30:3	15:1	0.133 ns
		35:1	2.92 ns
35S-ACS4	12:0	15:1	0.09 ns
		35:1	0.14 ns
AP3-ACS1	15:0	15:1	0.22 ns
		35:1	0.02 ns

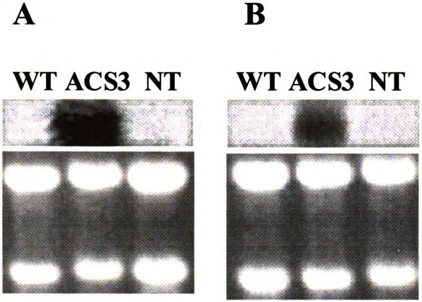


Figure 1. Expression of the heterologous *ACS* gene in (A) leaf tissue and in (B) male flower buds of T_1 transgenic plants carrying the *35S-ACS* construct. Top panels show total RNA isolated from non-transformed plants (WT), from *35S-ACS* transgenic plants (ACS3) and non-transgenic tetraploid plants (NT). The membranes were hybridized with the full-length ^{32}P-labeled petunia *ACS3*. The bottom panels show the rRNA stained with ethidium bromide to indicate equivalent loading.

Ethylene Evolution and ACC content

Leaves of ACS3 and ACS4 plants were sampled from main stem node positions 5 (at the onset of flowering), 10 (during male phase), and 15 (at the onset of female phase). The transgenic *35S-ACS* T_1 plants produced higher ethylene levels, as compared to non-transformed Hale's Best Jumbo plants (WT), at all three node positions (Figure 2A and B). Since it was necessary to excise the leaves at different plant ages, we can not directly test the effect of node position. However there appears to be a declining trend in amount of ethylene produced as the node position increases.

Apical growing points excised from lateral branches of transgenic plants, showed similar levels of ethylene evolution as those from control plants (Figure 3A). However, excised male and hermaphrodite buds of transgenic plants showed higher ethylene evolution compared to WT plants (Figure 3B and C). Interestingly, transgenic hermaphroditic buds produced approximately 10-fold higher ethylene than male buds (4 vs. 0.5ppm for ACS3 and 8-10 vs. 0.8ppm for ACS4). Although WT hermaphrodite buds also produced more ethylene than male buds, the difference was much less pronounced, ca 3-fold (1 vs. 0.3ppm). Ethylene production of non-transgenic tetraploid plants was equivalent to WT control (0.18ppm (NT) vs. 0.12 (WT) for the male buds and 0.26ppm (NT) vs. 0.41 (WT) for hermaphroditic buds). The ACC content measured in leaves, male buds and hermaphroditic buds was not significantly higher in ACS3 transgenic plants

Figure 2. (A) Ethylene evolution of excised leaves from node positions 5, 10 and 15 at 3 hours post excision and (B) at 6 hours post excision. The experiment was repeated twice, with total number of samples n=16 for leaf 5, n=18 for leaf 10, and n=5 for leaf 15 for the WT plants, n=18 for leaves 5 and 10, n=14 for leaf 15 for ACS3 transgenic plants, and n=12 for leaf 5, n=13 for leaf 10 and n= 9 for leaf 15 for ASC4 transgenic plants. Values are means (±SE) of the combined data.

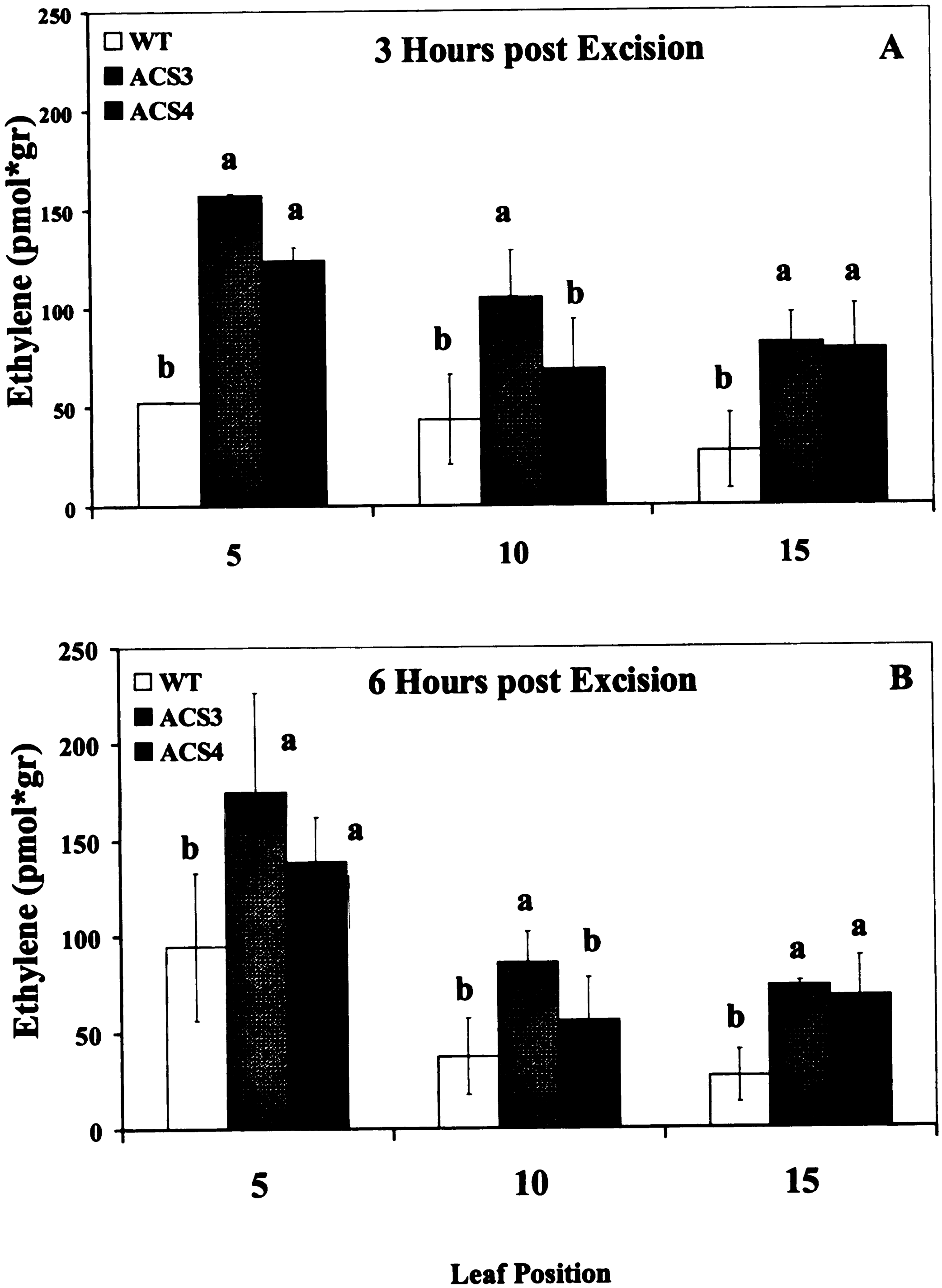
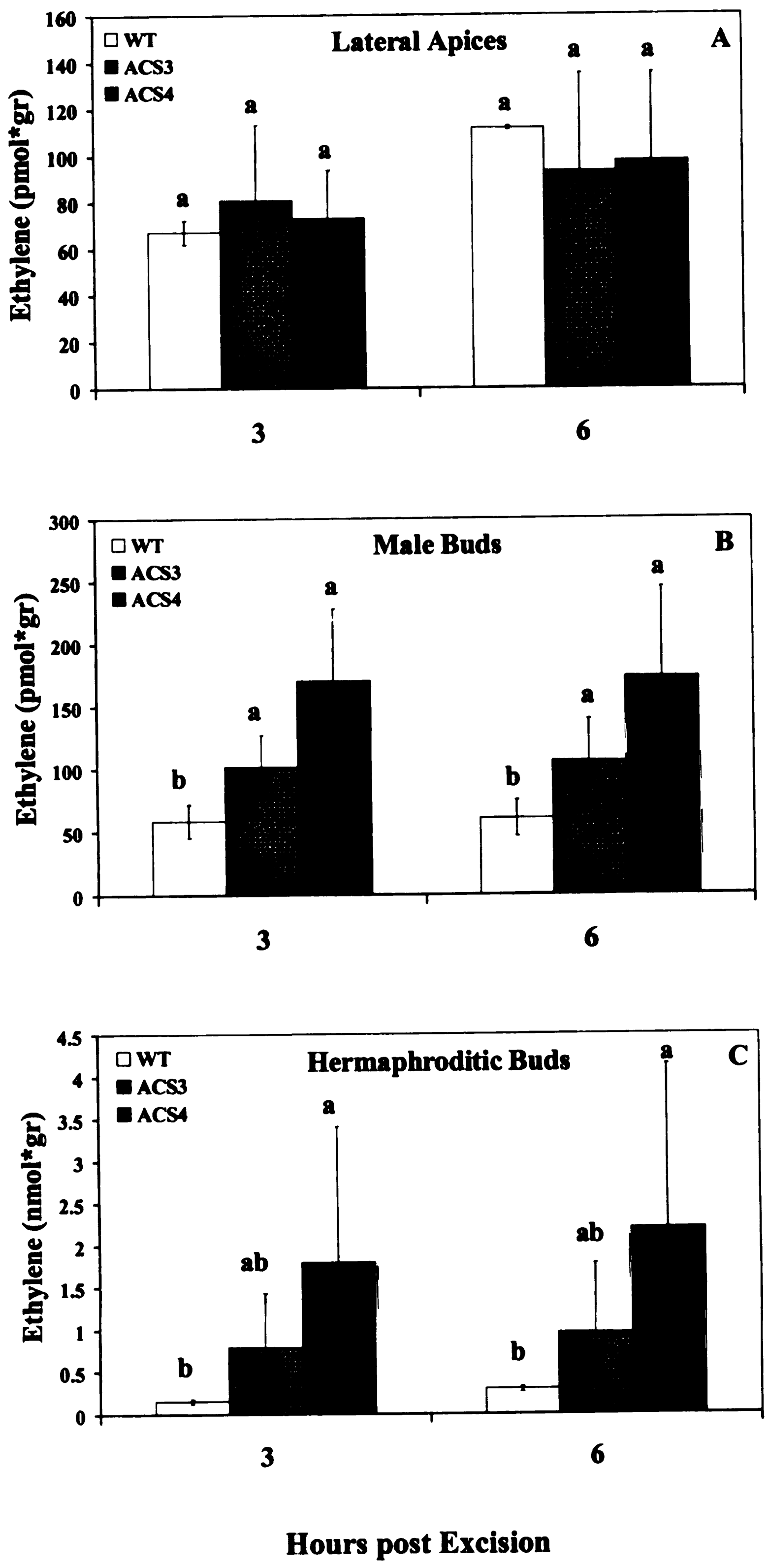

3 Hours post Excision
A
WT
ACS3
ACS4
Ethylene (pmol*gr)
250
200
150
100
50
0
5
10
15
a
b
6 Hours post Excision
B
Leaf Position

Figure 3. Ethylene evolution of transgenic *35S-ACS* melon plants. (A) Ethylene production of growing points excised from lateral branches, at 3 and 6 hours post excision. The experiment was performed twice, with a total number of samples 12 for the WT plants, 12 for line ACS3 per experiment, and 9 for line ACS4 transgenic plants. (B) Ethylene evolution of excised male flower buds. The experiment was repeated five times, with a total of n=17 for the WT plants, n=31 for the ACS3 plants, and n=18 for the ACS4 plants. (C) Ethylene evolution of excised hermaphroditic flower buds. The experiment was performed four times, with a total of n=12 for the WT plants, n=23 for the ACS3 plants and n=10 for the ACS4 transgenic plants. All data are means ±SE.


Lateral Apices
A
WT
ACS3
ACS4
Ethylene (pmol*gr)
Male Buds
B
Hermaphroditic Buds
C
Ethylene (nmol*gr)
3
6
Hours post Excision

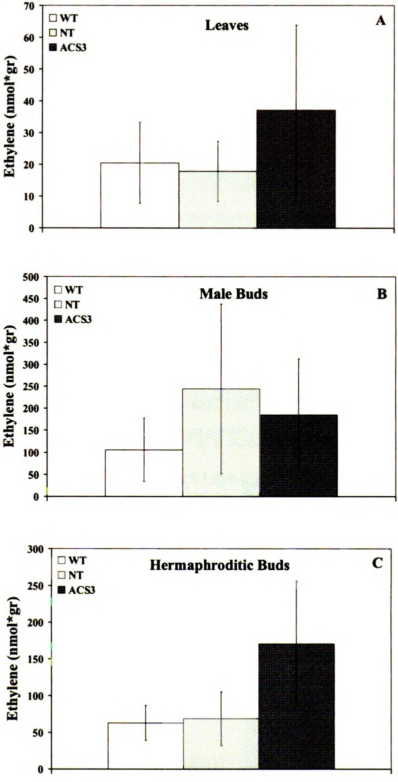
than wild type and non-transgenic tetraploid plants (Figure 4A, B and C). Even though the experiment needs to be repeated, because of the small number of samples, there appears to be a trend of increased ACC content in leaves and flower buds of the transgenic plants.

Sex Expression

The normal sex expression pattern along the main stem of andromonoecious melon plants is four to five vegetative nodes followed by a few nodes that carry only male buds, and finally production of nodes that carry both male and hermaphroditic buds. In our experimental conditions, the control plants formed the first hermaphroditic bud at approximately node 11, after approximately six male nodes (Figure 5A, 6A). To exclude the effect of tetraploidy on the sexual determination of the transgenic plants, transgenic ACS3 plants were compared with non-transgenic tetraploid plants (Figure 5).While plants of line ACS3 showed significant difference compared to both WT and NT plants for all the measurements of sex expression, the non-transgenic tetraploids were not significantly different from the WT controls (Figure 5A, B, C and D).

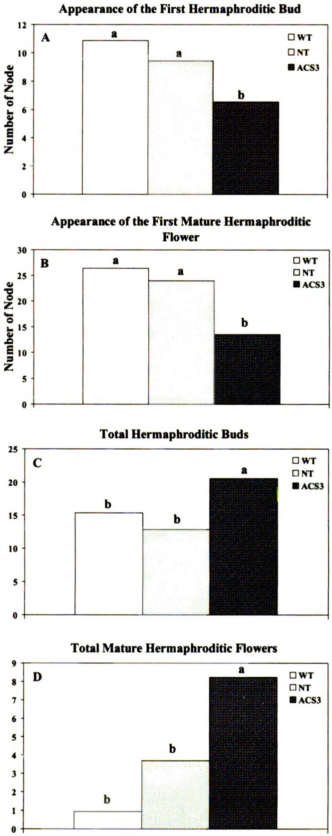
The earlier formed hermaphroditic buds often abort before anthesis so that the first perfect flower to reach anthesis generally occur 10-15 nodes later (Figure 5B, 6B). This pattern of flowering was modified in the transgenic ACS lines. Transgenic ACS plants formed the first hermaphroditic bud four to five nodes earlier compared to WT plants (Figure 5A, 6A). The time of appearance of the first male bud was not affected in the transgenic plants (data not shown), however where hermaphroditic buds occurred earlier, the male-only phase was reduced from an average of 4.2 nodes for the WT to an average of 2.4 for the ACS3 and 0.3 for the ACS4 plants.

