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VIA ALTERED ETHYLENE PRODUCTION AND PERCEPTION

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MODIFICATION OF SEX EXPRESSION IN TRANSGENIC MELON VIA ALTERED ETHYLENE PRODUCTION AND PERCEPTION

By

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ABSTRACT

MODIFICATION OF SEX EXPRESSION IN TRANSGENIC MELON VIA ALTERED ETHYLENE PRODUCTION AND PERCEPTION

By

Holly A. Little

Cucurbit species are noted for their diversity of sexual phenotypes which are subject to control by developmental, genetic, and hormonal factors. Understanding the basis of sex expression is of basic interest to elucidate underlying mechanisms of sex determination and is of applied interest as pistillate flowers are necessary for fruit production. Studies with exogenous hormones have indicated that ethylene is the primary hormone influencing sex determination in cucurbits, with increased ethylene causing increased femaleness. In this dissertation, I sought to: examine the role of ethylene perception by the developing flower in sex determination, test the ability to increase femaleness in the field through increased endogenous ethylene production, and examine possible ecological impacts of altered ethylene sensitivity or production. In the first study, melon (*Cucumis melo* L.) plants were transformed with the Arabidopsis dominant mutant ethylene receptor *etr1-1*, under the control of the petal/stamen targeted Arabidopsis *Apetela3* (*AP3*) promoter, or carpel/nectary targeted Arabidopsis *Crab's Claw* (*CRC*) promoter, to evaluate the effects of blocked ethylene perception in different regions of the developing flower. *CRC::etr1-1* melon plants showed increased femaleness as measured by earlier and increased pistillate bud production, and the formation of female rather than hermaphrodite buds. *AP3::etr1-1* melon plants showed

increased maleness as measured by almost exclusive staminate flower production and the presence of poorly developed carpels in the few pistillate buds that were produced. The results of these experiments suggest a model for sex determination in melon whereby ethylene perception by the stamen is required for carpel development while ethylene perception by the carpel is required for stamen development. In the second study, field grown transgenic melon constitutively overexpressing an *ACS* gene for ethylene biosynthesis showed earlier and increased production of mature pistillate flowers as well as earlier fruit set and increased sequential fruit set on the main stem. These results indicate that ethylene is important for pistillate bud development and also may suggest an additional role in signaling associated with patterns of fruit set. Lastly, published literature was examined to conduct a case study on modified ethylene production and perception in order to provide insight into possible secondary effects and ecological impacts of altered signaling pathways.

ACKNOWLEDGEMENTS

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I would like to express my gratitude to my fellow labmates, past and present, for their technical and moral support, particularly Zakir Ullah, Xiaofeng Wang, and Katerina Papadopoulou for teaching me many of the techniques I used for my research and Kaori Ando for being such a good friend. I owe a special acknowledgement to Sue Hammar, for providing an enormous amount of assistance with my research as well as a great friendship and support when I needed it.

Lastly but certainly not least, I wish to thank Joel for his friendship, understanding, and endless love and support; Jackson, for bringing a new dimension of joy to my life and helping me to put things in perspective; and Allie, for her energy and zest for life.

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Chapter 1

Literature Review: Sex Determination in *Cucumis*

Introduction

Most flowering plants produce hermaphroditic flowers in which both male and female gametes are produced by providing them with the capacity to self-fertilize. Although self-fertilization can be an advantage for a species, it also has the potential to result in loss of fitness arising from inbreeding depression. This may have been an evolutionary force for plant species to develop strategies to promote outcrossing (Grant et al., 1994; Dellaporta and Calderon-Urrea, 1993; Juarez and Banks, 1998; Ainsworth et al., 1998; Barrett, 2002; Tanurdzic and Banks, 2004). For hermaphroditic species, strategies to minimize inbreeding depression include differences in position of male and female reproductive organs, differences in the timing of maturity of male and female gametes, and genetic self-incompatibility (Dellaporta and Calderon-Urrea, 1993; Ainsworth et al., 1998; Barrett, 2002). Another mechanism, which has appeared in a number of plant genera, is unisexual species, such that flowers form only one gamete type. The most common unisexual types are monoecy and dioecy. Within a dioecious species, individual plants will produce exclusively either male or female flowers. Members of a monoecious species produce separate male (staminate) and female (pistillate) flowers on the same plant (Grant, 1994). Agronomically important dioecious species include papaya, spinach, yam, and asparagus; and monoecious species of agronomic importance include maize, cucumber, melon, castor bean, and hazelnut (Frankel and Galun, 1977). Elucidation of the genetics of sex determination is

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fundamental to further understanding of plant evolution as well as advancement of plant breeding efforts.

Among monoecious species, cucurbit species show tremendous diversity in sexual types and have become a valuable tool in research to further understand sex determination. Driven by the efforts of plant breeding programs to produce hybrid seed, *Cucumis sativus* (cucumber) became a model system for studies to understand sex determination in the 1950s and 1960s and remains the best understood and most studied (Tanurdzic and Banks, 2004). The most common sexual types in cucumber are gynoecious and monoecious, however androecious, andromonoecious, and hermaphroditic types also exist (Rudich, 1990). While not as well understood, a range of sex types can be produced in *Cucumis melo* (muskmelon), which is predominately andromonoecious. Research on sex expression in cucurbits has evaluated the roles of genetic, environmental, hormonal, and developmental factors.

Extensive research of the 1950's and 1960's established a solid foundation for much of the role of plant growth regulators, both endogenous and exogenous on cucurbit, particularly cucumber, sex expression, as well as effects of the environment which are generally thought to be mediated via hormones (Perl-Treves, 1999). Insight also was gained regarding the genetic basis of components controlling the various sexual phenotypes, which exist among cultivars (Perl-Treves, 1999). The advent of molecular technologies enables the possibility for further elucidation of the underlying genetic control of sex determination.

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Unisexual flower development in *Cucumis*

Despite having separate male and female flowers, at a young developmental age the floral buds of monoecious cucumber plants have both stamen and carpel primordia and are therefore considered bisexual (Atsmon and Galun, 1960). The subsequent inhibition of carpel primordia leads to a male flower while arrest of stamen primordia results in pistillate flower development. Similarly in andromonoecious melon, inhibition of carpel primordia results in a male flower while stamen and carpel primordia continue to develop in pistillate buds to form perfect flowers. Further investigation to determine the precise timing of sexual differentiation of cucumber buds showed morphological differences beginning at the 1mm stage while changes in the growth rate of respective reproductive organs begins prior to the visible difference (Goffinet, 1990).

Based on the ABC model of flower development, combination of the activities of three classes of homeotic genes (A, B, and C) are responsible for organ identity of the four whorls of the flower (Ng and Yanofsky, 2000; Jack, 2001). Through the evaluation of homeotic flower mutants of Arabidopsis and snapdragon, studies have shown that A genes control the development of the sepals; A and B genes, petals; B and C genes, stamens; and C genes, carpels. Study of homeotic mutants in cucumber has provided insight into the spatial control of unisexual flower development (Kater et al., 2001). One would predict petals and stamens to be replaced by sepals and carpels, respectively, in a class B mutant. However, female flowers of the class B mutant had sepals in the first two whorls, carpels in the fourth, and an undeveloped third whorl, while in node positions normally destined to males, flowers had sepals in the first two whorls, carpels in the third, and an undeveloped fourth whorl. These results suggest that the developmental fate of

stamens or carpels of the unisexual cucumber flower is dependent on their position and not their sexual identity.

Additional research has identified and evaluated the expression of MADS-box homeotic genes in developing male and female cucumber buds to investigate their possible roles in selective differentiation of sexual organs (Perl-Treves et al., 1998 and Ando et al., 2001). Three C-function Arabidopsis *AGAMOUS (AG)* homologs were isolated from cDNA libraries of male and female buds at the bisexual stage and designated *CAG1*, *CAG2*, and *CAG3* (Perl-Treves et al., 1998). *CAG1* and *CAG3* expression followed a pattern typical of C-function genes with transcript detection in the stamens, carpels, and nectary while *CAG2* exhibited carpel-specific expression. Since *CAG2* expression was not detected until visible sexual differentiation, it did not appear to play a role in the "designation" of female flowers. In another study of cucumber, differential display was used to identify a female specific MADS-box gene (*ERAF17*) (Ando et al., 2001). *ERAF17* was found to be expressed at the apex upon hormonal induction of female flowers, and its expression was maintained in tissue of female buds throughout development. It was suggested that *ERAF17* may act in a feminizing cascade.

A recent study focused on morphological, cellular, and molecular changes in the arrested inappropriate sexual organ in floral buds of cucumber (Hao et al., 2003; Bai et al., 2004). Detailed microscopic analysis revealed that divergence from a bisexual to unisexual flower begins following the initiation of the carpel in both male and female buds (Bai et al., 2004). Using a TUNEL assay, DNA damage was detected in cells of the primordial anther, coinciding with arrest in stamen development in female buds (Hao et al., 2003). This finding suggests a pathway of unisexual flower development in which

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further growth of the inappropriate organ is hindered by DNA damage resulted from the activity of a specific DNase. Evaluation of the arrest of carpels in male buds, showed stigma-like primordia but no differentiation of the ovary (Bai et al., 2004). The common features of the inappropriate reproductive organ arrest, such as the timing and targeting of spore-bearing parts, were suggested to indicate that a common signal may initiate the cascade of events leading to inhibited stamen development in pistillate buds and inhibited carpel development in staminate buds (Bai et al., 2004).

In monoecious *C. sativus* cultivars, flower development along the main shoot follows three phases of sex expression creating a 'physiological gradient' (Atsmon and Galun, 1962; Shifriss, 1961). During the first phase, staminate flowers are formed; during the second, a mixture of staminate and pistillate flowers; and during the third, pistillate flowers. A similar gradient is observed in andromonoecious *C. melo* in which an initial phase of vegetative nodes is followed by staminate nodes then a mixture of staminate and perfect flowers. Atsmon and Galun (1962) attempted to describe the 'physiological gradient,' suggesting that a growth substance varying in distribution between young and mature leaves along the shoot affect the sex of adjacent buds creating a gradient. Sex genotype, environment, and plant growth regulators influence the length of each phase (Perl-Treves, 1999).

Genetics of sex expression

Varying levels of femaleness or maleness exists among cucurbit genotypes giving rise to an array of sexual phenotypes. Among these phenotypes are gynoecious lines having strictly pistillate flowers, androecious lines having only staminate flowers,

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andromonoecious lines having both perfect and staminate flowers, and a variety of intermediate intersexual phenotypes.

Studies have shown that the majority of the sexual phenotypes in cucumber are controlled by three major genes, although minor sexual modifier genes do exist (Galun, 1961; Kubucki, 1969; Shifriss, 1961; Rudich et al., 1976; Perl-Treves, 1999). The *F* locus is a partially dominant gene that influences the degree of femaleness such that *F-* results in gynoecy. The *A* locus is associated with increased maleness such that plants with an *aaff* genotype are androecious and do not produce pistillate flowers (Rudich et al., 1976; reviewed in Perl-Treves, 1999). The *M* locus is a dominant gene that influences the development of stamens, whereby a *MM* or *Mm* genotype results in formation of a female flower while *mm* results in perfect flower formation (Kubicki, 1969; reviewed in Perl-Treves, 1999). Various sexual genotypes, particularly those homozygous dominant for the *F* locus, have been utilized commercially for inbred and hybrid seed production. The use of a female line for hybrid seed production ensures that the seed harvested from the maternal parent is the product of cross-pollination with a paternal line. Maintenance of gynoecious lines by self-pollination is possible through thehormonal induction of staminate flowers. Furthermore, the *F* locus has been extremely useful to breeders in order to generate and maintain gynoecious cultivars that provide an earlier and more uniform fruit-set (Rudich, 1990).

The genetic basis of sex expression in *C. melo* is thought to be controlled by interaction of at least three genes, *A*, *G*, and *M* (Kenigsbuch and Cohen, 1989; Roy and Saran, 1990). Interactions between *A* and *G* are proposed to be responsible for the inheritance of many of the sexual phenotypes, such that *A* inhibits the development of the

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androecium in female flowers while *G* inhibits the development of the gynoecium in male flowers (Roy and Saran, 1990). Therefore, genotype *A-G-* designates monoecy; *aaG-*, andromonecy; *A-gg*, gynomonoecy; and *aagg*, hermaphrodism. Inheritance studies of gynoecy in muskmelon have suggested that the *M* (maleness) locus in a homozygous recessive form is required for a complete and stable gynoecious phenotype; therefore, the genotype of gynoecious melon is *A-ggmm* (Kenigsbuch and Cohen, 1989). The lack of a locus analogous to the *F* locus in cucumber has made efforts to maintain stable breeding lines of gynoecious melon more difficult.

Hormonal control of sex expression

Another level of control of cucurbit sex expression is by plant hormones (Rudich, 1990). Exogenous application as well as changes in the endogenous level of a variety of plant hormones is capable of influencing sex determination. Auxin, brassinosteroids, and ethylene are growth regulators which promote pistillate flower formation, while gibberellins and ethylene inhibitors promote staminate flower production (Wittwer and Bukovac, 1959; Galun, 1959; Atsmon et al., 1968; McMurray and Miller, 1968; Rudich et al., 1969; Papadopoulou, 2002). Therefore, with appropriate timing and quantity, it is possible to shift sex in either direction by application of plant growth regulators.

The ability of exogenous treatment with gibberellins (GA) to modify sex in cucumber led researchers to suggest the possibility of a role of endogenous GA in the regulation of unisexual flower development (Atsmon et al., 1968). Treatment with GA was found to promote male flower initiation as well as prevent existing female buds from fully developing (Atsmon et al., 1968; Perl-Treves, 1999) while treatment with inhibitors

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of GA biosynthesis has been observed to promote femaleness (Yin and Quinn, 1995). In addition, evaluation of GA content in monoecious and isogenic gynoecious cucumber lines showed higher levels in monoecious plants, supporting the idea that endogenous GA is involved in sex determination (Atsmon et al., 1968).

The feminizing effect of exogenous auxin has been demonstrated in cucumber and melon (Galun et al., 1963; Galun et al., 196; Trebitsh et al., 1987; Rudich, 1990). In addition, endogenous levels of auxin from varying sex genotypes of cucumber plants have been evaluated and compared, and findings provided support for a hypothesized role of auxin as a regulator of sex in cucumber (Galun et al., 1965; Rudich et al., 1972). However, studies demonstrating the ability of auxin to stimulate ethylene production via induction of ethylene biosynthetic enzyme, ACC synthase (ACS), in plant tissues led to further characterization of the feminizing effect of auxin (Trebitsh et al., 1987). Treatment with auxin was found to stimulate ethylene in gynoecious and monoecious cucumber, and, in addition, the feminizing effect of exogenous auxin treatment could be blocked by treatment with inhibitors of ethylene action or biosynthesis while treatment of gynoecious cucumber with antiauxins failed to inhibit female flower formation. Therefore, there is strong evidence suggesting that the feminizing effect of auxin is mediated via ethylene.

Most recently, the feminizing effect of brassinosteroids (BR) on sex expression has been evaluated in cucumber, melon, and zucchini (Papadopoulou, 2002). Upon treatment of seedling apices with a synthetic BR, epibrassinolide, monoecious cucumber showed earlier and increased female bud formation, while sex expression of zucchini and melon plants was not affected. Evaluation of ethylene production demonstrated increased

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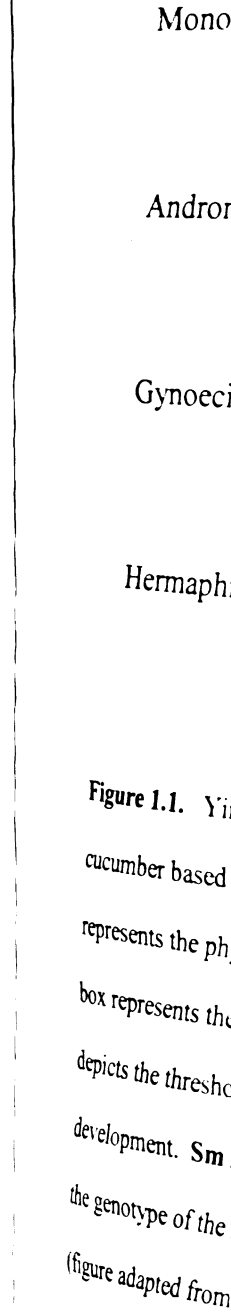
ethylene production from BR treated seedlings, suggesting that the effect on sex expression may be a result of increased ethylene production.