Figure 4. ACC content of transgenic *35S-ACS* plants as expressed by ethylene evolution. (A) Leaves. The experiment was repeated three times, with a total of n=5 for the WT plants, n=7 for the ACS3 plants, and n=12 for the non-transgenic tetraploid plants (NT). (B) Male flower buds. The experiment was repeated three times, with a total of n=6 for the WT plants, n=7 for the ACS3 plants, and n=12 for the non-transgenic tetraploid plants and n=12 for the non-transgenic tetraploid plants. (C) Hermaphroditic flower buds. The experiment was repeated three times, with a total of n=4 for the WT plants, n=6 for the ACS3 plants, and n=10 for the non-transgenic tetraploid plants. Values are the means of the combined data (±SE).



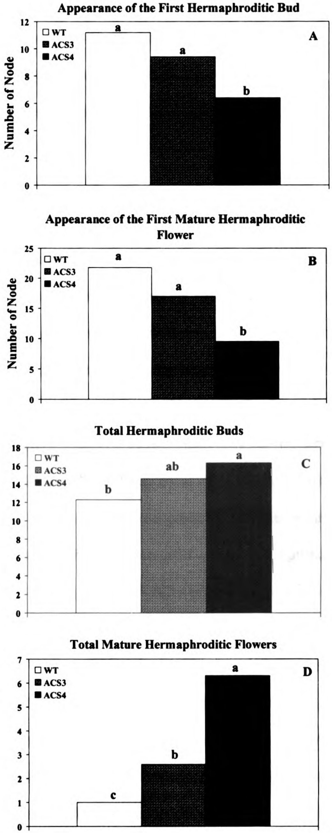
Leaves
A
Male Buds
B
Hermaphroditic Buds
C
WT
NT
ACS3
Ethylene (nmol*gr)

Figure 5. Sex expression pattern of transgenic T_1 plants carrying the *35S-ACS* construct and of tetraploid non-transgenic control plants (NT). (A) Node position on the main stem where the first hermaphroditic flower bud was formed. (B) Node position of the first hermaphroditic flower to reach anthesis. (C) Total number of hermaphroditic flower buds formed along thirty nodes of the main stem (D) Total number of hermaphroditic flowers that reached anthesis. The plants were scored for 30 nodes and the experiments were repeated three times with total number of samples n=16 for the WT plants, n=8 for ACS3 and n=16 for the NT (Duncan's Multiple Range Test, P=0.05).



Appearance of the First Hermaphroditic Bud
A
Number of Node
12
10
8
6
4
2
0
a
a
b
WT
NT
ACS3
Appearance of the First Mature Hermaphroditic Flower
B
Number of Node
30
25
20
15
10
5
0
a
a
b
WT
NT
ACS3
Total Hermaphroditic Buds
C
25
20
15
10
5
0
b
b
a
WT
NT
ACS3
Total Mature Hermaphroditic Flowers
D
9
8
7
6
5
4
3
2
1
0
b
b
a
WT
NT
ACS3

Figure 6. Sex expression pattern of transgenic T_1 plants carrying the *35S-ACS* construct. (A) Node position on the main stem where the first hermaphroditic flower bud was formed. (B) Node position of the first hermaphroditic flower to reach anthesis. (C) Total number of hermaphroditic flower buds formed along thirty nodes of the main stem (D) Total number of hermaphroditic flowers that reached anthesis. The plants were scored for 30 nodes and the experiments were repeated three times with total number of samples n=22 for the WT plants, n=22 for ACS3 and n=12 for ACS4 transgenic plants carrying the *35S-ACS* construct (Duncan's Multiple Range Test, P=0.05).



Appearance of the First Hermaphroditic Bud
Number of Node
WT
ACS3
ACS4
A
a
a
b
Appearance of the First Mature Hermaphroditic Flower
Number of Node
WT
ACS3
ACS4
B
a
a
b
Total Hermaphroditic Buds
WT
ACS3
ACS4
C
b
ab
a
Total Mature Hermaphroditic Flowers
WT
ACS3
ACS4
D
c
b
a

The first hermaphroditic flower to mature to anthesis also occurred at around node 10-15 (Figure 5B, 6B) vs. node 20-25 for the non-transgenic wild type and tetraploid plants. Overall, ACS plants produced more hermaphroditic nodes and a greater number of hermaphroditic flowers that reached anthesis compared to the control plants (Figure 5 and 6, C and D).

Discussion

Phenotypic evaluation of T_1 transgenic families carrying the *ACS* full-length cDNA clone from petunia, under control of a constitutive promoter, showed an effect on the endogenous ethylene levels produced by leaves and flower buds of the transgenic plants. Leaves, male and hermaphroditic flower buds of the transgenic plants produced higher ethylene levels, indicating that the heterologous *ACS* gene was functional and increased the plant potential for higher endogenous ethylene production. Consistent with previous studies ethylene evolution by hermaphroditic flower buds was higher than male buds (Rudich et al., 1976). This difference was accentuated in the transgenic plants where there was a 10-fold increase in ethylene production by the hermaphrodite vs. the male buds. Introduction of the heterologous *ACS* gene also significantly affected the sex expression pattern. Transgenic *35S-ACS* melons exhibited earlier appearance of hermaphroditic flower buds, a reduced male-only phase, and an increased number of hermaphrodite buds that reached anthesis. Thus, modification in endogenous ethylene production can alter floral development in transgenic melons.

At early stages of initiation and differentiation male, female or hermaphroditic buds are morphologically indistinguishable and carry both stamen and carpel primordia (Goffinet, 1990). Afterwards, stamen development is arrested in buds destined to be female flowers and similarly pistil development is paused in buds destined to be male flowers. Upon application of exogenous hormones, primordia destined to become unisexual cucumber or melon flowers may either convert to hermaphrodite or completely change to the opposite sex (Galun et al., 1963; McMurray and Miller, 1968; Pike and Peterson, 1969; Pike and Peterson, 1969; Karchi, 1970; Augustine et al., 1973; Tolla and Peterson, 1979; Yin and Quinn, 1995). The extent of conversion can be affected by genotype, stage of floral development at the time of application, and amounts of applied hormone. Increased femaleness in the transgenic ACS melons is consistent with effects of exogenous ethylene treatments in promoting pistil development.

Since higher levels of ethylene are associated with increased femaleness, increased ethylene evolution might be expected as the plant enters the pistillate flower production phase. Our studies indicated higher levels of ethylene production in the leaves when plants had visible male buds (5th leaf expanded) and approximately the 10th leaf was beginning to emerge. Although ethylene production has not generally been measured in leaves, several studies have examined ethylene evolution from excised apices of cucumber seedlings (Rudich et al., 1972; Makus, 1975; Makus et al., 1975; Rudich et al., 1976; Trebitsh et al., 1987; Yamasaki et al., 2000; Yamasaki et al., 2001). A peak in ethylene evolution occurred at the 4-5 leaf stage in apices of both monoecious and gynoecious cucumbers, but was 2-3 fold higher in the gynoecious apices (Yamasaki et al., 2000). Differences tended to be minimal at the first leaf stage, but increased during the first 24 days after germination to a maximum 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; Rudich et al., 1976; Yamasaki et al., 2000). *CS-ACS2* transcripts paralleled ethylene evolution and peak expression of ethylene receptor genes (e.g. *CS-ETR1, CS-ETR2* and *CS-ERS*) was observed in apices of 4-5 leaf stage gynoecious cucumbers (Kamachi et al., 1997; Yamasaki et al., 2000). Surprisingly, however, transcripts homologous to the *F* locus associated *ACS* gene, *CS-ACS1*, were not observed in the apices of 25 day old plants (Yamasaki et al., 2000), raising the question of the role of different *ACS* genes and the critical site for ACC or ethylene production. Studies of cucumber *ACO* genes, encoding ACC oxidase which is responsible for conversion of ACC to ethylene, showed a negative correlation between *CS-ACO2* and *CS-ACO3* transcript levels and gynoecy in the apices, but a positive correlation with gynoecy in the leaves (Kahana et al. 1999). These varying results for different *ACO* and *ACS* genes and different tissues suggest complexity of regulation of different members of the ethylene biosynthetic gene family.

There was not a difference in ethylene evolution of shoot tips excised from lateral branches of the transgenic *35S-ACS* plants relative to the control plants. This was surprising as other studies have shown high level expression of 35S-driven constructs in the apex (Williamson et al., 1989). Analyses of gene expression of *ACO*, led Kahana et al. (1999) to suggest that a negative feedback inhibition of *ACO* gene transcription may occur in cucumber apices in response to elevated ethylene, while a positive one may operate in the leaf. If a similar mechanism exists for melons, this may explain the higher ethylene evolution from transgenic *ACS* leaves, but failure to see elevated ethylene production from apices. Whether the *35S-ACS* gene was transcribed in the transgenic melon apices, or if lateral apices differ from the primary apex, or if *ACO* expression is a limiting factor, remains to be determined. However, at least in these lines, it appears that elevated ethylene production in the leaves and/or floral buds is sufficient to influence sex expression.

An interesting and unexpected effect of the *ACS* gene was on flower bud maturity. The first hermaphroditic bud to reach anthesis occurred earlier on the transgenic plants, and a higher percentage of the buds reached maturity rather than abscising prior to anthesis. This trait could have potential horticultural value by allowing for earlier fruit production, which would be an important feature in regions with a short growing season. These results also suggest that ethylene may be important not only for pistil initiation, but also to sustain further development of the female organ. The timing of ovary maturation and female gametophyte development during flower development varies among species, and in many cases is not completed until after pollination (O'Neill, 1997). Several lines of evidence have implicated a role for ethylene and ACC in ovary development and full maturation of ovules in a variety of species (O'Neill, 1997). The best characterized examples are in the orchid family where the ovary is immature prior to pollination, at which point differentiation and development are induced in response to ethylene produced by various floral organs (O'Neill, 1997; Bui and O'Neill, 1998). Whether expression of the introduced *ACS* gene and ACC or ethylene play a role in melon ovary development remains to be determined.

In summary, the above results with the *35S-ACS* melons indicate that ethylene production plays a central role in female flower organ development in melon. Constitutive expression of an additional copy of the *ACS* gene in transgenic andromonoecious melon plants induced earlier and increased formation of hermaphroditic flower buds and increased the number of hermaphroditic buds that reach maturity. Future research will be needed to understand the role of specific timing and location of ethylene production relative to the developing flower primordia.

References

An G, Ebert PR, Mitra A, Ha SB (1988) Binary vectors. Plant Mol Biol Manual **A3:** 1-19

Augustine JJ, Baker LR, Sell HM (1973) Female flower induction on androecious cucumber *Cucumis sativus* L. J. Amer. Soc Hort. Sci **98:** 197-199

Byers RE, Baker LR, Dilley DR, Sell HM (1972) Chemical induction of perfect flowers on a gynoecious line of muskmelon, *Cucumis melo* L. HortScience **913:** 321-3131

Byers RE, Baker LR, Sell HM, Herner RC, Dilley DR (1972) Ethylene: A natural regulator of sex expression of *Cucumis melo* L. Proc Natl Acad Sci **69:** 717-720

Den Nijs A, Visser D (1980) Induction of male flowering in gynoecious cucumbers (*Cucumis sativus* L.) by silver ions. Euphytica **29:** 237-280

Ezura HH, Amagai D, Yoshioka D, Oosawa K (1992) Highly frequent appearance of tetraploidy in regenerated melon plants, a universal phenomenon in tissue cultures of melon (*Cucumis melo*). Plant Sci **85:** 209-213

Fang G, Grumet R (1990) *Agrobacterium tumefasciens* mediated transformation and regeneration of muskmelon plants. Plant Cell Rep **9:** 160-164

Galun E, Yung Y, Lang A (1963) Morphogenesis of floral buds of cucumber cultured in vitro. Developmental Biology **6:** 370-387

Goffinet M (1990) Comparative ontogeny of male and female flowers of Cucumis sativus. *In*, pp 288-304

Hoekema A, Hooykaas PJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefasciens* Ti-plasmid plant genetics. Nature **303:** 179-180

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New Agrobacterium helper plasmids for gene transfer to plants. Transgenic Res. **2:** 208-218

Jack T, Fox GL, Meyerowitz EM (1994) Arabidopsis homeotic gene *APETALA3* ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. Cell **76:** 703-716

Johnson PR, Ecker JR (1998) The ethylene gas signal transduction pathway: A Molecular perspective. Annu. Rev. Genet. **32:** 227-254

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

Karchi Z (1970) Effects of 2-chloroethanephosphonic acid on flower types and flowering sequences in muskmelon. J Amer Soc Hort Sci **95:** 575-578

Kater MM, Franken J, Carney KJ, Colombo L, Angenent GC (2001) Sex determination in the monoecious species cucumber is confined to specific floral whorls. Plant Cell **13:** 481-493.

Kenigsbuch D, Cohen Y (1989) The inheritance of gynoecy in muskmelon. Genome **33:** 317-320

Makus DJ, Pharr DM, Lower R, L. (1975) Some morphogenic differences between monoecious and gynoecious cucumber seedlings as related to ethylene production. Plant Physiol **55:** 352-355

McMurray AL, Miller CH (1968) Cucumber sex expression modified by 2-chloroethanephosphonic acid. Science **162:** 1397-1398

Perl-Treves R (1999) Male to female conversion along the cucumber shoot: approaches to studying sex genes and floral development in *Cucumis sativus*. *In* CC Ainsworth, ed, Sex determination in plants. Bios Scientific Publishers, Oxford, UK, pp 189-215

Pike LM, Peterson CE (1969) Gibberellin A4/A7 for induction of staminate flowers on the gynoecious cucumber (*Cucumis sativus* L.). Euphytica **18:** 106-109

Robinson RW, Shannon S, La Guardia MD (1969) Regulation of sex expression in the cucumber. HortScience **19**

Rothstein SJ, Lahners KN, Lotstein RJ, Carozzi NB, Jayne SM, Rice DA (1987) Promoter cassettes, antibiotic-resistance genes, and vectors for plant transformation. Gene **53:** 153-161

Roy RP, Saran S (1990) Sex expression in the Cucurbitaceae. *In* C Jeffry, ed, Biology and utilization of the Cucurbitaceae. Cornell Univ. Press, Ithaca, NY, pp 251-268

Rudich J (1990) Biochemical aspects of hormonal regulation of sex expression in Cucurbits. *In* C Jeffry, ed, Biology and utilization of the Cucurbitaceae. Cornell Univ. Press, Ithaca, NY, pp 269-280

Rudich J, Baker LR, Scott JW, Sell HM (1976) Phenotypic stability and ethylene evolution in androecious cucumber. J Amer Soc Hort Sci **101:** 48-51

Rudich J, Halevy A, Kedar N (1969) Increase in femaleness of three cucurbits by treatment with ethrel, an ethylene releasing compound. Planta **86:** 69-76

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

Sambrook J, Russell DW (2001) Molecular Cloning A Laboratory Manual, Ed Third. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sitrit Y, Riov J, Blumenfeld A (1988) Interference of phenolic compounds with the 1-aminocyclopropane-1-carboxylic acid assay. Plant Physiol **86:** 13-15

Tolla GE, Peterson CE (1979) Comparison of gibberellin A4/A7 and silver nitrate for induction of staminate flowers in a gynoecious cucumber line. HortScience **14:** 542-544

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

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.

Williamson JD, Hirsh-Wyncott E, Larkins BA, Gelvin SB (1989) Differential accumulation of a transcript driven by the CaMV 35S promoter in transgenic tobacco. Plant Physiol. **90:** 1570-1576

Yadav RC, Saleh MT, Grumet R (1996) High frequency shoot regeneration from leaf explants of muskmelon. Plant Cell Tiss. Org. Cult. **45:** 207-214

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 (2000) The ethylene-regulated expression of CS-ETR2 and CS-ERS genes in cucumber plants and their possible involvement with sex expression in flowers. Plant Cell Physiol **41:** 608-616.