One possible mechanistic model for the regulation of sex expression is the one hormone model proposed by Yin and Quinn (1992, 1995). According to this model, there are both male and female receptors for a hormone to inhibit one sex and induce the other, whereby differing sensitivity or levels of expression of the receptors respond to a range of hormone concentrations to regulate sex expression (Figure 1.1). By this model of one hormone producing a dual response of inhibition of one sex and induction of the other, Yin and Quinn (1995) identified ethylene (which will be described in greater detail in the next section) and not GA as the 'sex hormone' for cucumber by evaluating different genotypes and their various hormone treatments. Furthermore, results showing that the combined GA/ethylene treatment had results similar to ethylene treatment alone, led them to conclude that ethylene had a more direct effect on sex expression and must, therefore, be the 'sex hormone' (Yin and Quinn, 1995). According to their explanation, femaleness is induced by an ethylene level higher than a threshold of female sensitivity (Sf), while any level below Sf results in the lack of female organ development. Conversely, maleness is inhibited by an ethylene level higher than a threshold of male sensitivity (Sm), while levels lower than Sm result in induction of male organ development. The mechanism of two major loci controlling sex expression in cucumber, namely *F-f* and *M-m*, which have long been regarded as critical loci in sex determination can be interpreted based on this model (Galun, 1961; Kubucki, 1969; Shifriss, 1961). The *M-m* gene is suggested to control the male sensitivity level such that the *M* allele represents Sm at a lower level of hormone concentration (higher sensitivity)



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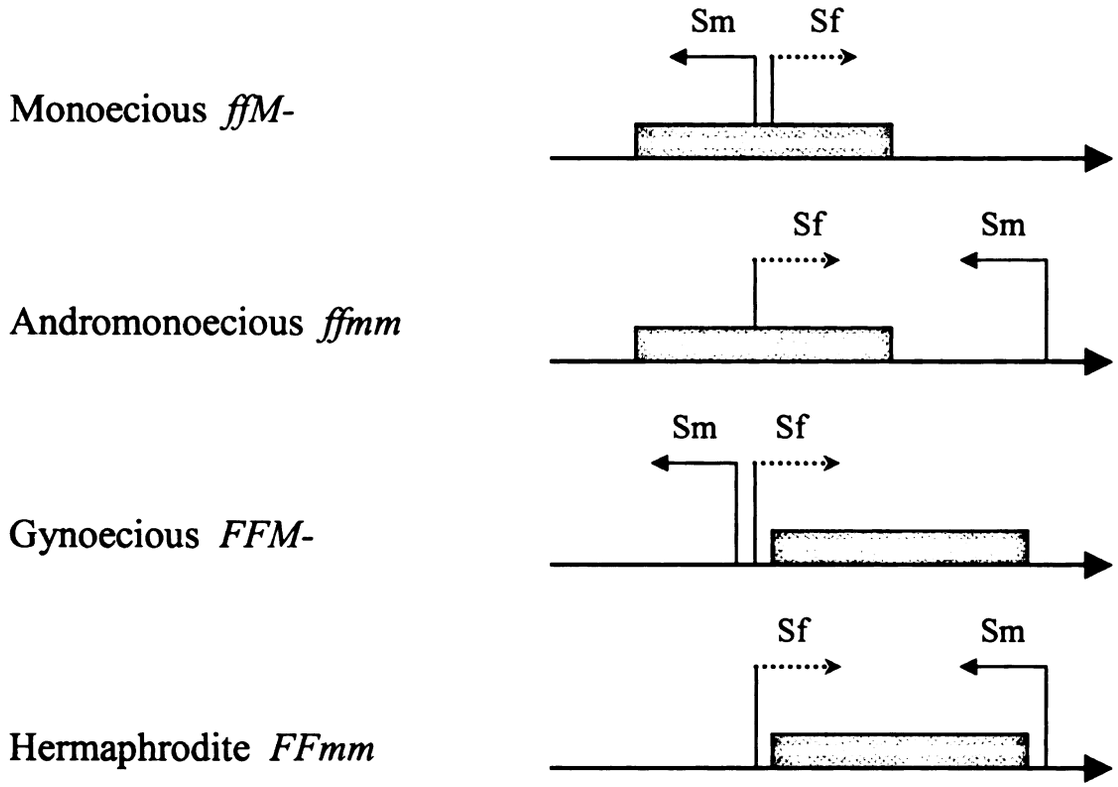

Figure 1.1. Yin and Quinn model for the effects of ethylene on sex expression in cucumber based on the predicted roles of the *F* and *M* loci. The long horizontal arrow represents the physiologically possible range of ethylene concentration. The rectangular box represents the range of ethylene actually produced, as controlled by the *F* locus. **Sf** depicts the threshold of ethylene production which must be exceeded to promote carpel development. **Sm** represents the threshold of ethylene sensitivity, which is dependent of the genotype of the *M* locus, and must be exceeded to inhibit stamen development. (figure adapted from Yin and Quinn, 1995)

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while the *m* allele represents Sm at a higher level of hormone concentration (lower sensitivity). The *F-f* gene is suggested to control the normal range of ethylene for a particular genotype such that the *F* allele leads to a higher level of ethylene and the *f* allele to a lower level. Further interaction of the *F*-locus with the *m* and *a* loci has been suggested to contribute to the range of sexual phenotypes seen in cucumber (Pierce and Wehner, 1990).

Role of ethylene in sex expression

The feminizing effect of ethylene on sex determination in cucumber and melon has been of topic of extensive study that is still under investigation. Application of the ethylene-releasing compound, 2-chloroethanephosphonic acid (ethrel), to plants is a useful method for increasing the number of female flowers in monoecious and andromonoecious lines (Robinson et al., 1969; Rudich et al., 1969). Conversely, application of inhibitors of ethylene perception, such as silver ions, induces staminate flower formation in gynoecious cucumber and hermaphrodite flowers in gynoecious melon and is, therefore, used to self-pollinate and maintain female genotypes (Den Nijs and Visser,1980; Owens, 1980). In addition, application of exogenous aminoethoxyvinyl glycine (AVG) or α-aminoxyacetic acid (AOA) which inhibit ethylene biosynthesis, has been shown to increase maleness in cucumber and melon plants (Rudich, 1990; Atsmon and Tabbak, 1979).

With the value of such applications recognized by breeders, considerable effort has focused on evaluating the effectiveness of treatment timing and quantity with regard to their subsequent effect on sex (Rudich, et al., 1969; Byers et al., 1972; Den Nijs and

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Visser, 1980; Iwahori et al., 1970; McMurray and Miller, 1968; Tolla and Peterson et al., 1979). While no effect of sex was observed following treatment with ethrel at the cotyledon stage, treatment of monoecious cucumber seedlings with 50 ppm at the first leaf stage reduced the appearance of the first pistillate flower from node 13 to 4 (Iwahori et al., 1970). In contrast, treatment of gynoecious cucumber seedlings at the fourth leaf stage with 50 ppm silver nitrate was sufficient for induction of staminate flowers at the fourth node and the four subsequent nodes (Tolla and Peterson, 1979). Additional treatments and/or increased concentration did not result in earlier staminate flower appearance but did prolong the phase of staminate flower production, suggesting that the earlier nodes had developed beyond the critical stage for sex determination. Furthermore, these studies have demonstrated that depending on the timing on application, the node positions affected varies, indicating that there are critical times in development when hormone treatment can have an effect. More recently, microscopic evaluation of the subsequent differentiation of developing monoecious and gynoecious cucumber buds upon treatment with ethylene-releasing ethephon or ethylene production inhibitor, AVG, demonstrated that the influence of ethylene on sex determination is concurrent with the timing of stamen primordia differentiation (Yamasaki et al., 2003a).

Early experiments to establish whether or not a relationship existed between endogenous ethylene and sex determination in cucurbits consisted of quantification of ethylene production by gas chromatography in various sex genotypes of cucumber and melon. Studies comparing ethylene production from apices of monoecious and gynoecious cucumber seedlings have shown two- to three-fold higher ethylene levels associated with the increased femaleness of gynoecious lines (Rudich et al., 1972, 1976;

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Makus et al., 1975). Despite the failure to observe a similar difference between sex genotypes in melon, when melon seedlings were grown under hypobaric conditions to reduce endogenous gas levels, gynoecious melon showed increased maleness, suggesting that endogenous ethylene is important in promoting femaleness (Byers et al., 1972).