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

CHAPTER 2

The *Arabidopsis etr1-1* gene confers ethylene insensitivity and prevents hermaphroditic flower formation in transgenic muskmelon plants.

Abstract

The plant hormone ethylene influences many aspects of plant growth and development, including sex expression in cucurbits. The objective of this work was to determine the effect of modified ethylene perception in female flower development in melon. Transgenic andromonoecious and gynoecious muskmelon plants were produced using the dominant mutant ethylene receptor gene *etr1-1* from *Arabidopsis*. Constitutive expression of the *etr1-1* gene resulted in several ethylene insensitive phenotypes including decreased rooting ability, higher ethylene production by male flower buds, decreased rate of pedicel abscission of excised transgenic flower buds, and failure to respond to exogenous ethylene. The observed phenotypes verify the ability of the heterologous *Arabidopsis etr1-1* gene to cause ethylene insensitivity in melons. Expression of the *etr1-1* gene also prevented pistillate flower formation in both andromonoecious and gynoecious melon lines. The phenotypic observations indicate that the ability to perceive ethylene is required for promotion of femaleness at the time of sex determination.

Introduction

Plant species of the *Cucurbitaceae* family show numerous patterns of sex expression and so have been used to study genetic and physiological mechanisms of sex determination (Roy and Saran, 1990; Perl-Treves, 1999). Heritable sex patterns can range from hermaphroditic (plants produce hermaphroditic or perfect flowers, with both male (stamens) and female (pistils) reproductive organs), to andromonoecious (male flowers are formed early in development followed by hermaphroditic flowers), to monoecious (male flowers are produced first followed by a predominantly female phase), to gynoecious (only pistillate or female flowers are produced). Andromonoecy is the most common pattern in muskmelon genotypes (Roy and Saran, 1990).

Expression of the sex patterns can be modified by hormonal and environmental factors (Roy and Saran, 1990). The main hormonal factor affecting sex determination appears to be ethylene, which is able to promote pistil development, and in some species suppress anther formation (Rudich, 1990; Yin and Quinn, 1995). Application of ethylene or ethylene releasing agents promoted female tendency by increasing pistillate flower production in monoecious cucumber and squash, pistillate flowers in androecious cucumber (Augustine et al., 1973) and hermaphroditic flower in andromonoecious muskmelon plants (Rudich et al., 1969). Conversely, inhibition of ethylene synthesis or action induced the development of male and hermaphroditic flowers in gynoecious cucumber and muskmelon plants, respectively (Byers et al., 1972; Byers et al., 1972). In addition, 2-3 fold higher levels of endogenous ethylene have been associated with gynoecious sex pattern in cucumber and muskmelon (Byers et al., 1972; Rudich et al., 1972; Rudich et al., 1976). In cucumber it has been hypothesized that the difference between monoecy and andromonoecy resulting in female vs. hermaphrodite flowers is due to differences in ethylene perception that cause suppression of anther formation (Yin and Quinn, 1995).

Ethylene is a gaseous plant hormone that regulates a diverse range of processes throughout plant growth and development including senescence, abscission, fruit ripening, and root development (Bleecker and Kende, 2000). The ethylene biosynthetic pathway has been characterized as a two-step process from S-adenosylmethionine (SAM)→ 1-aminocyclopropane-1-carboxylic acid (ACC)→ ethylene. In the last decade research has focused on the study of ethylene perception and signal transduction, facilitated in large part by the identification of ethylene perception mutants (Bleecker and Kende, 2000).

In the presence of ethylene, dark grown seedlings show the triple response, reduced hypocotyl and root elongation, swelling of the hypocotyls, and accentuation of the apical hook. These characteristics are not observed in ethylene insensitive *Arabidopsis* seedlings that have a dominant mutation in the *ethylene receptor1* (*etr1*) gene (Chang et al., 1993). Isolation and characterization of the *ETR1-1* gene showed similarity to the bacterial two-component signal transduction system, which implies the involvement of the gene in ethylene signal transduction, acting most possibly as the ethylene receptor (Chang et al., 1993). Further characterization showed that one domain of the C-terminus of the *etr1-1* gene functions as a histidine kinase *in vitro* (Gamble et al., 1998) and the second is a response-regulator (Chang et al., 1993). The three hydrophobic domains of the N-terminus are responsible for membrane localization, dimerization (Schaller et al., 1995) and show ethylene binding activity (Schaller and Bleecker, 1995; Hall et al., 1999).

Expression of the mutant ethylene receptor *etr1-1* gene, under the control of the constitutive 35S *Cauliflower Mosaic Virus* (*CaMV*) promoter, abolished the ability of transgenic *Arabidopsis* plants to perceive ethylene, as assessed by failure to repress the triple response when germinated in the dark and in the presence of ethylene (Chang et al., 1993). Transgenic tomato plants constitutively expressing the *etr1-1* gene from *Arabidopsis* also showed ethylene insensitivity (Wilkinson et al., 1997). Seedlings failed to exhibit the triple response phenotype when germinated in dark in the presence of ACC. Flower senescence and abscission process was altered on transgenic plants, so that they remained attached for a long time after pollination. In addition, transgenic fruits showed delayed maturation, remaining yellow for a long time, before turning red. Similarly, transgenic petunia plants expressing the heterologous *etr1-1* gene also exhibited lack of responsiveness to ethylene (Wilkinson et al., 1997). Transgenic flowers produced higher levels of ethylene than control flowers. Senescence of flowers after hand-pollination was delayed and excised flowers did not respond to ethylene treatment whereas control flowers senesced in 24 hours (Wilkinson et al., 1997).

Since studies with exogenous ethylene have indicated a role of ethylene in sex determination in cucurbits, the objective of this work was to determine the effect of modified ethylene perception in sex expression of melon flowers. The mutant *Arabidopsis etr1-1* gene was introduced into transgenic andromonoecious and gynoecious muskmelon plants and verified to confer ethylene insensitivity. Our results indicate that ethylene perception plays a central role in female flower organ development.

Materials and Methods

Plasmid Construction

The *etr1-1* cDNA clone (Schaller et al., 1995), kindly provided by Dr E. Schaller, Univ Wisconsin, Madison, WI) was transferred into the pGA643 plant transformation vector (An et al., 1988) as a *BamH1* fragment including the full coding region, downstream of the constitutive cauliflower mosaic virus *35S* promoter. The construct was introduced into the *Agrobacterium tumefasciens* strain EHA105 (Hoekema et al., 1983) and used for plant transformation.

Plant Transformation

Transgenic melon plants were generated by *Agrobacterium*-mediated cotyledon transformation according to the method of Fang and Grumet (1990). The andromonoecious melon cv. Hale's Best Jumbo (Chesmore Seed Company, St Joseph, MO) and the gynoecious WI-998 (kindly provided by Dr. J. Staub Univ Wisconsin) were used for transformation with the *etr1-1* gene. The regenerated plantlets (T_0) were selected on kanamycin (200mg/L), evaluated for presence of the kanamycin resistance gene *neomycin phosphotransferase* (*NPTII*) by polymerase chain reaction (PCR) (as described below) and then self-pollinated to produce progeny (T_1), which were used for phenotypic evaluations. In case of the WI-998 T_0 plant, female flowers were pollinated with wild type WI-998 pollen (male flowers induced by 6mM silver thiosulfate spray).

PCR and Northern analysis

Genomic DNA from leaf tissue was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Amplification by Taq DNA polymerase (GIBCO Invitrogen Co, Carlsbad, CA) was performed in 1.5mM $MgCl_2$, for 5 min at 95°C, 2 min at 62°C and 1 min at 72°C for 1.5min cycle, followed by 1min at 95°C, 1min at 62°C and 1.5 min at 72°C for 35 cycles and for the last cycle 5min at 72°C. Total RNA from leaf tissue was isolated with the RNeasy Plant Mini Kit (Qiagen). RNA blot analysis was performed with 10 μg (per lane) total RNA. Following electrophoresis and blotting to Hybond N+ membrane (Amersham Pharmacia) using standard procedures (Sambrook and Russell, 2001), RNA was hybridized with radiolabeled cDNA probe encoding the *etr1-1* gene. Hybridization was done in PerfectHyb^{TM} Plus (SIGMA) at 65°C overnight, and washed in 0.5X SSC, 0.1% SDS and 0.01% sodium pyrophosphate at 65°C for 30 min and with 0.2XSSC, 0.1% SDS and 0.01% sodium pyrophosphate at 65°C for 30 min.

Ethylene Experiments

Seeds of T_1 plants were imbibed overnight at 30°C in dark, in plastic petri dishes (100X15mm) with wet Whatman paper #2 and then planted in 23cm diameter plastic pots containing commercial soil mix (sphagnum peat 70-80%, pH 5.5-6.5) (Baccto, Michigan Peat Company, Houston, TX) in the greenhouse, under natural photoperiod, at average temperature of 25°C. The plants were fertilized twice per week with 300ppm of the commercial fertilizing mix Peter's Special 20-20-20.

Young, fully expanded leaves with 1cm long petiole were excised with a razor blade, weighed and placed individually in 250ml plastic containers (Magenta) with a Whatman filter paper #2 moistened with 2ml of distilled water (dH_2O). The containers were tightly closed with covers fitted with a hole sealed with a piece of electric tape and silicon and then sealed with parafilm. Headspace gas samples were taken at 2, 4, 6 and 12 hours with an airtight syringe (1cc) and analyzed in a gas chromatograph (HACH CARLE series 100AGC, Linear 1200 recorder), with an alumina column and flame ionization detector. The experiment was conducted four times using a completely randomized design, with 3-7 replicates per genotype and per experiment.

Similarly, male buds (1-2 days before anthesis) were excised with a razor blade, weighed and placed individually in 5ml glass vials, containing 2 discs (6mm) of Whatman filter paper #2 and 30µl dH_2O. The vials were tightly closed with rubber lids and incubated for 3 hours, when headspace gas samples were taken and analyzed as above. The experiments were conducted four times according to completely randomized design with 6-10 replicates per genotype and per experiment.

Ethylene Response

Male buds were excised and treated as above. The buds were treated with air or 3ppm of ethylene gas (from a 100ppm mix that was prepared in the lab and compared to a certified standard) and were evaluated daily for abscission of the pedicel. The experiment was repeated seven times with 3-5 replicates per genotype and per treatment.

Rooting Experiments

Lateral shoots with two expanded leaves were excised with a razor blade, treated with rooting hormone (RooTone, Black Leaf Products, Louisville, KY) and placed in plastic containers (10x10x7.5cm), filled with sand. The pots were put in plastic re-closable bags and then placed in a growth chamber to root under a 18/6-light/dark cycle and 24/20°C temperature cycle, for three weeks.

The sand was rinsed off the developed roots, and the maximum root length measured. Then the roots were excised, weighed and dried in 65 °C drying oven for three days, to obtain dry weight. The experiments were conducted three times according to a completely randomized design), with a total of 15 for H-WT, 7 for H-NT6 and 17 for H-etr6 transgenic plants.

Sex Expression

The T_1 plants were evaluated for the time of appearance of the first hermaphroditic flower bud, the total number of hermaphroditic and male buds formed, and the number of buds that reached anthesis, along 30 nodes of the main stem. The experiments were conducted four times according to a completely randomized design, with 7-19 replicates per genotype and per experiment, except for the H-NT genotype where fewer plants were available.

Results

Characterization of transgenes

Several transgenic *etr1-1* plants were regenerated from tissue culture. Transgenic progeny were obtained from two plants; one from the andromonoecious genotype (Hale's Best Jumbo) and one from the gynoecious genotype (WI-998). Hales-*etr1-1*, T_0 plants showed andromonoecious sexual pattern producing hermaphroditic flowers, while the WI-998 T_0 plant produced only female flower buds. T_1 Hale's Best Jumbo progeny were obtained from self-pollination from one Hale's transgenic, and from F_1 progeny from cross-pollination of the WI-998 T_0 plant with non-transformed WI-998. The propagation ratios (3:1 for the T_1 and 1:1 for the hybrid progeny) were consistent with a single insertion site in each case (Table 1). Northern analysis verified expression of the introduced *etr1-1* gene in transgenic Hales etr6 (H-etr6) (Figure 1). The etr1-1 message was not detected in wild type (H-WT) or non-transgenic T_1 segregant (H-NT6) plants. As has been observed frequently for melon plants regenerated from tissue culture (Yadav et al., 1996), the Hale's-*etr1-1* plants were tetraploid. The WI-998 plant progeny were diploid.

Table 1. Segregation analysis of the T_1 progeny of 35S-*etr1-1* trangenic plants and the F_1 progeny of the W-etr transgenic plant. H-etr6: transgenic *etr1-1* T_1 plants line 6 of the andromonoecious cv. Hale's Best Jumbo, and W-etr: transgenic plants *etr1-1* F_1 plants of the gynoecious genotype WI-998.

Lines	PCR-Positive:Negative	Expected ratios	χ^2
H-etr6	51:14	3:1	0.42ns
W-etr	4:5	1:1	0.11ns

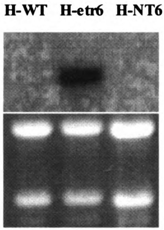


Figure 1. Expression of the heterologous *etr1-1* gene in leaf tissue of T_1 transgenic melon plants. The top panel shows total RNA isolated from non- transformed Hale's Best Jumbo plants (H-WT), from transgenic *etr1-1* T_1 plants line 6 (H-etr6), and from non-transgenic segregant progeny from the H-etr6 parent (H-NT6), hybridized with the full length ^{32}P-labeled *Arabidopsis etr1-1* gene. The bottom panel shows the rRNA stained with ethidium bromide to indicate equivalent loading.

Phenotypic evaluation for ethylene insensitivity

Transgenic *etr1-1* melon plants showed several phenotypes associated with ethylene insensitivity. Vegetative cuttings from lateral branches of T_1 transgenic plants showed reduced and delayed rooting ability as evidenced by the decreased maximum root length, fresh weight and dry weight, compared to the non-transformed Hale's Best Jumbo plants (H-WT) and non-transgenic segregants (H-NT) from the same family (Figure 2), indicating specific effect of the introduced gene. Transgenic seedlings also had reduced root growth in commercial potting mix and older plants were more prone to wilting (data not shown).

Although differences in ethylene evolution were not apparent in the leaves (Figure 3A), excised flower buds of transgenic plants showed significantly higher ethylene evolution (ANOVA, P=0.05), compared to control and H-NT plants (Figure 3B).

Pedicel abscission of excised male buds was monitored as an indication of ethylene insensitivity. Pedicels of H-etr6 transgenic male buds, excised 1-2 days before anthesis, abscised an average of a half to one day later (3.8 days post excision) than the H-WT (3.0) and H-NT6 buds (3.4 days post excision) (Figure 4). H-WT and H-NT6 buds responded to the presence of exogenous ethylene by hastened abscission; pedicels of control buds abscised one day earlier compared to buds in air (2.1 and 2.6 days post excision, respectively). However, ethylene treated H-etr6 transgenic male buds abscised the same time (3.5 days) as in air, indicating insensitivity to the exogenous ethylene presence. Differences between genotypes (H-etr6 vs. H-WT and H-NT6) are significant in both air and presence of ethylene (ANOVA, P<0.01).