The understanding of ethylene biosynthesis has led to further evaluation of the feminizing effect. Ethylene is synthesized from S-adenosyl methionine (AdoMet) in a two step process which includes cyclization to 1-aminocyclopane carboxylate (ACC), mediated by ACC synthase (ACS), followed by an oxidation step mediated by ACC oxidase (ACO) (Adams and Yang, 1979). The level of ACC synthase has been proposed as a key factor in regulating ethylene biosynthesis (Yang and Hoffman, 1984). Both *ACS* and *ACO* are generally encoded by multigene families in which different genes are induced by a variety of environmental and developmental stimuli, such as: auxin, ethylene, wounding, fruit ripening, pathogens, and stress (Zarembinski and Theologis, 1994; Bleeker and Kende, 2000).

The overall consensus of the key role of ethylene in the sex determination of cucumber, and advancements in the understanding of biochemistry and regulation of ethylene production, have led to further investigation to link the two together at a molecular level. Work in cucumber by Trebitsh et al. (1997) provided evidence for a direct relationship between the *F*-locus and ethylene levels. They reported the identification of a partial ACC synthase sequence in cucumber (*CS-ACS1*), which upon further analysis of near isogenic lines by Southern blot hybridization revealed the presence of an additional copy of ACC synthase (*CS-ACS1G*) present only in gynoecious cucumber lines. Linkage analysis indicated co-segregation of *CS-ACS1G* with the *F*-

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locus with 0% recombination leading the authors to speculate the possibility of the duplicated ACC synthase being the *F*-locus. Recent molecular characterization of the *F* locus using RFLP (restriction fragment length polymorphisms) analysis further confirmed the presence of a duplication of *CS-ACS1* in gynoecious genotypes, *CS-ACS1G* (Mibus and Tatlioglu, 2004). Subsequent cloning and sequence evaluation suggest that variation in the promoter region of CS-*ACS1G* is responsible for its exclusive expression in genotypes with a dominant *F* allele (Mibus and Tatlioglu, 2004).

Results published by Kamachi et al. (1997) reported the cloning of the three distinct cDNAs encoding ACC synthase (*CS-ACS2, CS-ACS3, CS-ACS4*) in cucumber. Since previous research indicated a strong correlation between the evolution of ethylene from apices and femaleness (Rudich et al., 1972, 1976), Kamachi et al. (1997) analyzed expression of the three ACC synthase genes at the apices of monoecious and gynoecious cucumber. Among the three ACS genes, only *CS-ACS2* mRNA was detected at the apices in which female flowers were developing while *CS-ACS3* (with sequence identical to Trebitsh *CS-ACS1*) and *CS-ACS4* were not detected.

Further research focused on unraveling the correlation of *CS-ACS1 (CS-ACS1G)* and *CS-ACS2* with sex determination in cucumber by examining their expression in the apices of isogenic monoecious and gynoecious lines (Kamachi et al., 2000). While *CS-ACS1* and *CS-ACS2* transcripts were detected at the apices of the gynoecious line, only *CS-ACS2* transcript could be detected in that of the monoecious line. In addition, *CS-ACS2* was limited to floral buds that would develop into female flowers. These results suggested that ethylene produced as a result of *CS-ACS2* expression was responsible for

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ACC oxidase, the enzyme that catalyzes the final step of ethylene formation, has also been studied at a molecular in an effort to interpret its role in sex expression (Kahana et al., 1999; Perl-Treves et al., 2000). Kahana et al (1999) reported the cloning of three distinct cDNAs of ACC oxidase (*ACO-1, ACO-2, ACO-3*), which are all expressed in the leaves and shoot apices of cucumber plants. Further evaluation of *ACO2* showed higher transcript levels in the first unfolded leaf of gynoecious and hermaphrodite plants, which one may predict were associated with the higher ethylene evolution of female genotypes relative to the lower transcipt levels measured in monoecious and androecious plants. However, *ACO2* transcript levels measured in the shoot apices showed an inverse correlation to the sexual genotype; therefore, the monoecious and androecious plants had higher *ACO2* levels in their apices. The authors concluded that these findings might indicate that the shoot apex is not the site critical for ethylene synthesis or gene products other than ACO (such as ACS) are responsible for increased ethylene levels in female genotypes (Kahana et al., 1999; Perl-Treves et al., 2000).

Studies also have focused on gaining insight into the signal transduction mechanism of ethylene on the promotion of femaleness in cucumber (Yamasaki et al., 2000). The signal transduction pathway of ethylene has been well studied in *Arabidopsis thaliana* by evaluating mutants with altered perception or response to ethylene (Bleeker and Kende, 2000; Steptanova and Ecker, 2000; Wang et al., 2002). A multigene family of ethylene receptors (in Arabidopsis *ETR1*, *ETR2*, *ERS1*, *ERS2*, and *EIN4*) was identified by this approach, which upon cloning and characterization was found to share a

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high degree of homology to bacterial two-component signal transduction proteins that contain histidine kinase (sensor) and receiver (response regulator) domains on the same polypeptide (Bleeker et al., 1988; Chang et al., 1993). Since ethylene must be perceived in order to trigger a downstream ethylene response, studies evaluating the expression of ethylene receptors as well as implementing receptor mutants to confer reduced ethylene binding are useful for furthering the understanding of processes in plants that are affected by ethylene (Bleeker and Kende, 2000).

To this end, ethylene receptor homologues, *CS-ETR1*, *CS-ETR2*, and *CS-ERS* were cloned from cucumber and their expression pattern analyzed in monoecious and gynoecious cucumber (Yamasaki et al., 2000). While transcript levels of all three genes were elevated in gynoecious relative to monoecious lines, differences in *CS-ETR2* and *CS-ERS* were more pronounced and followed a time course of expression in shoot apices analogous to that of *CS-ACS2*, ethylene production, and female flower development. In addition, upon treatment with ethrel or AVG, *CS-ETR2* and *CS-ERS* exhibited subsequent changes in expression reflecting their regulation by ethylene. Furthermore, since receptor binding of ethylene initiates a signaling cascade, *CS-ETR2* and *CS-ERS* may be important in the signal transduction involved in cucumber sex determination.

In a related study, the expression patterns of *CS-ACS2*, *CS-ETR2*, and *CS-ERS* provided insight into the underlying function of the *M* locus in cucumber sex determination (Yamasaki et al., 2001). As already described, loss-of-function at the M locus confers bisexual flower development, such that in andromonoecious cucumber (*mmff*) treatment with ethylene induces pistil development but does not inhibit stamen development. In a comparison of monoecious (*M-ff*), gynoecious (*M-F-*) and

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andromonoecious (*mmff*) cucumber genotypes, ethylene treatment resulted in increased *CS-ETR2*, *CS-ERS*, and *CS-ACS2* transcript levels in *M-ff* and *M-F-* plants but not in *mmff*. These findings suggest that response to ethylene is reduced in *mmff* plants and supports implication of the *M* locus in the mediation of ethylene signals.

To further elucidate the relationship between tissues producing and perceiving ethylene, a series of experiments characterized the localization of *CS-ACS2*, *CS-ETR1*, *CS-ETR2*, and *CS-ERS* transcripts within cucumber flower buds throughout development by *in situ* hybridization analysis (Yamasaki et al., 2003a). Since overlap of *CS-ETR2* and *CS-ACS2* mRNA was observed in pistil regions of monoecious and gynoecious buds, it was suggested that for the induction of pistils, tissues involved in ethylene production and perception are identical. In addition, in monoecious buds, since *CS-ACS2* message was detected in petals, while *CS-ETR1* and *CS-ERS* were detected in the stamens, it was suggested that for development of stamens, cells responsible for ethylene production and perception need not be identical.

Another study has evaluated the environmental regulation of ethylene-related gene expression and its correlation to sex expression as short day conditions have been observed to promote femaleness (Yamasaki et al., 2003b). The peak of ethylene production and expression of *CS-ACS2*, *CS-ACS4*, and *CS-ERS* were found at the mid point of 8 or 16 hour light period in monoecious and andromonoecious cucumber demonstrating a photoperiodic rhythm. However, in monoecious cucumber, the peak of ethylene evolution and *CS-ACS2* was greater under short-day conditions than long-days, but such a phenomenon was not seen in andromonoecious plants. These results provide

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Objectives of the thesis research

As is suggested in the preceding literature review, even though ethylene has been demonstrated to play an important role as a "sex hormone" in muskmelon, much of the studies to elucidate the role of ethylene in sex determination of cucurbits have focused on cucumber and have relied on correlative studies of exogenous ethylene treatment or analyses of ethylene-related gene expression. With reliable methodology available in our laboratory to produce transgenic melon, the opportunity exists to directly modify endogenous ethylene production and perception in order to further evaluate its role in the determination of sex.

The signal transduction pathway of ethylene has been studied in *Arabidopsis thaliana* by evaluating mutants with altered perception or response to ethylene. The characteristic of the ethylene receptor mutant, *etr1-1*, to act as a dominant negative mutant causing ethylene insensivity (Bleeker et al., 1988; Chang et al., 1993), enables its use as a tool to evaluate processes in plants that are affected by ethylene. Recent work in our laboratory, studying transgenic melons haboring the *etr1-1* receptor mutant driven by a constitutive (*35S*-CaMV) promoter, showed phenotypes characteristic of decreased ethylene sensitivity, including: poor root formation, delayed flower senescence and abscission, and failure to respond to exogenous ethylene (Papadopoulou, 2002). In addition, Papadopoulou (2002) showed that *35S::etr1-1* melon fail to produce pistillate

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buds, demonstrating that the perception of ethylene is critical for development of female floral organs.

Therefore, one objective of my research was to further evaluate the requirement of ethylene perception by specific regions of the developing floral primordia for subsequent sex determination in melon. Constructs for transformation were prepared to alter ethylene perception in stamens or carpels. The *Apetela3* promoter, which was isolated from *Arabidopsis thaliana,* is targeted to primordial cells destined to become the petals and stamens (Irish and Yamamoto, 1995). The *Crab's Claw* promoter, which was also isolated from *Arabidopsis thaliana,* is specifically expressed in the carpels and nectaries of gynoecial primordia from their inception (Bowman and Smyth, 1999).

Predicted results were based on the resulting changes in sex expression in wild-type melon and cucumber upon the addition of ethylene. In melon, sex expression changes from a phase of staminate to a phase of perfect flower production (Perl-Treves, 1999), suggesting that carpel development is induced while stamen development is unaffected. In cucumber, sex expression changes from a phase of staminate to pistillate flower production (Perl-Treves, 1999), suggesting that carpel development is induced while stamen development is suppressed. Therefore, in the flower bud of melon, one can speculate that perception of ethylene is critical in the carpel while in cucumber, perception is critical in both the carpel and stamen. Table 1.1 summarizes the hypothesized results of experiments using plants transformed with constructs of the *etr1-1* mutant and the subsequent effects on sex expression.

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Table 1.1. Summary of *etr1-1* constructs and predicted effects on sex expression in transgenic melon.

wild-type	Staminate to perfect 1. carpel development 2. stamens unaffected
35S-etr1-1 (constitutive)	Staminate only 1. no carpel development 2. stamens unaffected
AP3-etr1-1 (petal/stamen)	Staminate and Perfect 1. carpel development 2. stamens unaffected
CRC-etr1-1 (carpel/nectary)	Staminate only 1. no carpel development 2. stamens unaffected

My second objective was to further evaluate the effects of increased endogenous ethylene production on sex expression in melon. Recently, an andromonoecious (producing male and hermaphroditic flowers) melon genotype, Hale's Best Jumbo, was transformed to constitutively express an *ACS* (1-aminocyclopropane-1-carboxylic acid synthase) gene from petunia in order to study the effect of modified endogenous ethylene production in melon (Papadoupoulou et al., 2005). Greenhouse studies showed increased ethylene evolution from leaves and floral buds of transgenic plants confirming that the overexpressed *ACS* conferred increased ethylene biosynthesis. Evaluation of the pattern of the sex expression along the main stem showed both earlier and increased numbers of hermaphroditic buds relative to non-transgenic controls, consistent with ethylene playing a key role in the commitment to form pistillate flowers. In performing these experiments, it was observed that most hermaphrodite buds abort before reaching anthesis. Interestingly, however, an increased percentage of hermaphroditic buds on the transgenic

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plants reached full anthesis, suggesting a role for ethylene in hermaphrodite bud development as well as hermaphrodite bud initiation. To further substantiate greenhouse observations on sex determination and flower development and to continue characterization of the transgenic melon to include the influence of modified ethylene levels on fruit production, I conducted a field evaluation of the *35S-ACS* melon lines.

The third objective of this thesis was to provide a case study of the secondary effects on plant phenotypes associated with genetically modified ethylene signaling, with an emphasis on effects that may be relevant in terms of risk assessment. For example, ethylene production and perception have been modified to alter plant physiological characteristics including ripening, senescence, and flowering. These alterations can in turn result in secondary effects such as, altered stress and disease response as well as changes in aroma, floral characteristics, and growth habit. Such secondary effects may, in turn, alter the likelihood of gene flow into neighboring non-transgenic populations and/or alter reproductive fitness of arising hybrids in the event of gene flow and so may be important to consider from an environmental risk assessment standpoint.

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Chapter 2

Modified sex expression in transgenic melon expressing the mutant ethylene receptor, *At-etr1-1*, under the control of floral targeted promoters

Abstract

The diversity of sexual phenotypes observed within cucurbit species is influenced by environmental, hormonal, developmental, and genetic factors. Ethylene is regarded as a key hormonal regulator in the sex determination of andromonoecious melon (*Cucumis melo* L.) plants such that application of ethylene causes a shift from staminate to hermaphrodite flower production. These effects of exogenous ethylene treatment suggest that ethylene is critical for carpel development, but does not affect stamen development. In this study, I sought to determine the critical sites of ethylene perception in the developing flower bud. A previous study of transgenic melon plants constitutively expressing the Arabidopsis dominant mutant ethylene receptor, *etr1-1*, displayed phenotypes consistent with ethylene insensitivity, and evaluation of their sex expression pattern showed almost exclusively male flower production. To further decipher the role of ethylene perception in melon sex determination, melon was transformed with *etr1-1* gene constructs under the direction of *Apetala3* (*AP3*) and *Crab's Claw* (*CRC*) promoters which target developing stamens and petals, and carpels and nectaries in Arabidopsis, respectively. Greenhouse characterization of sex expression and floral morphology provided surprising results. *CRC::etr1-1* melon plants showed increased femaleness as measured by earlier and increased pistillate bud production, and the formation of female

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rather than hermaphrodite buds. *AP3::etr1-1* melon plants showed increased maleness as measured by nearly exclusive staminate flower production and the presence of poorly developed carpels in the few pistillate buds that were produced. The results of these experiments suggest a model for sex determination in melon whereby ethylene perception by the stamen is required for carpel development, while ethylene perception by the carpel is required for stamen development.

Introduction

The Cucubitaceae family, which is noted for diversity in sex expression phenotypes, has served as a model for understanding of processes involved in sex determination. Cucurbit species exhibit various combinations of hermaphrodite, male, and female flowers which can influence outcrossing behavior and fruit set timing and patterns. Cucumber (*Cucumis sativus* L.) is typically monoecious, in which a phase of male flowers precedes a phase of both male and female flowers; however, gynoecious (all female), androecious (all male), andomonoecious (male followed by perfect flowers), and hermaphroditic types also exist (Rudich, 1990; Perl-Treves, 1999). Melon (*C. melo*), which is typically andomonoecious, also exhibits a variety of sexual phenotypes. These various sexual phenotypes are under the control of genetic, developmental, hormonal, and environmental factors. The underlying molecular processes are now beginning to be understood.