Figure 2. Rooting ability of vegetative cuttings excised from lateral branches of T_1 transgenic plants. Average fresh weight (gr) (A), dried weight (gr) (B) and maximum length (cm) (C) of adventitious roots at three weeks post excision. The experiment was repeated three times, with a total of 15 for H-WT, 7 for H-NT6 and 17 for H-etr6 cuttings. Values are the means of the combined data (±SE). (D) Adventitious root formation. H-WT: non- transformed Hale's Best Jumbo plants, H-NT6: non-transgenic segregant progeny from the H-etr parent line 6, and H-etr6: transgenic *etr1-1* T_1 plants line 6.

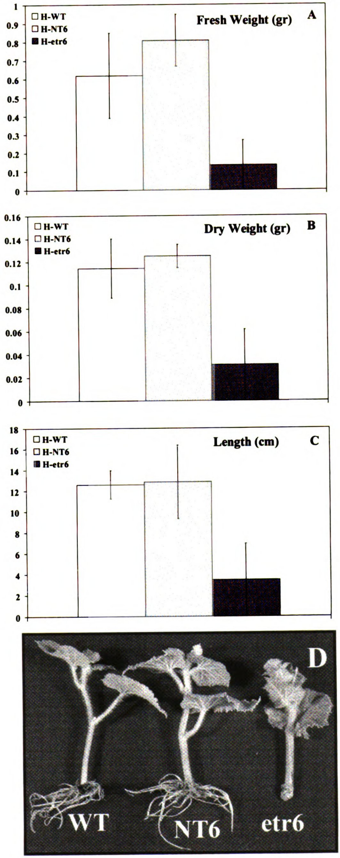
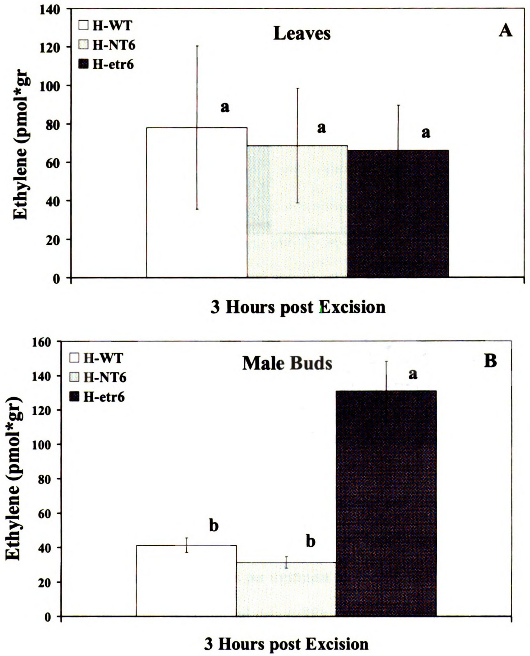

H-WT
H-NT6
H-etr6
Fresh Weight (gr)
A
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Dry Weight (gr)
B
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
Length (cm)
C
18
16
14
12
10
8
6
4
2
D
WT
NT6
etr6

Figure 3. (A) Average ethylene evolution of transgenic leaves in pmoL and gr of tissue, at 3 hours post excision. The experiment was repeated four times, with a total of 20 for H-WT, 8 for H-NT6, and 26 for H-etr6 samples. Values are the means of the combined data (±SE) (ANOVA and orthogonal contrast). (B) Average ethylene evolution of male flower buds, at 3 hours post excision. The experiment was performed four times with 8-10 wild buds per experiment for H-WT and 4 buds per experiment for H-NT6 and H-etr6. H-WT: non- transformed Hale's Best Jumbo plants, H-NT6: non-transgenic segregant progeny from the H-etr parent line 6, and H-etr6: transgenic *etr1-1* T_1 plants line 6. Values are the means of the combined



Leaves
A
H-WT
H-NT6
H-etr6
Ethylene (pmol*gr
3 Hours post Excision
Male Buds
B
Ethylene (pmol*gr)
3 Hours post Excision

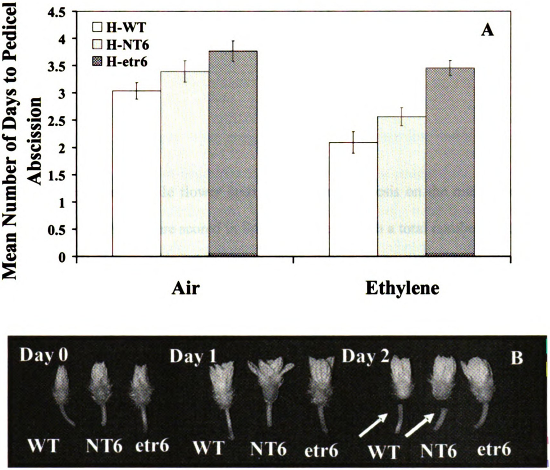


Figure 4. Response of flower buds to exogenous ethylene. (A) Average number of days to flower pedicel abscission, in air and in presence of 3ppm ethylene. The experiment was repeated seven times with 4 buds per treatment for H-WT, H-NT6 and H-etr6 plants. Values are the means of the combined data (±SE). Orthogonal contrast analysis showed significant differences between the control and the transgenic flowers. (B) Flower pedicel abscission and senescence in air. H-WT: non- transformed Hale's Best Jumbo plants, H-NT6: non-transgenic segregant progeny from the H-etr parent line 6, and H-etr6: transgenic *etr1-1* T_1 plants line 6.

Table 2. Percentage of male flower buds that reached anthesis on the main stem of T_1 transgenic plants. Plants were scored in four experiments with a total number of 43 for H-WT, 14 for H-NT6, and 51 for H-etr6. H-WT: non- transformed Hale's Best Jumbo plants, H-NT6: non-transgenic segregant progeny from the H-etr parent line 6, and H-etr6: transgenic *etr1-1* T_1 plants line 6.

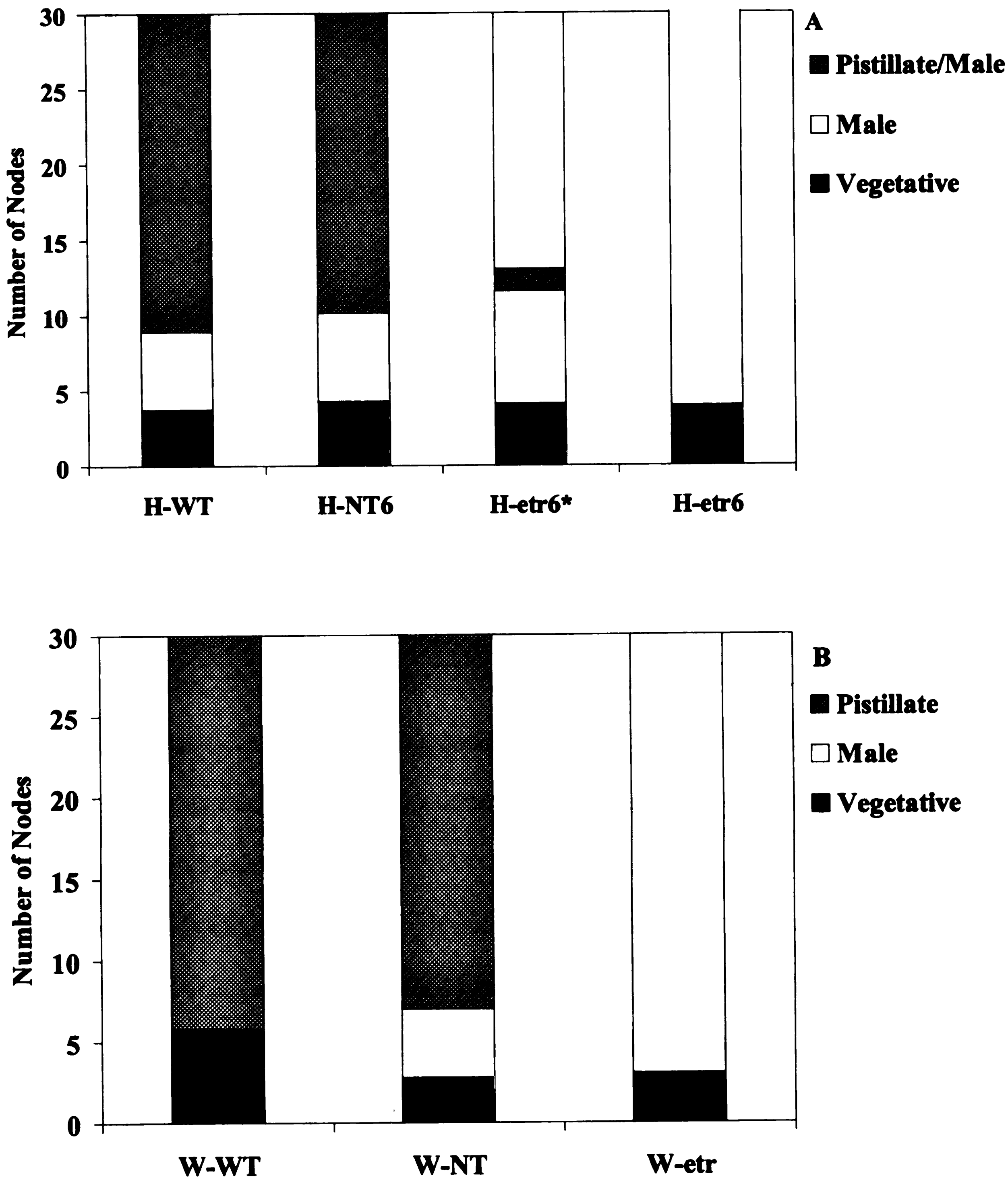
Genotypes	Percent of Mature Male Flowers
H-WT	67.5
H-NT6	77.6
H-etr6	97.6

Consistent with abscission of excised buds, flower buds formed on the transgenic plants were more likely to develop to anthesis, rather than abort. All the male buds produced on transgenic melons developed fully, while only 67.5% of the control buds reached anthesis (Table 2).

Sex Expression

The transgenic plants were monitored for sex expression pattern. All plants produced approximately four vegetative nodes prior to flower production. For wild type and non-transgenic segregants the next 5-7 nodes produced only male flowers, followed by initiation of the hermaphroditic flower phase, which includes a mixture of both hermaphroditic and male buds at each node with a least one hermaphroditic bud per node (Figure 5). The majority (86%) of transgenic plants, however, failed to produce any hermaphroditic flower buds; during the observation period of 30 nodes of the main stem (Figure 5). Some plants were even observed until the 40^{th} node and still did not form hermaphroditic buds. There was a small number (7/51) of transgenic plants that formed 1-2 hermaphroditic nodes at approximately the time that the H-WT and the H-NT6 plants formed their first hermaphroditic bud; but then converted back to production of only male buds. Non-transformed plants produce hermaphroditic flower buds in almost every node along the lateral branches. Although sex pattern was scored only along the main stem, transgenic plants scarcely produced hermaphroditic flowers on lateral branches. We also observed that the female buds formed occasionally on the *etr1-1* transgenic plants reached anthesis, whereas only 11% of the female buds on H-WT plants developed fully. Evaluation of the sex expression phenotype of the F_1 segregating progeny, of WI-998 X WI-998 T_0 *etr1-1*, showed that introduction of the *etr1-1* gene into a gynoecious melon genotype caused the formation of male flower buds instead of only pistillate buds

Figure 5. (A) Production of vegetative and flowering nodes along the main stem of transgenic T_1 plants derived from cv. Hale's Best Jumbo (andromonoecious). Each node produces multiple buds and is scored as pistillate when at least one pistillate bud is formed. H-WT: non- transformed Hale's Best Jumbo plants (n=43); H-NT6: non-transgenic segregant progeny from the H-etr parent line 6 (n=14); H-etr*6: transgenic plants that produced 1-2 pistillate flowers (n=7); H-etr6: transgenic *etr1-1* T_1 plants line 6 (n=44). The experiment was repeated four times. Values are the means of the combined data. (B) Production of vegetative and flowering nodes along the main stem of an F1 segregating population of transgenic plants derived from WI-998-*etr1-1* T_0 plant crossed by WI-998 genotype plants (gynoecious). W-WT: WI-998 gynoecious plants (n=5), W-NT: non-transgenic segregants within the family (n=5), W-etr: transgenic plants (n=4). The experiment was performed once due to limited seed availability.



30
25
20
15
10
5
0
Number of Nodes
H-WT
H-NT6
H-etr6*
H-etr6
A
Pistillate/Male
Male
Vegetative
30
25
20
15
10
5
0
Number of Nodes
W-WT
W-NT
W-etr
B
Pistillate
Male
Vegetative

(Figure 5). The segregating W-NT plants produced a few male buds early in their development. We also have observed this phenomenon occasionally also in the wild type WI998 plants.

Discussion

The results presented here show that the dominant mutant *Arabidopsis* ethylene receptor gene, *etr1-1*, conferred ethylene insensitive related phenotypes and failure to respond to exogenous ethylene. Among the ethylene-insensitive related phenotypes were increased ethylene evolution, reduced root formation and delayed flower bud pedicel abscission. Furthermore, unlike the control flower buds, transgenic *etr1-1* flowers treated with ethylene did not respond by accelerated pedicel abscission.

Excised male flower buds of the transgenic plants showed higher ethylene evolution than the H-NT and the H-WT plants. Elevated ethylene production was also observed in leaves of *etr1-1 Arabidopsis* plants (Schaller et al., 1995) and in hand-pollinated transgenic *etr1-1* expressing petunia flowers (Wilkinson et al., 1997). The phenomenon was attributed to disruption of a feedback mechanism that controls ethylene biosynthesis (Wilkinson et al., 1997).

Among the effects of ethylene, it has been implicated as a positive regulator of root growth and root hair development (Tanimoto et al., 1995). Ethylene biosynthesis inhibitors repressed root formation in tomato cotyledon and lavandin micro shoots explants *in vitro*, indicating a requirement for ethylene to induce adventitious root formation (Mensuali-Sodi et al., 1995). Similarly, inhibitors of ethylene synthesis and perception inhibited root hair development of *Arabidopsis* (Tanimoto et al., 1995). Cuttings from transgenic *etr1-1* petunia plants, grown in sand, also showed reduced root formation, suggesting that endogenous ethylene responsiveness is necessary for adventitious root formation (Clark et al., 1999). Our results are consistent with these observations. Vegetative cuttings of transgenic *etr1-1* melon plants showed reduced and delayed formation of adventitious roots.

Ethylene insensitivity of the transgenic plants was also manifested by increased percentage of flower buds that reached anthesis prior to abscission and the delayed pedicel abscission of excised male buds compared to the H-WT and the H-NT plants. Furthermore, exogenous ethylene accelerated pedicel abscission of H-WT and H-NT plants, but did not alter the response of the transgenic plants. These results are consistent with observations with transgenic *etr1-1* tomatoes and petunias (Wilkinson et al., 1997). Transgenic tomato flowers showed delayed senescence and abscission, and even though the increase in ethylene evolution normally observed after pollination is higher for transgenic *etr1-1* petunia flowers, the transgenic flowers were slower to senesce and abscise, and failed to respond to exogenous ethylene with accelerated senescence.

In these studies we demonstrate that the heterologous *etr1-1* ethylene receptor gene from *Arabidopsis* also can modify sex expression pattern of andromonoecious and gynoecious melons. Formation of hermaphroditic flower buds was largely abolished in the transgenic *etr1-1* plants. These results indicate the importance of ethylene perception for pistil formation in melon, and are consistent with the effects of exogenous inhibitors of ethylene action, which cause a delay in the pistillate flower phase (Byers et al., 1972; Byers et al., 1972).