Sex expression in both cucumber and melon is influenced by at least three major genes. In cucumber, sex is largely controlled by the *F*, *M*, and *A* loci (reviewed in Perl-Treves, 1999). The partially dominant *F* (female) locus has been used commercially to

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develop gynoecious cultivars, which provide earlier and more uniform fruit-set (Lower and Nienhuis, 1990). The *M* locus affects the development of stamens in pistillate floral buds such that *M-* confers the development of unisexual, female buds while *mm* confers hermaphrodite buds (reviewed in Perl-Treves, 1999). The A locus is associated with increased maleness such that a plant with an *aaff* genotype produces staminate flowers exclusively. Although three major loci are also primarily responsible for inheritance of sexual phenotypes in melon (Kenigsbuch and Cohen, 1989; Roy and Saran, 1990), they are not completely analogous, as an equivalent locus to the semi-dominant *F* locus in cucumber conferring gynoecy has not been identified.

Developmentally, sex expression in cucumber and melon typically follows a 'physiological gradient' such that a phase of staminate flowers is followed by a mixture of staminate and pistillate flowers in monoecious cucumber or a mixture of staminate and bisexual flowers in andromonoecious melon (Atsmon and Galun, 1962; Shifriss, 1961). Despite this observed chronology in flower sex, in early stages of development all initiated floral buds contain the four whorls (sepals, petals, stamen, and carpels) typical of a bisexual flower (Goffinet, 1990; Kater et al., 2001; Yamasaki et al, 2003; Hao et al., 2003; Bai et al., 2004). The subsequent arrest of carpel primordia, while stamen development continues, leads to a male flower; similarly, the arrest of stamen primordia, while carpel development continues, results in a bisexual flower. An evaluation of floral homeotic mutants of cucumber revealed that sex determination is dependent on the whorl position rather than sexual identity, such that the prevention of carpel development in male flowers results from suppression of differentiation in whorl 4, where carpels

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Detailed morphological studies of male and female cucumber bud development have divided the process of floral development from floral meristem initiation to anthesis into 12 stages to determine the precise timing of sexual differentiation (Hao et al., 2003; Bai et al., 2004). Divergence from a bisexual to unisexual flower begins to become apparent morphologically at stage 6 in both male and female buds following the initiation of carpel primordia (Bai et al., 2004). In male flowers, the arrest of carpel primordia begins immediately following its inception (Bai et al., 2004). Despite some subsequent differentiation of the inappropriate carpel to form the nectary and stigma-like primordia, no differentiation of the ovary was observed. In female flowers, differentiation of a filament and anther is observed, followed by the detection of DNA damage in the primordial anther (Hao et al., 2003). It was suggested that the response to the signal conferring arrest of inappropriate male or female floral organs may differ because carpel differentiation follows that of stamens (Bai et al., 2004).

The effects of hormones on sex determination of cucurbits, particularly cucumber have received considerable attention (Rudich, 1990; Perl-Treves, 1999). Auxin, ethylene, and brassinosteroids have been shown to promote femaleness while gibberellins promote maleness (Wittwer and Bukovac, 1958; Galun, 1959; Atsmon, 1968; McMurray and Miller, 1968; Rudich et al., 1969; Papadopoulou, 2002). However, ethylene appears to be the primary hormonal component affecting sex expression with other hormones acting via ethylene. Application of ethylene or ethylene-releasing compounds has been demonstrated to increase pistillate flower production in cucumber, muskmelon, and

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squash while treatment with inhibitors of ethylene biosynthesis or action confers an increase in maleness (Rudich, 1990; Perl-Treves, 1999). Since treatment at the 4-leaf stage does not confer changes in sex until node 8-10, it has been proposed that there are critical stages in development at which ethylene can modulate sex determination (Iwahori et al., 1970; Tolla and Peterson, 1979; Den Nijs and Visser, 1980). Morphological studies to characterize the stage of floral development affected by treatment with ethylene releasing compounds or inhibitors of ethylene biosynthesis, indicated that the stage just preceeding, or immediately following, differentiation of stamen primordia, is critical for sex determination (Yamasaki et al., 2003).

Further support for the feminizing effect of ethylene is provided by measurements of ethylene evolution, which showed 2-3 fold higher ethylene production from apices of gynoecious cucumber seedlings compared to monoecious and andromonoecious seedlings (Byers et al., 1972; Rudich et al., 1976). Additional studies have provided molecular support for ethylene's role in promoting pistillate flower development. In cucumber, increased mRNA levels of a gene encoding a key ethylene biosynthetic enzyme, ACC synthase (ACS), (*CS-ACS2*) has been shown to coincide with female flowering in cucumber (Kamachi et al., 1997, 2000; Yamasaki et al., 2001), and gynoecious lines were found to carry an additional copy of the *CS-ACS1* gene (*CS-ACS1G*) co-segregating with the *F* locus (Trebitsh et al., 1997; Mibus and Tatlioglu et al., 2004). Our studies in melon demonstrated that overexpression of an *ACS* gene conferred increased femaleness as measured by earliness and number of hermaphrodite buds (Papadopoulou et al., 2005)

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Yin and Quinn (1992, 1995) proposed that the developmental fate of male and female organs in cucumber is affected by the level of, and perceptiveness to, ethylene. Therefore, perception of ethylene by male and female primordia could inhibit one sex and induce the other independently, and differing sensitivity or receptor levels could respond to different ranges of hormone concentration. It has been proposed that the cucumber *M* locus controls sensitivity to ethylene and the *F* locus controls ethylene levels (Yin and Quinn, 1995). The *A* locus in melon is also proposed to influence the development of the stamens in pistillate flowers such that an *A-* genotype confers the inhibition of stamen development while *aa* allows stamen development resulting in hermaphrodite bud development (Roy and Saran, 1990). Therefore, the *A* locus in melon may be analogous to the *M* locus in cucumber.

Genetic and molecular studies in *Arabidopsis* have elucidated the role of ethylene receptors in the transduction of the ethylene signal (Bleecker and Kende, 2000; Steptanova and Ecker, 2000; Wang et al., 2002). A family of membrane-localized receptors homologous to bacterial two-component histidine kinases is responsible for perception of ethylene. The five ethylene receptors in Arabidopsis (ETR1, ETR2, ERS1, ERS2, and EIN4) act to repress downstream ethylene responses in the absence of ethylene and become inactivated by binding ethylene to de-repress ethylene responses. Thus, increased expression can lead to decreased sensitivity to ethylene and reduced expression can lead to constitutive ethylene response (Hua and Meyerowitz, 1998).

Evaluation of the relationship between ethylene perception and sex determination in cucumber included the isolation of three ethylene receptor homologs (*CS-ETR1*, *CS-ETR2*, and *CS-ERS*) and analysis of expression patterns in monoecious (*ffM-*),

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gynoecious (*F-M-*), and andromonoecious (*ffmm*) genotypes (Yamasaki et al., 2000; 2001). *CS-ETR2* and *CS-ERS* were expressed at higher levels in shoot apices of gynoecious than monoecious seedling (Yamasaki et al., 2000). In addition, treatment of gynoecious shoot apices with the ethylene biosynthesis inhibitor AVG, resulted in down-regulation of *CS-ETR2* and *CS-ERS* while treatment of monoecious apices with ethylene-releasing compound, Ethrel, led to increased accumulation of *CS-ETR2* and *CS-ERS* mRNA, demonstrating their regulation by ethylene. In contrast, induction of ethylene receptor expression was not observed in an andromonoecious genotype in response to Ethrel treatment (Yamasaki et al., 2001). The absence of receptor induction in the andromonoecious genotype in response to ethylene provided support for the relationship between the *M* locus and ethylene response as proposed by the Yin and Quinn model (1995). It was proposed that reduced ethylene perception by the stamen of *mm* genotypes may be insufficient for inhibition of stamen development in pistillate buds, leading to development of hermaphrodite buds (Yamasaki et al., 2001).

In situ hybridization studies evaluating the expression of ethylene receptors (*ERS*, *ETR1*, *ETR2*) and biosynthetic gene (*ACS2*) following initiation of all four floral whorls, the time critical for divergence to a unisexual flower, provided further insight into the relationship between ethylene-producing and ethylene-perceiving tissues (Yamasaki et al., 2003). Buds from monecious plants at the stage following initiation of primordia for all four floral whorls showed expression of *ETR1* in both stamen and pistil primordia and *ERS* in stamen primordia; *ETR2* was expressed just below the pistil primordia and in the adaxial side of petals. In contrast, at the same developmental stage, buds from andromonoecious plants showed expression of *ETR1* only in the region just below pistil

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primordia and no detectable expression of *ERS* and *ETR2*. The lack of receptor expression detected in the stamens of andromonoecious buds was suggested to prevent response to the ethylene signal which would typically inhibit stamens in pistillate buds.