Recent studies have identified putative ethylene receptors in melon and cucumber (Yamasaki et al., 2000). In cucumber expression of ethylene receptor homologs *CS-*

ETR1, *CS-ETR2* and *CS-ERS* was correlated with sex expression. Higher levels were observed in shoot apices of a gynoecious vs. monoecious genotype, and higher accumulation of *CS-ETR2* and *CS-ERS* transcripts was observed in gynoecious cucumber at the 4-5 leaf stage (Yamasaki et al., 2000). Ethylene application increased expression of *CS-ETR1*, CS-*ETR2* and *CS-ERS* in apices of both monoecious (*M-ff*) and gynoecious (*M-F-*) cucumber genotypes, while application of AVG (aminoethoxyvinyl glycine), an ethylene synthesis inhibitor decreased expression of *CS-ETR1*, *CS-ETR2* and *CS-ERS* in the gynoecious genotype, suggesting regulation of receptor levels by endogenous ethylene levels (Yamasaki et al., 2000; Yamasaki et al., 2001). It has been suggested that andromonoecious genotypes are less responsive to ethylene, and that the *m* locus, which conditions monoecy vs. andromonoecy in cucumber, is involved in ethylene perception leading to inhibition of stamen development (Rudich, 1990; Yin and Quinn, 1995). Ethylene treatment increased female flower formation in a monoecious genotype, but was not sufficient to inhibit stamen development in an andromonoecious genotype, and did not cause an increase in expression of the ethylene receptor homologs (Yamasaki et al., 2001). Although putative ethylene receptor genes, *Cm-ETR1* and *Cm-ERS*, also have been identified in melon (Sato-Nara et al., 1999), they have not been characterized in association with sex expression.

In summary these experiments demonstrate that the dominant mutant ethylene receptor gene, the Arabidopsis *etr1-1* gene, conferred ethylene insensitivity in the heterologous species, melon. The transgenic melons also exhibited markedly altered patterns of sex expression. The inability of transgenic plants to perceive ethylene was correlated with a reduction or absence of hermaphroditic flower bud formation, indicating that responsiveness to ethylene is required for promotion of femaleness at the time of sex determination. These results presented here provide a direct demonstration of the requirement for ethylene perception to promote hermaphroditic flower formation in melon.

References

An G, Ebert PR, Mitra A, Ha SB (1988) Binary vectors. Plant Mol. Biol. Manual **A3:** 1-19

Augustine JJ, Baker LR, Sell HM (1973) Female flower induction on androecious cucumber *Cucumis sativus* L. J. Amer. Soc. Hort. Sci. **98:** 197-199

Bleecker AB, Kende H (2000) Ethylene: a gaseous signal molecule in plants. Annu. Rev. Cell Dev. Biol. **16:** 1-18

Byers RE, Baker LR, Dilley DR, Sell HM (1972) Chemical induction of perfect flowers on a gynoecious line of muskmelon, *Cucumis melo* L. HortScience **913:** 321-3131

Byers RE, Baker LR, Sell HM, Herner RC, Dilley DR (1972) Ethylene: A natural regulator of sex expression of *Cucumis melo* L. Proc. Natl. Acad. Sci. USA **69:** 717-720

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) Arabidopsis ethylene-response gene *ETR1*: similarity of product to two- component regulators [see comments]. Science **262:** 539-544

Clark DG, Gubrium EK, Barrett JE, Nell TA, Klee HJ (1999) Root formation in ethylene-insensitive plants. Plant Physiol. **121:** 53-60.

Gamble RL, Coonfield ML, Schaller GE (1998) Histidine kinase activity of the *ETR1* ethylene receptor from Arabidopsis. Proc. Natl. Acad. Sci. U S A **95:** 7825-7829.

Hall AE, Chen QG, Findell JL, Schaller GE, Bleecker AB (1999) The relationship between ethylene binding and dominant insensitivity conferred by mutant forms of the *ETR1* ethylene receptor. Plant Physiol. **121:** 291-300

Mensuali-Sodi A, Panizza M, Tognoni F (1995) Endogenous ethylene requirement for adventitious root induction and growth in tomato and lavandin microcuttings *in vitro*. Plant Growth Regul. **17:** 205-212

Perl-Treves R (1999) Male to female conversion along the cucumber shoot: approaches to studying sex genes and floral development in *Cucumis sativus*. *In* CC

Ainsworth, ed, Sex determination in plants. Bios Scientific Publishers, Oxford, UK, pp 189-215

Roy RP, Saran S (1990) Sex expression in the Cucurbitaceae. *In* DM Bates, RW Robinson, C Jeffry, eds, Biology and utilization of the Cucurbitaceae. Cornell Univ. Press, Ithaca, NY, pp 251-268

Rudich J (1990) Biochemical aspects of hormonal regulation of sex expression in Cucurbits. *In* DM Bates, RW Robinson, C Jeffry, eds, Biology and utilization of the Cucurbitaceae. Cornell Univ. Press, Ithaca, NY, pp 269-280

Rudich J, Baker LR, Scott JW, Sell HM (1976) Phenotypic stability and ethylene evolution in androecious cucumber. J. Amer. Soc. Hort. Sci. **101:** 48-51

Rudich J, Halevy AH, Kedar N (1969) Increase in femaleness of three cucurbits by treatment with ethrel, an ethylene releasing compound. Planta **86:** 69-76

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

Sambrook J, Russell DW (2001) Molecular Cloning A Laboratory Manual, Ed Third. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sato-Nara K, Yuhashi KI, Higashi K, Hosoya K, Kubota M, Ezura H (1999) Stage- and tissue-specific expression of ethylene receptor homolog genes during fruit development in muskmelon. Plant Physiol. **120:** 321-330.

Schaller EG, Bleecker AB (1995) Ethylene-binding sites generated in yeast expressing the *Arabidopsis ETR1* gene. Science **270:** 1809-1811

Schaller GE, Ladd AN, Lanahan MB, Spanbauer JM, Bleecker AB (1995) The ethylene response mediator *ETR1* from Arabidopsis forms a disulfide- linked dimer. J. Biol. Chem. **270:** 12526-12530.

Tanimoto M, Roberts K, Dolan L (1995) Ethylene is a positive regulator of root hair development in *Arabidopsis thaliana*. Plant J. **8:** 943-948.

Wilkinson JQ, Lanahan MB, Clark DG, Bleecker AB, Chang C, Meyerowitz EM, Klee HJ (1997) A dominant mutant receptor from Arabidopsis confers ethylene insensitivity in heterologous plants. Nature Biotechnol. **15:** 444-447.

Yadav RC, Saleh MT, Grumet R (1996) High frequency shoot regeneration from leaf explants of muskmelon. Plant Cell Tiss. Org. Cult. **45:** 207-214

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 (2000) The ethylene-regulated expression of *CS-ETR2* and *CS-ERS* genes in cucumber plants and their possible involvement with sex expression in flowers. Plant Cell Physiol. **41:** 608-616.

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

CHAPTER 3

Effect of brassinosteroids and the maize *TASSELSEED 2* gene on sex expression of cucurbit species.

Abstract

Brassinosteroids (BRs), are a recently recognized group of plant hormones, that can affect a number of plant developmental processes, including promotion of cell division and expansion, vascular differentiation, reproductive development, senescence, photomorphogenesis and enhancement of plant tolerance to stress. Our results show that application of BRs to monoecious cucumber plants also can influence sex expression by inducing early female flower formation and increasing the number of female buds formed along the main stem. Sex expression of zucchini and melon plants was not affected by BR treatment. Further investigation revealed that BRs increase ethylene evolution when applied to the growing point of intact seedlings, suggesting that the effect on femaleness can be attributed to increased ethylene production. Application of ethephon that released a low level of ethylene equivalent to that caused by BRs caused increased femaleness in cucumber but not in zucchini. The maize sex determination gene *TASSELSEED2* (Calderon-Urrea and Dellaporta, 1999) shows sequence similarity to bacterial short-chain alcohol dehydrogenases, both gibberellins (GAs) and BRs have been suggested as possible *TS2* substrates. We sought test whether expression of the maize sex determination gene *TS2* would influence sex expression in andromonoecious melon plants. Presence of the heterologous gene *TS2* did not affect either earliness or the number of hermaphroditic flowers formed along the main stem.

Introduction

Flowering habit of species in the *Cucurbitaceae* family is mainly monoecious or andromonoecious (Perl-Treves, 1999). Monoecious species, such as cucumber (Augustine et al., 1973) and squash (Chrominski and Kopcewicz, 1972), bear separate male and female flowers on the same plant. During plant development, male flowers are produced first, followed by a phase of predominantly female flowers. Similarly, andromonoecious species such as melons (Byers et al., 1972) produce male flowers followed by a phase of bisexual (or hermaphroditic) flowers (Roy and Saran, 1990). Sex determination of individual flower buds is a response to genetic, environmental and hormonal factors (Perl-Treves, 1999). At early stages of flower initiation male and female buds are morphologically indistinguishable, all four whorls (sepals, petals, stamens and carpels) are initiated, and subsequently either male or female organs develop. Studies with homeotic mutants of cucumber indicate the position within the developing flowers bud is critical for determination of whether the specific floral organ will develop (Kater et al., 2001).

Several plant hormones, including ethylene, auxins and gibberellins (GAs) have been shown to influence flower sex expression in cucurbits. Application of ethylene or ethylene releasing compounds increased pistillate flower production in monoecious cucumber, squash and muskmelon (Rudich et al., 1969, 1972; Augustine et al., 1973). Consistent with the exogenous effect, excised apices and flower buds of gynoecious cucumber plants produced 1.5 to 2.5 fold higher ethylene levels compared to monoecious and andromonoecious genotypes, suggesting a correlation between sex expression and endogenous ethylene production (Byers et al., 1972; Rudich et al., 1972; Rudich et al., 1976). We observed that transgenic melon expressing ACS (1-amino-cyclopropane-1-carboxylic acid synthase), a key enzyme in ethylene biosynthesis, formed perfect flowers sooner. In contrast, inhibitors of ethylene biosynthesis and action can induce staminate flower development (Byers et al., 1972; Den Nijs and Visser, 1980; Takahashi and Jaffe, 1984), and have been used as a tool by breeders to facilitate seed production of gynoecious genotypes (Tolla and Peterson, 1979). Transgenic melon expressing the Arabidopsis mutant ethylene perception gene, *etr1-1*, failed to produce female flowers. Hormonal application studies have suggested that ethylene plays a dual role in sex expression in cucumber, by both promoting female and inhibiting male flower formation (Yin and Quinn, 1995).Application of the auxin indole-3-acetic acid (IAA) on cucumber flower buds at the pre-sexual stage also resulted in female flower formation (Galun et al., 1963). However this effect has been largely attributed to increased ethylene production that occurred in response to the IAA application (Takahashi and Jaffe, 1984). Furthermore, the observations that endogenous levels of IAA were found to be lower in gynoecious than monoecious cucumber and squash genotypes (Chrominski and Kopcewicz, 1972; Trebitsh et al., 1987), and that treatment of gynoecious plants with anti-auxin compounds did not induce staminate flower formation (Trebitsh et al., 1987), lend additional support to a secondary role of auxins.

Exogenous gibberellins (GAs) exhibit the opposite effect of ethylene and have been shown to induce formation of male flowers on leaf axils normally occupied by female flowers (Tolla and Peterson, 1979). Higher amounts and activity of GAs were reported in monoecious compared to gynoecious lines (Atsmon et al., 1968; Freidlander et al., 1977). GAs are also involved in sex differentiation of maize, a role which is supported by the GA-deficient mutant phenotype of *an1* (*anther ear1*) and *d* genes (Bensen et al., 1995; Azpiroz et al., 1998). Mutations in these genes leading to reduced GA levels cause increased maleness exhibited by stamen development in ear florets, resulting in hermaphrodite flowers, whereas pistil abortion in tassel florets is unaffected (Bensen et al., 1995). In contrast, mutation in the *TASSELSEED 2* (Calderon-Urrea and Dellaporta, 1999) gene leads to the formation of female flowers in the normally male tassel (DeLong et al., 1993). The proposed function of *TS2* is to trigger a programmed organ death that results in gynoecium abortion in the tassel and the suppression of the lower floret development in the ear (Calderon-Urrea and Dellaporta, 1999). GAs have been suggested as potential substrates of the proposed steroid alcohol dehydrogenase activity of TS2 (DeLong et al., 1993). However, the double mutant phenotype *ts2/d1*, where hermaphrodite flowers are formed both in tassel and ear, suggests the possibility that *TS2* works independently from GAs (Ainsworth et al., 1998).

Recently, the brassinosteroids (BRs), a new group of polyhydroxysteroid plant hormones, has received increasing attention. BRs, initially isolated from *Brassica napus* pollen, are found in a variety of plant species, including monocotyledonous, dicotyledonous, green alga and a fern, and can be isolated from seeds, fruits, shoots, leaves and flower buds, at endogenous levels sufficient to promote physiological effects (Sasse, 1997; Clouse and Sasse, 1998). A role for endogenous BRs in plant growth and development was demonstrated recently, via the identification of *Arabidopsis thaliana* mutants defective in BR biosynthesis and response, which provided strong evidence that BRs play an essential role in several aspects of plant growth and development (Altmann, 1999; Li and Chory, 1999). Biological activity of applied BRs, at nanomolar (Ainsworth et al., 1998) or micromolar (μM) levels, was reported in various bioassay systems designed for GAs, auxins and cytokinins (Takatsuto et al., 1983; Altmann, 1999). BRs have been shown to promote cell division and expansion, vascular differentiation, reproductive development, senescence, photomorphogenesis and enhance plant tolerance to stress (Suge, 1986; Clouse et al., 1992; Clouse et al., 1996; Kauschmann et al., 1996; Li et al., 1996; Szekeres et al., 1996; Nomura et al., 1997; Yamamoto et al., 1997; Azpiroz et al., 1998; Clouse and Sasse, 1998; Mathur et al., 1998; Oh and Clouse, 1998). Promotion of ethylene biosynthesis by BRs via increased ACS (1-aminocyclopropane-1-carboxylic acid synthase) activity in excised mung bean hypocotyls also has been reported (Arteca et al., 1988). BRs also have been proposed as potential substrates of the *TS2* gene since anthers are rich in BRs and two BR deficient mutants (*det2* and *cyp90*) show reduced male fertility (Li et al., 1996; Szekeres et al., 1996).

Given the range of activities of BRs, and the role of several hormones in influencing sex determination in cucurbits, we were interested in testing whether exogenous BRs would exert an effect on sex differentiation of cucurbits. Therefore, the purpose of the present study was to examine the potential involvement of BRs in sex differentiation of cucumber, melon and zucchini plants, and to dissect the effect of exogenous BR from ethylene. Our results indicate that application of BRs to cucumber cause earlier and increased female flower production, and that the effect of BRs may be mediated, at least in part, via increased ethylene production. We also sought to determine whether the *TS2* gene would influence the sex determination process of heterologous andromoecious melon plants expressing the gene constitutively or tissue specifically in petals and stamens.

Materials and Methods

Genotypes

The effect of BR was tested on monoecious cucumber cultivar Straight Eight (S8) (Hollar Seeds, Rocky Ford, CO), the gynoecious breeding line GY14 (kindly provided by Greg Tolla, Seminis Seeds, Tifton, GA) and the monoecious zucchini cv Black Beauty (Willhite, Poolville, TX). The andromonoecious melon cv. Hales' Best Jumbo (Chesmore Seed Company, St Joseph, MO) was used for transformation with the *TS2* gene.

Brassinosteroid Experiments

Seeds were imbibed overnight at 30°C and planted in 23cm diameter pots containing commercial soil mix (sphagnum peat 70-80%, pH 5,5-6,5) (Baccto, Michigan Peat Company, Houston, TX). Plants were grown in the greenhouse, under natural photoperiod, at average temperature of 25°C, and fertilized twice per week with 300ppm of the commercial fertilizing mix, Peter's Special 20-20-20. The synthetic BR, epibrassinolide (epi-BL) (22R, 23R, 24R-2α, 3α, 22,23-Tetrahydroxy-B-homo-7-oxa-5α-ergostan-6-one) (Sigma Chemical Co, St Louis, MO) was applied by pipetting 250μl solution at a concentration of 0.1, 1 or 10μM solution (BR dissolved in H_20), onto the apical meristem and the developing leaf. The first application was made at the first true leaf stage (mean diameter ~5cm), two subsequent treatments were applied at 3-day intervals.

The experiments were conducted according to a randomized complete block design (RCB), with 10 replications per treatment. The effect of epi-BL on sex determination was expressed as the number of female buds formed in 20 or 25 flowering nodes and the number of the first female node from the base.

Ethylene Experiments

Seeds were sterilized in LD solution (Alcide LD 10: 1:1 Disinfectant, Alcide Corporation, Norwalk, CT) for 10 minutes, rinsed with distilled water three times, and transferred in 250ml containers (Magenta) containing medium with 0.4% Murashige and Skoog basal salt (Sigma, St. Louis, MO), 3% sucrose, 0.8% agar, adjusted to pH 5.7-5.8, and placed under 16hours light at 25°C. Eight to 10 days old seedlings, when the cotyledons were fully expanded and first true leaf just emerging, were treated with 20µl of 0, 1 and 10µM epi-BL. Headspace gas samples were taken with an airtight syringe at 0, 6, 12, 24, 48, and 72 hours post application. The samples were analyzed in a gas chromatographer (HACH CARLE series 100AGC, Linear 1200 recorder, with an alumina column and flame ionization detector). The same experimental procedure was used to determine the concentration of ethephon ((2 chloroethyl) phosphonic acid) that releases the same amount of ethylene as that caused by application 10µM epi-BL. Ethephon was applied at concentrations of 500, 50 and 5ppm. The 5ppm ethephon concentration released comparable levels of ethylene as 10µM epi-Bl and so was used for subsequent experiments.

Influence of the *TS2* gene on sex expression of melons.

Plasmid Construction

The *TS2* cDNA clone (De Long *et al.,* 1993, kindly provided by Dr. A. Calderon-Urrea, Yale Univ.) was transferred into the pBSKS^{+} plasmid (Stratagene, La Jolla, CA), as an *EcoRI* fragment, and then transferred as a *HindIII-BamHI* fragment into the pGA643 plant transformation vector (An et al., 1988) downstream of the constitutive cauliflower mosaic virus *35S* promoter. Similarly, the floral specific *APETALA 3* (*AP3*) promoter from Arabidopsis (kindly provided by Dr. V. Irish, Yale Univ.) was transferred as a *HindIII-XbaI/NheI* fragment upstream of the *TS2* gene into the pBSKS$^+$ plasmid. The *35S* terminator, from the plasmid pCIB710 (CIBA-GEIGY, Research Triangle Park, NC) (Rothstein et al., 1987), was introduced as a *BamHI-XbaI* fragment, downstream of the *TS2* gene. Then the whole construct was transferred as *KpnI-XbaI* fragment into the pCIB10 (CIBA-GEIGY, Research Triangle Park, NC) (Rothstein et al., 1987) plant transformation vector. Both constructs were introduced into *Agrobacterium tumefasciens* strain EHA105 (Hoekema et al., 1983).

Plant Transformation

Transgenic melon plants were constructed by cotyledon transformation according to the method of Fang and Grumet (Roy and Saran, 1990). The regenerated plantlets were (T_0) selected on kanamycin (200mg/Lt), evaluated for presence of the kanamycin resistance gene *neomycin phosphotransferase* (*NPTII*) by polymerase chain reaction (PCR), (as described below) and then were self-pollinated to produce progeny (T1). The T1 generation plants were evaluated for the time of appearance of the first perfect flower bud and the total perfect buds formed along 30 nodes of the main stem.

PCR and Northern analysis

Genomic DNA from leaf tissue was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Amplification by Taq DNA polymerase (GIBCO Invitrogen Co, Carlsbad, CA) was performed in 1.5mM $MgCl_2$, for 5 min at 95°C, 2 min at 62°C and 1 min at 72°C for 1.5min cycle, followed by 1min at 95°C, 1min at 62°C and 1.5 min at 72°C for 35 cycles and for the last cycle 5min at 72°C. RNA was extracted from plant tissue using the TRIZOL RNA isolation reagent (GIBCO Invitrogen

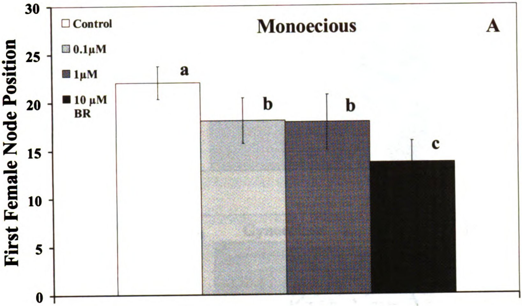
Co, Carlsbad, CA). For the Northern blot 15μg RNA were loaded on the gel and the analysis was performed following standard procedures (Sambrook and Russell, 2001).

Results

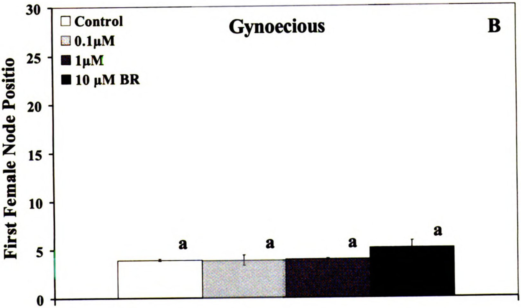
Sex Expression

Of the three species tested, cucumber, melon, and zucchini, cucumber appeared to be the most sensitive to BR application. Ten and 100μM of epi-BL caused a significant decrease (ANOVA, $P \leq 0.001$) in the node position of the first female flower bud of monoecious cucumber, from an average of 24 for the control to 14 with 10μM epi-BL (Figure 1A). Applied epi-BL also caused an increase (ANOVA, $P \leq 0.001$) in the number of female flowers formed in 25 flowering nodes from the base of the plant (Figure 1C). There appeared to be a progressive effect on both time to appearance of the first female flower and the number of female flowers as epi-BL concentration increased. The 100μM concentration occasionally caused damage to the growing points, and so is not included in this analysis. For all future experiments 10μM epi-BL was used. In addition, as might be expected from gynoecious cucumbers that already produce female buds at the first flowering node (usually node 4), epi-BL treatment did not influence the earliness or the number of female buds of gynoecious cucumber plants (Figure 1B and D). Application of epi-BL did not significantly alter the sex expression pattern, earliness or number of female flowers of melon plants (data not shown) and did not alter number of female flowers in monoecious zucchini (Figure 1E).

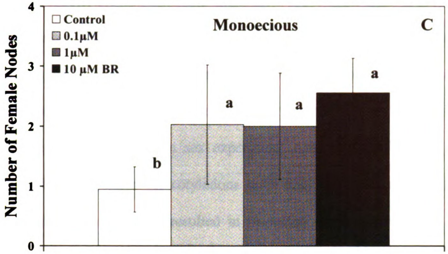
Figure 1. Appearance of the first female bud on monoecious (A) and gynoecious (B) cucumber, and the number of the female buds formed on monoecious (C) and gynoecious (D) cucumber plants along 25 nodes of the main stem. Each value is the mean of 4 experiments (±SE) with 9 replicates per experiment for the monoecious cucumber and of 3 experiments (±SE) with 5, 9 and 9 replicates, respectively, for the gynoecious cucumber. Equivalent trends were observed in each experiment. Significant differences were observed for the effect of BR on the first female node position and the number of female flowers for the monoecious cucumber (ANOVA and orthogonal contrast). (E) Number of female flower buds formed on monoecious zucchini. The experiment was repeated three times. Values are the means of combined data (± SE).



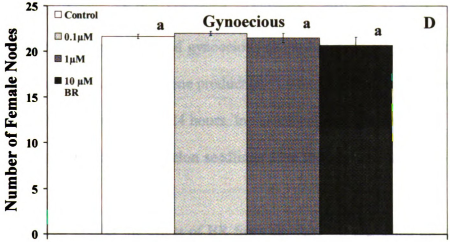
Monoecious
A
Control
0.1µM
1µM
10 µM BR
First Female Node Position
30
25
20
15
10
5
0
a
b
b
c



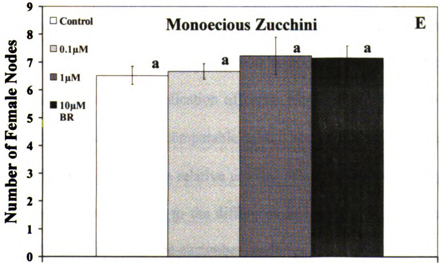
Gynoecious
B
Control
0.1µM
1µM
10 µM BR
First Female Node Positio
30
25
20
15
10
5
0
a
a
a
a



Monoecious
C
Control
0.1μM
1μM
10 μM BR
Number of Female Nodes
4
3
2
1
0
a
a
a
b



Gynoecious
D
Control
0.1μM
1μM
10 μM BR
Number of Female Nodes
25
20
15
10
5
0
a
a
a

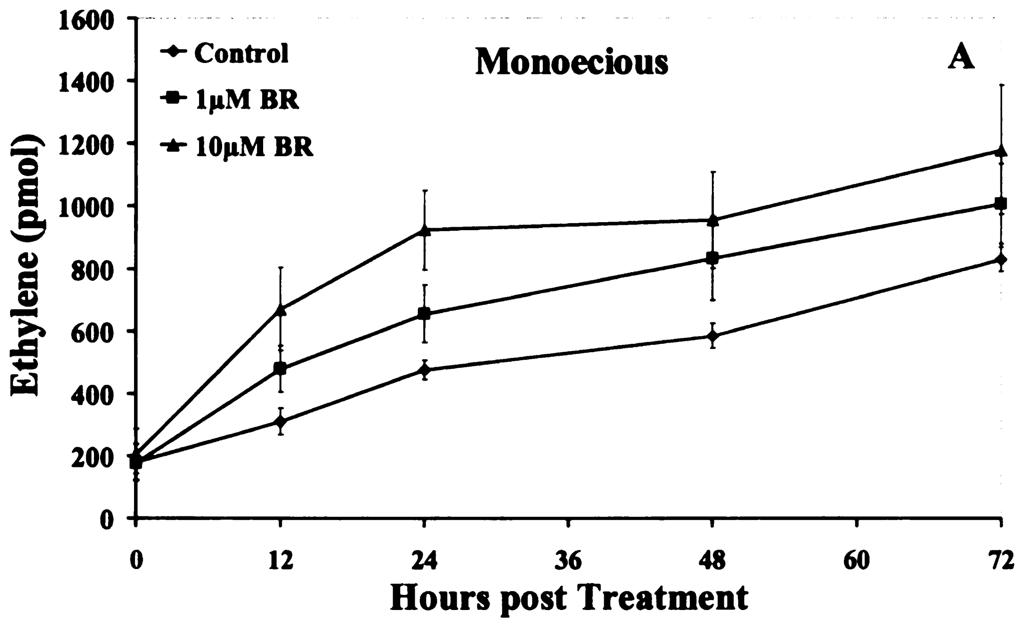


Monoecious Zucchini
E
Control
0.1μM
1μM
10μM BR
Number of Female Nodes
9
8
7
6
5
4
3
2
1
0
a
a
a
a

Relationship between BR treatment and ethylene

Exogenous BR can increase ethylene production in excised mung bean hypocotyls via increased ACS activity (Arteca et al., 1988); therefore we sought to determine whether BR could increase ethylene production in intact plants and whether this might be related to the observed effect on sex expression. Application of epi-BL to cucumber seedlings, at the time when the cotyledons were fully expanded and the first leaf was developing (ca 8-10 days old), resulted in increased ethylene evolution, compared to water treated control, in both monoecious and gynoecious cucumber genotypes (Figure 2A and B). Ethylene evolution increased and then leveled off at about 24 hours after treatment. In both monoecious and gynoecious cucumber cultivars, higher levels of epi-BL result in higher levels of ethylene production; 1.4 and 1.7 fold for 1μM and 2 fold and 3 fold for 10μM, respectively, at 24 hours. Increased ethylene production of 2-3 fold also was observed for zucchini and melon seedlings after the application of epi-BL (data not shown).

To further dissect the effect of BR from ethylene we identified the concentration of ethephon that induces ethylene production similar to levels induced by 10μM epi-BL. A range of concentrations 500, 50 and 5ppm was tested. The standard level applied for sex conversion of cucumber in the greenhouse is 50ppm (Augustine et al., 1973). For both cucumber and zucchini application of 5ppm ethephon was found to give a 2 fold increase in ethylene production comparable to that observed with 10μM epi-BL (Figure 3A and B). The difference in the relative amount of ethylene produced by zucchini and cucumber seedlings is attributed to the difference in biomass, as the zucchini seedlings weigh about 4 times more than the cucumber seedlings.



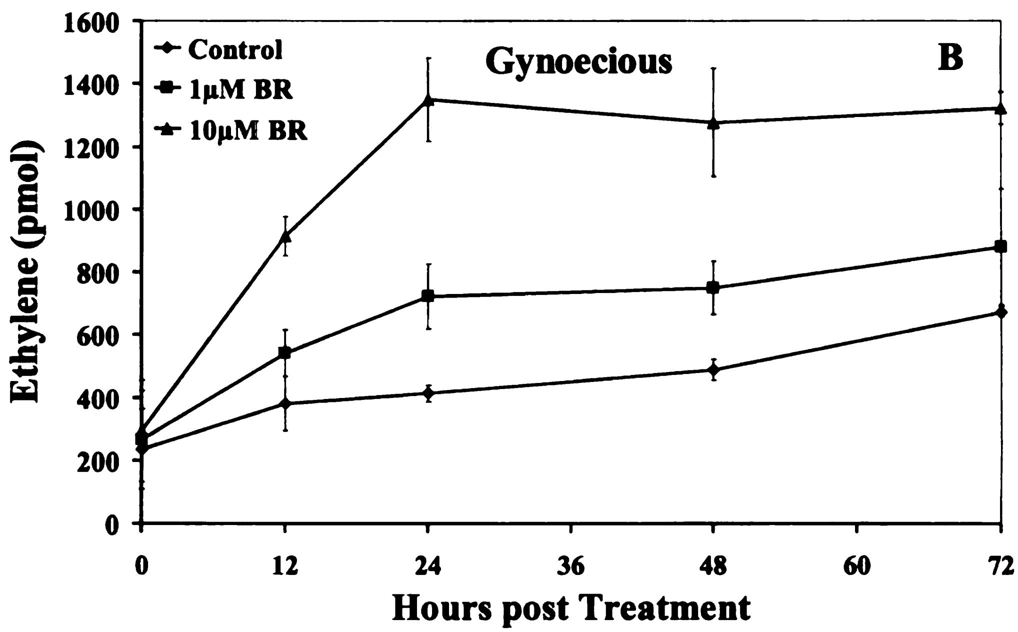


Figure 2. Ethylene evolution of monoecious (A) and gynoecious (B) cucumber seedlings treated with 1µM and 10µM epi-BL. Each point is the mean of 3 experiments (± SE) with 5, 4 and 4 replicates (containers) per treatment and 6 seedlings per container. Similar trends were observed in all 3 experiments; BR treatment caused a significant increase in ethylene evolution for both monoecious and gynoecious cucumber seedlings (ANOVA).