Further evidence for the importance of ethylene perception for pistillate flower formation was obtained from recent studies of andromonoecious and gynoecious melon transformed with the mutant Arabidopsis *etr1-1* gene (Papadopoulou et al., 2002). The receptor mutant *etr1-1,* which is unable to bind ethylene, confers a dominant ethylene-insensitive phenotype as a result of its inability to turn off the repression of ethylene responses. Constitutive expression of *etr1-1* caused phenotypes associated with reduced ethylene sensitivity, including reduced adventitious rooting, delayed flower senescence, and the inability to respond to exogenous ethylene, indicating that *etr1-1* is able to confer ethylene insensitivity in the heterologous melon system. In addition, the *35S::etr1-1* melons showed an altered sex expression pattern such that the formation of carpel-bearing buds was largely abolished. These studies demonstrated that ethylene perception is necessary to promote bisexual flower development in melon.

In this study, I sought to investigate the effect of targeted inhibition of ethylene perception. Andromonoecious melon was transformed with *etr1-1* under the direction of Arabidopsis floral-organ specific *Crab's Claw* (*CRC*) and *Apetela3* (*AP3*) promoters. In Arabidopsis, *Crab's Claw* (*CRC*) encodes a regulator of carpel and nectary development which is expressed specifically in primordia of these organs from their inception (Bowman and Smyth, 1999; Lee et al., 2005). The class B floral identity homeotic gene *Apetala3* (*AP3*) is expressed in cells destined to become petals and stamens (Irish and Yamamoto, 1995).

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The predicted sex expression patterns of *AP3::etr1-1* and *CRC::etr1-1* melon plants were based on the observed effects of exogenous ethylene treatment in cucumber and melon. In cucumber, ethylene treatment promotes the transition from a phase of staminate to pistllate flower production (Rudich, 1990; Perl-Treves, 1999), suggesting that carpel development is induced while stamen development is suppressed. In contrast, analogous treatment of melon results in a shift from staminate to bisexual flower production (Rudich, 1990), suggesting that carpel development is induced while stamen development is unaffected. The production of virtually all staminate flowers by *35S::etr1-1* melon plants (Papadopoulou et al., 2002) was consistent with this proposed effect of ethylene, such that the inability to perceive ethylene in carpels prevented their development while blocked perception in the stamens had no effect. Therefore, as shown in Figure 2.1, ethylene insensitivity in the stamens and petals conferred by *AP3::etr1-1* was expected to have no effect on sex expression in melon while the carpel insensitivity conferred by *CRC::etr1-1* was expected to prevent carpel development, resulting in staminate flower production. Phenotypic characterization of sex expression of the transgenic *CRC::etr1-1* and *AP3::etr1-1* provided the unexpected, opposite observation of female rather than bisexual flowers on *CRC::etr1-1* plants, and almost exclusive staminate flower production on *AP3::etr1-1* plants. These results and a revised model for the role of ethylene perception in sex determination of melon will be discussed.

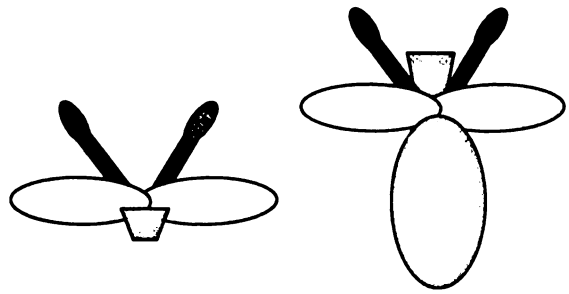
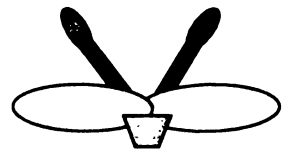
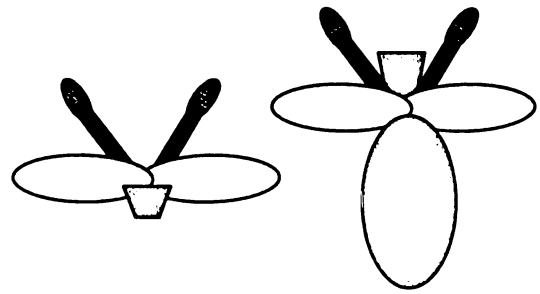
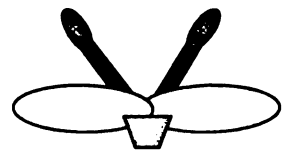
Genotype	Expected Result	
Wild-type	Staminate and bisexual 1. Carpel development 2. Stamens unaffected	
35S-etr1	Staminate only 1. No carpel development 2. Stamens unaffected	
AP3-etr1	Staminate and bisexual 1. Carpel development 2. Stamens unaffected	
CRC-etr1	Staminate only 1. No carpel development 2. Stamens unaffected	

Figure 2.1. Expected sexual phenotype of transgenic melon lines based on effects of ethylene treatment of wild type andromonoecious melon.

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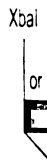
Materials and Methods

Preparation of Plasmid Constructs

CRC::etr1-1: The *35S* promoter was removed from pCIB710 (CIBA-GEIGY, Research Triangle Park, NC) (Rothstein et al., 1987) as an *XbaI-BamHI* fragment to form pCIB710Δ*35S*. The *CRC* promoter was cut from 8-12pbj-60 (kindly provided by John Bowman, University of California, Riverside) as an *XbaI-BamHI* fragment and ligated into pCIB710Δ*35S* to form pCIB710-*CRC*. Arabidopsis *etr1-1* cDNA (Bleecker et al., 1988) was cut from pBS-*etr1-1* as a *BamHI* fragment and cloned into pCIB710-*CRC*. Colonies were screened for proper *etr1-1* orientation based on an *XbaI* site internal to the *etr1-1* gene. The selected pCIB710-*CRC-etr1-1* clone was digested with *PstI-EcoRI* to move the *CRC-etr1-1* fragment into the binary *Agrobacterium* transformation vector pCambia2300 (CAMBIA, Australia.) (Figure 2.2).

AP3::etr1-1: The *AP3* promoter (Jack et al., 1994) was PCR amplified with 5' primer CCTCTAGAAAGCTTAAGAATTATAGTAGC (RG176) to introduce a *XbaI* site at the 5'end and 3' primer CCCGGATCCATTCTTCTCTCTTTGTTTAATC (RG177) to introduce a *BamHI* site at the 3'end. The PCR fragment was cloned into pGEM with pGEM®-T-Easy Vector System (Promega Corporation, Madison, WI). The promoter was then cut from pGEM as an *XbaI-BamHI* fragment and ligated into pCIB710Δ*35S*. *etr1-1* was cloned into pCIB710-*AP3* as a *BamHI* fragment. Colonies were screened for proper *etr1-1* orientation based on the internal *etr1-1 XbaI* site. The selected pCIB710-*AP3-etr1-1* clone was digested with *PstI-EcoRI* to move *AP3-etr1-1* into pCambia2300 (Figure 2.2).



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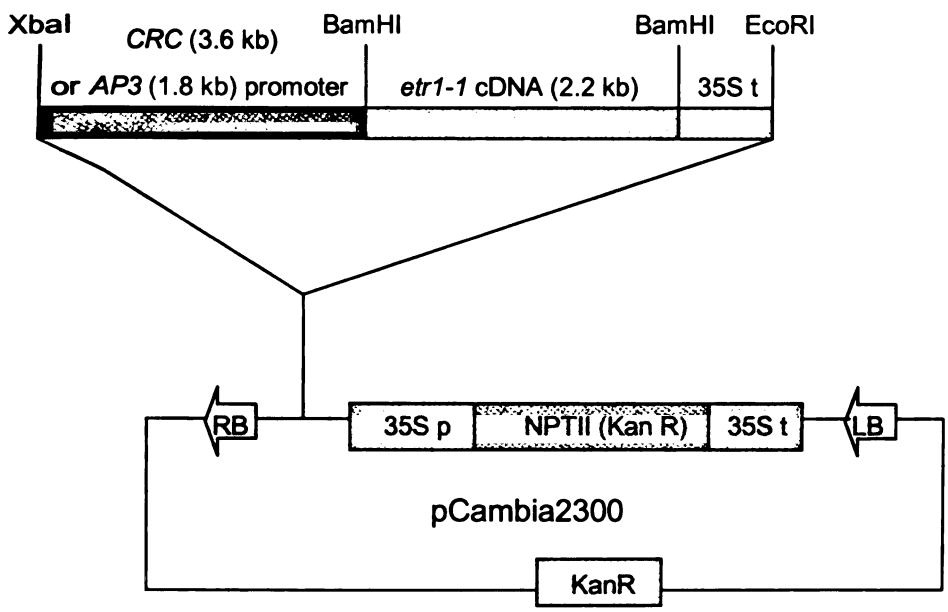

Figure 2.2. Schematic of binary vector construct *CRC::etr1-1/AP3::etr1-1* in binary plasmid vector pCambia2300 as an *XbaI-EcoRI* fragment.