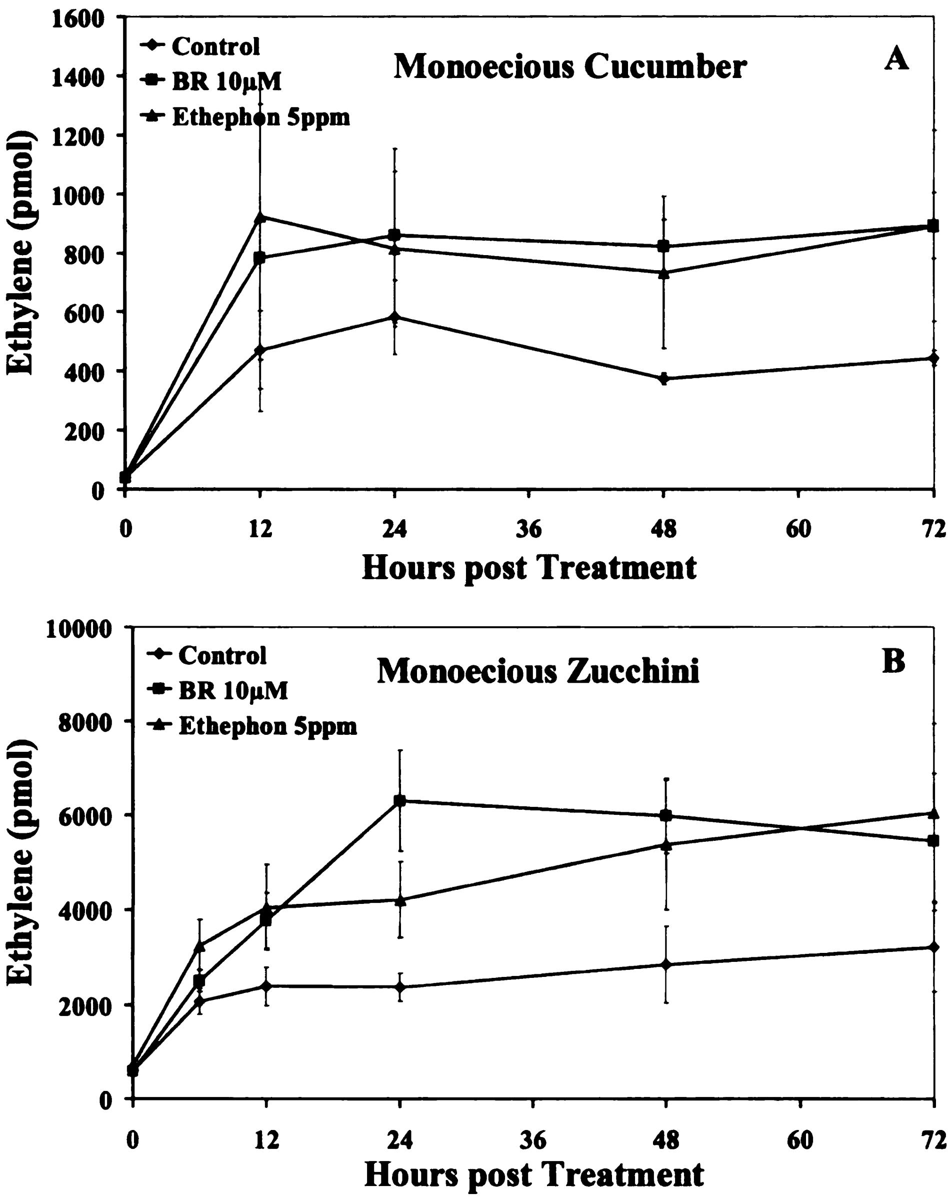
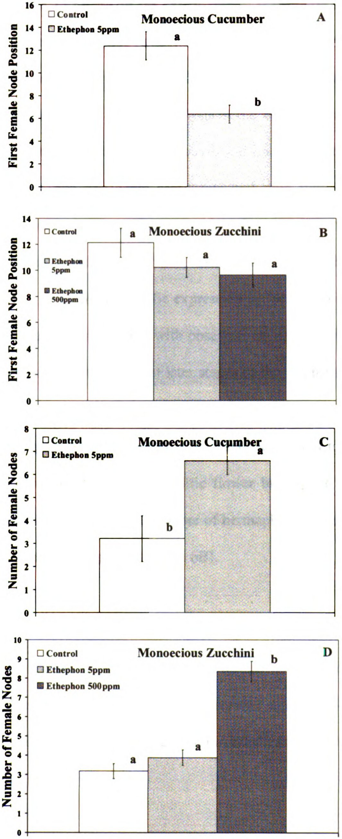


Figure 3. Ethylene evolution of monoecious cucumber (A) and zucchini (B) seedlings treated with 10μM epi-BL and 5ppm ethephon. Each point is the mean of 3 experiments (± SE) with 5, 4, and 5 replicates (containers) per treatment and 6 seedlings per container for cucumber and the mean of 3 experiments with 5 replicates (containers) per treatment and 6 seedlings per container for zucchini. Similar trends were observed in all 3 experiments; BR and ethephon treatments caused significant increase in ethylene evolution from the control, but not from each other (ANOVA).

Subsequently, a set of experiments was conducted to compare the effect of BR and ethephon applied at concentrations that release comparable amount of ethylene, on sex expression of cucumber and zucchini plants. The low ethephon treatment caused a significant decrease (ANOVA, $P \leq 0.002$) in the position of the first pistillate node of monoecious cucumber plants and number of pistillate flowers compared to the control plants (ANOVA, $P \leq 0.002$) (Figure 4A), indicating that the low level of ethylene produced by epi-BL was sufficient to increase femaleness in monoecious cucumber. Consistent with previous results, however, where epi-BL did not increase female flower production on monoecious zucchini plants, The low ethylene treatment (5ppm) also was not sufficient to induce earlier formation of female buds compared to the control plants (ANOVA, $P \leq 0.009$) or to increase number of female buds in zucchini (ANOVA, $P \leq 0.001$) (Figure 4B and D). Even though the higher ethylene treatment (500ppm) did not affect the time of appearance of the first female flower, it resulted in significant increase (ANOVA, $P \leq 0.0001$) of female flowers in 20 nodes compared to the control plants.

Figure 4. Effect of ethephon treatment on sex expression in cucumber and zucchini plants. Appearance of the first female bud on monoecious cucumber (A) and zucchini (B) plants, and the number of the female buds formed on monoecious cucumber along 25 nodes of the main stem (C) and on monoecious zucchini plants (D) for 20 nodes. Each value is the mean of 4 experiments (± SE) with 9, 10, 8 and 7 replicates per treatment for the cucumber results and the mean of 4 experiments (± SE) with 10 replicates per treatment for the zucchini results. Similar trends were observed in all experiments. Significant differences were observed for the first female node position and the number of female buds formed for the monoecious cucumber (ANOVA). In zucchini the 5ppm ethephon treatments were not sufficient to induce earliness, however the 500ppm treatment significantly increased the number of female buds formed (ANOVA).


Monoecious Cucumber
A
Control
Ethephon 5ppm
First Female Node Position
0
2
4
6
8
10
12
14
16
a
b
Monoecious Zucchini
B
Control
Ethephon 5ppm
Ethephon 500ppm
First Female Node Position
0
2
4
6
8
10
12
14
a
a
a
Monoecious Cucumber
C
Control
Ethephon 5ppm
Number of Female Nodes
0
1
2
3
4
5
6
7
8
a
b
Monoecious Zucchini
D
Control
Ethephon 5ppm
Ethephon 500ppm
Number of Female Nodes
0
1
2
3
4
5
6
7
8
9
10
a
a
b

Influence of the maize *TS2* gene on sex expression of transgenic melons.

The *TS2* gene from maize was introduced into an andromonoecious melon genotype to test its role as a sex determination gene in a heterologous system. Expression of the *TS2* gene was verified by northern analysis of the T_0 plants and T_1 progeny (Figure 5). As expected, *TS2* was expressed in both leaves and flower buds of the transgenic *35S-TS2* plants but not in non-transgenic segregants and wild type plants. *TS2* was not expressed in leaves of transgenic plants carrying the *AP3-TS2* construct, but it was expressed in both male and female buds, demonstrating that the *Arabidopsis AP3* promoter was able to drive floral specific expression in the heterologous melon system. Expression in the ovaries is consistent with observations in *Arabidopsis* flowers, where the *AP3* promoter remains active even at later stages of development. Comparable levels of expression were observed in young buds of the *35S-TS2* and the *AP3-TS2* plants. The presence of the *TS2* gene, either constitutive or floral specific did not significantly affect time of appearance of the first hermaphroditic flower bud of the T_1 generation plants (Figure 6A) and did not affect the total number of hermaphroditic buds formed, along the main stem of transgenic melon plants (Figure 6B).

Discussion

The role of BRs on the reproductive development of higher plants has not been extensively studied. Earlier study showed that BRs did not influence flower induction when applied on the short-day plant *Perilla frutescence* under non-inductive conditions and only slightly enhanced the flowering of non-vernalized long-day *Raphanus sativus* plants (Suge, 1986).

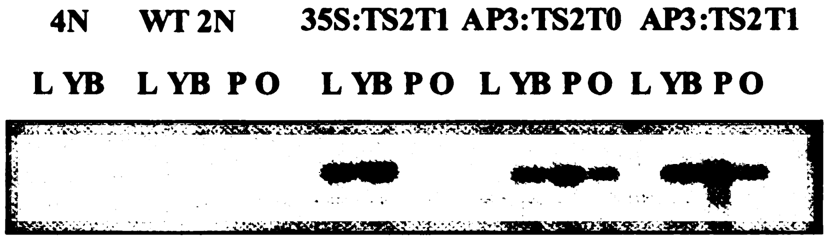
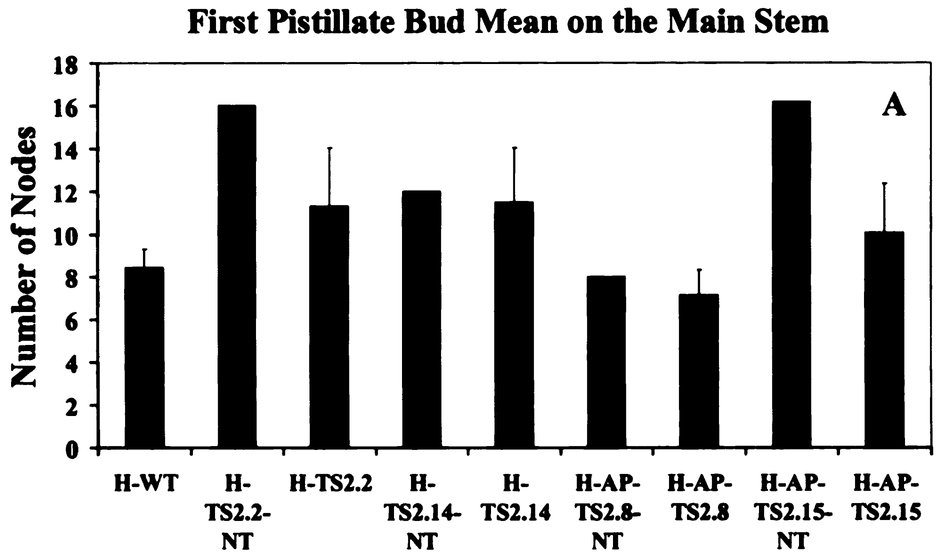


Figure 5. Northern blot analysis for the expression of *TS2* gene driven by the *35S* or the *AP3* promoter (4N=non-transgenic tetraploid, WT=Hales wild type, *35S-TS2* and *AP3-TS2* transgenic plants, L= leaf, YB= young male bud, P and O= petals and ovaries of pistillate buds, respectively).



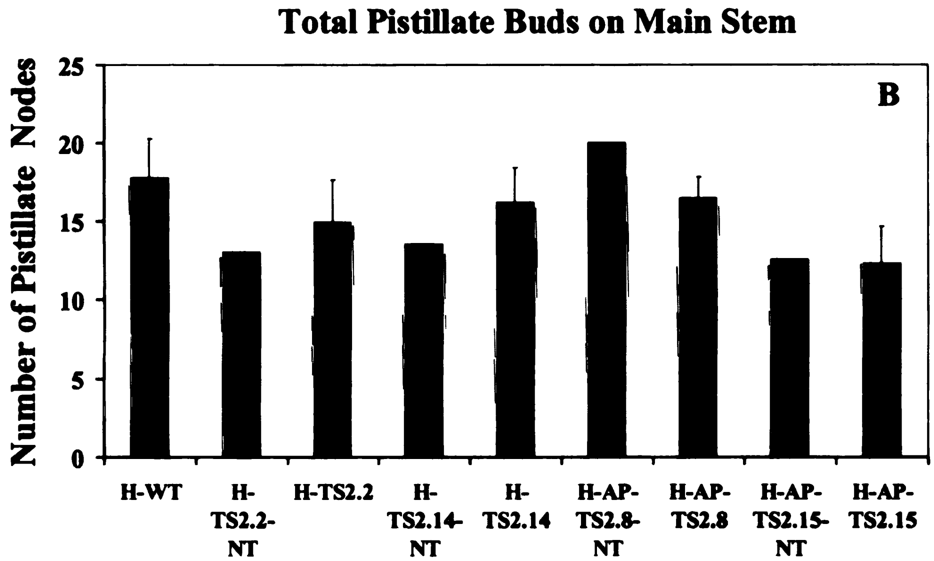


Figure 6. Effect of the *TS2* gene on sex expression of andromonoecious melon plants expressed as the node position of the first pistillate bud (A), the node carrying the first mature pistillate flower (B), and the total pistillate nodes (C) along the main stem (±SE). H-WT: untransformed Hales' Best Jumbo control plants (n=20), H-TS2.2: *35S-TS2* T_1 transgenic plants line 2 (n= 16), H-TS2.12: *35S-TS2* T_1 transgenic plants line 12 (n= 24), H-AP-TS2.8: *AP3-TS2* T_1 transgenic plants line 8 (n=12), and H-AP-TS2.15: *AP3-TS2* T_1 transgenic plants line 15 (n=13). All columns labeled with NT represent non-transgenic segregant progeny within each transgenic line.

Recently, BRs have been implicated in reproductive development based on the phenotype of *det2* and *cpd* Arabidopsis mutants, which show reduced male fertility due to inhibition of pollen elongation during germination (Szekeres et al., 1996). Based on BRs hydroxysteroid structure and localization to the stamens, BRs were also proposed as potential substrates of the *TASSELSEED2* (Calderon-Urrea and Dellaporta, 1999) gene, which encodes a putative short-chain alcohol hydroxysteroid dehydrogenase and is essential for sex determination in maize, specifically for the female primordia abortion (Calderon-Urrea and Dellaporta, 1999). Application of BRs to male inflorescence of the cucurbit plant *Luffa cylindrica* resulted in formation of hermaphroditic and female flowers (Suge, 1986). The higher doses caused the formation of deformed flower sepals and the reproductive meristems at the top of the inflorescence were converted into vegetative shoots.

This report demonstrates that application of epi-BL on monoecious cucumber plants caused earlier and increased production of female buds, indicating a possible involvement of BRs in floral sex differentiation in this species. Our experiments however, did not show an increase in femaleness in zucchini or melon in response to applied BR. The observed differences among the cucurbit species may reflect differences in responsiveness or sensitivity to BR between the different species.

Another possible explanation for the difference in response among these cucurbit species is that BR acts indirectly, via increased ethylene production, and that the level of ethylene produced is sufficient to increase femaleness in monoecious cucumber, but not zucchini or melon. Earlier work showed that exogenous BRs promote ethylene production in excised mung bean hypocotyls via induction of *ACS* gene (Arteca et al., 1988). The experiments presented here verify ability of BR to also increase ethylene production in intact plants, and suggests that the observed increase in femaleness might be attributed to increased ethylene availability. However, even melon and zucchini, which did not show increased femaleness in response to epi-BL treatment, showed a similar-fold increase in ethylene production in response to epi-BL treatment.

The different species showed different sensitivity to ethylene consistent with their responsiveness to epi-BL. While the 5ppm ethephon treatment was sufficient to cause early female flower formation and an increase in the female flower production in monoecious cucumber it was not sufficient to significantly increase femaleness in zucchini. Thus, a likely explanation for the different responses of cucumber and zucchini to epi-BL is indirectly due to their differential sensitivity to ethylene. Indeed, levels of ethephon used in the literature to increase femaleness in zucchini are in the range of 250ppm to 500 and 1000mg/lt (More and Seshadri, 1998) vs 50ppm for cucumber (Augustine et al., 1973).

A similar relationship has been shown for the effect of auxin in femaleness in cucurbits. High levels of endogenous auxin have been related to female tendency (Galun et al., 1964), and application of auxin increased femaleness (Takahashi and Jaffe, 1984) and ethylene evolution in cucumber plants (Trebitsh et al., 1987). However, application of auxin-competitors did not alter female flower formation (Trebitsh et al., 1987), indicating that the observed effect of auxin on sex expression is ethylene mediated (Trebitsh et al., 1987).

Influence of maize *TS2* gene on sex expression of transgenic melons.

The constitutive or floral specific expression of the *TS2* gene did not affect sexual differentiation of flower buds in transgenic melon plants. GAs were originally proposed as *TS2* substrates due to their steroid structure and their involvement in sex determination in corn (DeLong et al., 1993). Endogenous GA activity at the apical meristem peaked at day 17 after emergence, and then fell rapidly. Minimal activity was observed at anthesis in the tassel (Rood and Pharis, 1980). Phenotypic evaluation of GA-deficient mutants as *an1* and *d* corn mutants clarified the role of GAs in sex determination in corn (Bensen et al., 1995). Lack of GAs in these mutants caused reduced plant height, delayed maturity, and an increase in maleness, converting the female florets in the ear to hermaphroditic, indicating that GAs are required for anther primordia abortion in female buds (Bensen et al., 1995).

In cucurbits, high endogenous GA levels have been associated with monoecious sexual pattern (Atsmon et al., 1968; Freidlander et al., 1977), and GA application induced male bud formation in gynoecious melon lines (Rudich, 1990). If *TS2* normally functions to decrease the levels of endogenous GA in corn so that only male florets develop in the tassel, expression in melon might be predicted reduce GA levels and cause an increase in female bud formation. In addition, based on the effect of GAs on vegetative tissue a reduction in plant height and internode length would be expected. However, *TS2* expression in andromonoecious melon did not alter the sexual pattern and did not affect the internode length (data not shown). These results may be due to lack of the specific GA substrate for *TS2* in melon, lack of sufficient effect on GA levels to influence phenotype, or may be consistent with the maize *ts2/d1* double mutant phenotype, which suggests that *TS2* functions in an independently of GAs.

BRs have also been proposed as possible *TS2* substrates, based on their steroid structure, location in the anther cells, and the reduced male fertility of the *Arabidopsis* BR-deficient mutants. If BR is the substrate, the failure of *TS2* to influence sex expression in transgenic melons is consistent with the lack of effect of BR application on the plant sexual pattern. Another explanation is that the *TS2* substrate is not either GA or the BR, but yet to be identified, and possibly that is not present in melon plants.

In summary, our results show that exogenous BR increased femaleness in cucumber and also caused increased ethylene production. Application of ethephon that releases equivalent amount of ethylene to the BR caused similar effect on monoecious cucumber sex expression and supports the hypothesis that the observed effect of exogenous epi-BL on sex expression is ethylene mediated.

References

Ainsworth C, Parker J, Buchanan-Wollaston V (1998) Sex determination in plants. *In* Curr. Topics Devel. Biol., Vol 38. Academic Press, San Diego, pp 167-223

Altmann T (1999) Molecular physiology of brassinosteroids revealed by the analysis of mutants. Planta **208:** 1-11.

An G, Ebert PR, Mitra A, Ha SB (1988) Binary vectors. Plant Mol. Biol. Manual **A3:** 1-19

Arteca RN, Bachman JM, Mandava NB (1988) Effects of Indole-3-acetic acid and brassinosteroid on ethylene biosynthesis in etiolated mung bean hypocotyl segments. J. Plant Physiol. **133:** 430-435

Atsmon D, Lang A, Light EN (1968) Contents and recovery of gibberellins in monoecious and gynoecious cucumber plants. Plant Physiol. **43:** 806-810

Augustine JJ, Baker LR, Sell HM (1973) Female flower induction on androecious cucumber *Cucumis sativus* L. J. Amer. Soc. Hort. Sci. **98:** 197-199

Azpiroz R, Wu Y, LoCascio JC, Feldmann KA (1998) An Arabidopsis brassinosteroid-dependent mutant in blocked in cell elongation. Plant Cell **10:** 219-230

Bensen RJ, Johal GS, Crane VC, Tossberg JT, Schnable PS, Meeley RB, Briggs SP (1995) Cloning and characterization of the maize *an1* gene. Plant Cell **7:** 75-84

Byers RE, Baker LR, Sell HM, Herner RC, Dilley DR (1972) Ethylene: A natural regulator of sex expression of *Cucumis melo* L. Proc. Natl. Acad. Sci. **69:** 717-720

Calderon-Urrea A, Dellaporta SL (1999) Cell death and cell protection genes determine the fate of pistils in maize. Development **126:** 435-441

Chrominski A, Kopcewicz J (1972) Auxins and gibberellins in 2-chloroethylphosphonic acid-induced femaleness of *Cucurbita pepo* L. Zeitschrift fur Pflanzenphysiologie **68:** 184-189

Clouse SD, Langford M, McMorris TC (1996) A brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. Plant Physiol. **111:** 671-678.

Clouse SD, Sasse JM (1998) Brassinosteroids: Essential regulators of Plant Growth and Development. Ann. Rev. Plant Physiol. Mol. Biol. **49:** 427-451

Clouse SD, Zurek DM, McMorris TC, Baker M (1992) Effect of brassinolide on gene expression in elongating soybean epicotyls. Plant Physiol. **100:** 1377-1383

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

Den Nijs APM, Visser DL (1980) Induction of male flowering in gynoecious cucumbers (*Cucumis sativus* L.) by silver ions. Euphytica **29:** 237-280

Fang G, Grumet R (1990) *Agrobacterium tumefasciens* mediated transformation and regeneration of muskmelon plants. Plant Cell Rep. **9:** 160-164

Freidlander M, Atsmon D, Galun E (1977) Sexual differentiation in cucumber: Abscisic acid and gibberellic acid of various sex genotypes. Plant Cell Physiol. **18:** 681-691

Galun E, Izhar S, Atsmon D (1964) Determination of relative auxin content in hermaphrodite and andromoniecious *Cucumis sativus* L. Plant Physiol. **40:** 321-326

Galun E, Yung Y, Lang A (1963) Morphogenesis of floral buds of cucumber cultured in vitro. Develop. Biol. **6:** 370-387

Hoekema A, Hooykaas PJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefasciens* Ti-plasmid plant genetics. Nature **303:** 179-180

Kater MM, Franken J, Carney KJ, Colombo L, G.C. A (2001) Sex determination in the monoecious species cucumber is confined to specific floral whorls. Plant Cell **13:** 481-493

Kauschmann A, Jessop A, Koncz C, Szekeres M, Willmitzer L, Altmann T (1996) Genetic evidence for an essential role of brassinosteroids in plant development. Plant J. **9:** 701-713

Li J, Nagpal P, Vitart V, McMorris TC, Chory J (1996) A role for brassinosteroids in light-dependent development of Arabidopsis. Science **272:** 398-401.

Li L, Chory J (1999) Brassinosteroid actions in plants. J. Exp. Bot. **50:** 275-282

Mathur J, Molnar G, Fujioka S, Takatsuto S, Sakurai A, Yokota T, Adam G, Voigt B, Nagy F, Maas C, Schell J, Koncz C, Szekeres M (1998) Transcription of the Arabidopsis CPD gene, encoding a steroidogenic cytochrome P450, is negatively controlled by brassinosteroids. Plant J. **14:** 593-602.

More TA, Seshadri VS (1998) Genetic Studies. *In* NM Nayar, TA More, eds, Cucurbits. Sci Publishers Inc, Enfield, pp 129-154

Nomura T, Naakayama M, Reid JB, Takeuchi Y, Yokota T (1997) Blockage of brassinosteroid biosynthesis and sensitivity causes dwarfism in garden pea. Plant Physiol. **113:** 31-37

Oh MH, Clouse SD (1998) Brassinolide affects the rate of cell division in isolated leaf protoplasts of *Petunia Hybrida*. Plant Cell Rep. **17:** 921-924

Perl-Treves R (1999) Male to female conversion along the cucumber shoot: approaches to studying sex genes and floral development in *Cucumis sativus. In* CC Ainsworth, ed, Sex determination in plants. Bios Scientific Publishers, Oxford, UK, pp 189-215

Rood SB, Pharis RP (1980) Changes of endogenous gibberellin-like substances with sex reversal of the apical inflorescence of corn. Plant Physiol. **66:** 793-796

Rothstein SJ, Lahners KN, Lotstein RJ, Carozzi NB, Jayne SM, Rice DA (1987) Promoter cassettes, antibiotic-resistance genes, and vectors for plant transformation. Gene **53:** 153-161

Roy RP, Saran S (1990) Sex expression in the Cucurbitaceae. *In* DM Bates, RW Robinson, C Jeffry, eds, Biology and utilization of the Cucurbitaceae. Cornell Univ. Press, Ithaca, NY, pp 251-268

Rudich J (1990) Biochemical aspects of hormonal regulation of sex expression in Cucurbits. *In* DM Bates, RW Robinson, C Jeffry, eds, Biology and utilization of the Cucurbitaceae. Cornell Univ. Press, Ithaca, NY, pp 269-280

Rudich J, Baker LR, Scott JW, Sell HM (1976) Phenotypic stability and ethylene evolution in androecious cucumber. J. Amer. Soc. Hort. Sci. **101:** 48-51

Rudich J, Halevy AH, Kedar N (1969) Increase in femaleness of three cucurbits by treatment with ethrel, an ethylene releasing compound. Planta **86:** 69-76

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

Sambrook J, Russell DW (2001) Molecular Cloning A Laboratory Manual, Ed Third. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sasse JM (1997) Recent progress in brassinosteroid research. Physiol. Plant. **100:** 696-701

Suge H (1986) Reproductive develoment of higher plants as influenced by brassinolide. Plant Cell Physiol. **27:** 199-205

Szekeres M, Nemeth K, Koncz-Kalman Z, Mathur J, Kauschmann A, Altmann T, Redei GP, Nagy F, Schell J, Koncz C (1996) Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. Cell **85:** 171-182.

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

Takatsuto S, Yazawa N, Ikekawa N, Takematsu T, Takeushi Y, Koguchi M (1983) Structure-activity relationship of brassinosteroids. Phytochemistry **22:** 2437-2441

Tolla GE, Peterson CE (1979) Comparison of gibberellin A4/A7 and silver nitrate for induction of staminate flowers in a gynoecious cucumber line. HortScience **14:** 542-544

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

Yamamoto R, Demura T, Fukuda H (1997) Brassinosteroids induce entry into the final stage of tracheary element differentiation in cultured Zinnia cells. Plant Cell Physiol. **38:** 980-983.

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

CONCLUSIONS AND FUTURE RESEARCH

The goals of the present study included the constitutive and tissue specific modification of endogenous ethylene production of melon plants, the alteration of plant responsiveness to ethylene and the study of the influence of these modifications on sex expression pattern of andromonoecious melons. Another goal was to test the potential role of the hormone brassinosteroid on sex specification of monoecious cucumber plants.

To accomplish the first objective, transgenic plants, expressing the heterologous *ACC synthase* (*ACS*) gene from carnation constitutively, or tissue specifically, in stamens and petals, were produced and the effect on sex determination was evaluated. Constitutive expression of the *ACS* gene increased ethylene evolution of leaves and flower buds, induced early formation of the first hermaphroditic flower bud on the main stem, increased the number of nodes carrying hermaphroditic flower buds and increased number of the hermaphroditic buds that reached anthesis, relative to the non-transformed plants. The phenotypic observations indicate that the heterologous *ACS* gene affected flower production of melon plants. These results may have horticultural significance in that early female flower production would result in early fruit production, an important genetic feature especially for areas with relatively short growing season. The next steps involve phenotypic evaluation of the transgenic lines and test of the stability of the phenotype in field growing conditions.

For the second goal, transgenic plants expressing the heterologous ethylene receptor gene, *etr1-1*, from *Arabidopsis thaliana* were produced and the sexual pattern was evaluated. Constitutive expression of the *etr1-1* gene resulted in several ethylene insensitive phenotypes including higher ethylene production by male flower buds, decreased rooting ability and decreased rate of pedicel abscission of excised transgenic flower buds, either in air or in the presence of ethylene. The observed phenotypes verify the ability of the *Arabidopsis etr1-1* gene to cause ethylene insensitivity in melons. Expression of the *etr1-1* gene also prevented hermaphroditic flower formation compared to the non-transformed plants and the non-transgenic segregants within a family, in both andromonoecious and gynoecious melon lines. The phenotypic observations indicate that the heterologous *etr1-1* confers ethylene insensitivity in melon and that the ability to perceive ethylene is required for promotion of femaleness at the time of sex determination. Based on these results and on the one-hormone and two receptors hypotheses, where ethylene is able to both suppress stamen development and promote pistil development, interest for future research includes the identification of ethylene perception site(s), within the flower buds, that are critical for determination of sex, by expression of the *etr1-1* gene in specific tissues, in the stamens and in the pistils.

To pursue the third goal, monoecious cucumber plants were treated with different levels of the synthetic hormone epi-brassinolide (epi-BL). Application of epi-BL to monoecious cucumber plants influenced sex expression by inducing early pistillate flower formation and increasing the number of pistillate buds formed along the main stem. Melon and squash plants did not show an increase in femaleness. Further investigation revealed that epi-BL increased ethylene evolution when applied to the growing point of intact seedlings, suggesting that the effect on femaleness may be attributed to increased ethylene production. Application of ethephon that released a low level of ethylene equivalent to that caused by epi-BL caused increased femaleness in cucumber, but not in squash, suggesting that the effect of epi-BL on femaleness in cucumber is ethylene mediated. Further research to separate the effect of ethephon and epi-BL could include production of transgenic cucumber expressing the *etr1-1* gene and then application of BR to test whether the hormone treatment exerts an effect on sex determination or not.