Plant Transformation

The pCambia-*CRC-etr1-1* and *–AP3-etr1-1* constructs were introduced into *Agrobacterium tumefaciens* strain C58 (Deblaere et al.,1985) for subsequent transformation into melon. Andromonoecious muskmelon, Hale's Best Jumbo (Hollar Seed, Rocky Ford, Colorado), was transformed via *Agrobacterium*-mediated transformation of cotyledon tissue via the method of Fang and Grumet (1990) with revisions based on the cucumber transformation method of Tabei et al. (1998), as follows. Seeds were decoated, sterilized in a 15% (v/v) bleach solution (5.25% sodium hypochlorite) with a drop of Tween-20 for 10 minutes, rinsed three times with sterile distilled H_2O, and then germinated in darkness at room temperature in a Petri plate with 5 mL sterile dH_2O for 17-19 hours.

Cotyledons from the partially germinated seeds were separated and cut into four pieces. The explants were soaked for 10 minutes in a diluted suspension (1:10 in LB) of an *Agrobacterium* culture grown overnight, blotted dry on sterile filter paper, and transferred to M1 media (MS media (BIO101) with 5 μM BAP, 1 μM ABA, and 2.5 g /L Phytogel (Sigma)). Plates were cultured for 3 days at 26°C in darkness. Explants were rinsed three times with dH_2O, blotted dry on sterile filter paper, transferred to shoot induction media, M2 (M1 with 400 mg/L timentin). M2 plates were cultured under growth room conditions (26°C) for 7 days before transferring to M3 media (M1 with 400 mg/L timentin and 200 mg/L kanamycin) then returned to the growth room. After 4-5 weeks, regenerated shoots were separated from explants and cultured on MS media with 2μM BAP, 400 mg/L timentin, 200 mg/L kanamycin, and 8 g/L agar in Magenta boxes (Magenta Corp., Chicago, IL) for selection and shoot elongation. Unbleached shoots

were transferred to MS media with 400 mg/L timentin and 8 g/L agar in Magenta boxes for rooting under growth room conditions. Plantlets were transplanted to Baccto soil as soon as roots appeared. Potted plantlets were sealed in bags for one week before slowly acclimating to lower humidity conditions and transferring to the greenhouse.

Regenerated plants (T_0) were evaluated for the presence of the neomycin phosphotransferase protein by NPTII ELISA (Agia®, Elkhart, Indiana) and the *etr1-1* gene by polymerase chain reaction (PCR) (described below). PCR-positive T_0 plants were transferred to the greenhouse and self-pollinated to produce T_1 progeny for further evaluation.

PCR analysis

The Wizard Genomic Plant DNA Purification Kit (Promega Corporation, Madison, WI) was used to extract genomic DNA from leaf tissue of melon. PCR was used to amplify the inserted *etr1-1* transgene using *etr1-1* specific 5' and 3' primers (5'-GGGGAGGTGGTCGCTGTGA (RG305) and 5'-GCTCATGGGACACAACTCGG (RG306), respectively). *CRC::etr1-1* was amplified with CRC specific 5' primer 5'-CTTGCAATCCCTAGCCAG (RG 299) and 3' *etr1-1* primer RG306. *AP3::etr1-1* was amplified with *AP3* specific 5' primer 5'-GCTTTGGTCCCCCTCTTTTACC (RG300) and 3' *etr1-1* primer RG306. The amplification reactions of *etr1-1* were carried out with Taq DNA polymerase (Invitrogen, Carlsbad, California) in 3.0 mM $MgCl_2$ for 1 cycle of 2 minutes at 95°C followed by 40 cycles of 95°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute, then 1 cycle of 5 minutes at 72°C (Sambrook and Russell, 2001). Amplification reactions of *CRC::etr1-1* and *AP3::etr1-1* were carried out as with *etr1-1* but with the following program: 1 cycle of 2 minutes at 95°C followed by 40 cycles of

95°C for 50 seconds, 58°C for 50 seconds, and 72°C for 2:20 minutes, then 1 cycle of 5 minutes at 72°C.

Northern Hybridization Analysis

Fresh young leaf tissue was collected from T_1 greenhouse grown melon plants to evaluate *etr1-1* transgene expression. RNA was isolated from 100 mg fresh leaf tissue frozen in liquid nitrogen using Concert™ Plant RNA Reagent (Invitrogen, Carlsbad, California). RNA quantification and purity was determined by absorbance at 260 nm and by gel electrophoresis. Total RNA (15 µg) was electrophoresed on a 1.5% agarose formaldehyde gel and blotted to nylon membrane (Hybond-N+; Amersham-Pharmacia Biotech Ltd, England) (Sambrook and Russell, 2001). The membrane was hybridized overnight at 61°C with a digoxigenin-UTP labeled RNA probe of *etr1-1* (Roche Diagnositics, Germany). Stringency washes and detection via chemiluminescent visualization with CDP-Star were carried out following protocols developed for the DIG system (Roche Diagnostics, Germany). RNA also was isolated from young male and female buds (3-5 mm) and older male buds (10-12 mm) from greenhouse grown plants. Petals were excised from the older male buds with a razor blade and designated as "petals" while the remaining portion of the male bud containing stamens, nectaries, and floral cup was designated "lower-bud."

Ethylene Measurements

Young leaves (approximately 4-7 cm wide) were removed from lateral branches of greenhouse grown plants with a razor blade, weighed, and placed in a 50 ml syringe containing ¼ piece of Whatman #2 filter paper (90 mm) moistened with 200 µL distilled water (dH_2O). The syringe plunger was adjusted to 30 mL, and the end of the syringe

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was fitted with a rubber septum and sealed with parafilm. Gas samples were removed using an airtight syringe (1mL) inserted through the rubber septa into the airspace of the 50mL syringe. Samples were analyzed by gas chromatography (Hach Carle series 100GC, Linear 1200 recorder) with an activated alumina column and flame ionization detector. Likewise, male buds (1-2 days pre-anthesis) were removed from lateral branches with a razor blade, weighed, placed in 10 mL syringes containing a 1 cm square of Whatman #2 filter paper moistened with 40 μL H_2O, plunger adjusted to 5mL, and the opposite end fitted with a rubber septa and parafilm. Leaf and male bud samples were collected three times from four randomly sampled plants per genotype.

Sex Expression

Plants for all experiments were grown in 35 cm diameter plaster pots containing commercial Baccto soil mix (Michigan Peat C., Houston, TX). Plants were fertilized once per week with 300 ppm of 20-20-20 fertilizer mix. Experiments were conducted between March and October, during seasons of adequate light to observe flowering patterns.

Each node of the mainstem of greenhouse grown melon plants was evaluated for the presence of staminate and/or carpel-bearing buds. The development of each bud was followed to note if anthesis was reached, and, for aborted carpel-bearing buds, the size reached prior to senescing. Plants were observed for 30 nodes.

All experiments were conducted in a randomized complete block design with 5-8 replicates per gentoype. Each genotype was tested in at least 4 experiment with the exception of *35S::etr1-1, AP3::etr1-1* line 3, and non-transgenic tetraploid controls. The specific genotypes and number of replicates are indicated in the figure legends.

Floral Morphology Evaluation

Flower buds were removed from greenhouse grown plants with a razor blade. Buds were cut longitudinally and viewed under a dissecting microscope fitted with a digital camera (Olympus Optical Co., Melville, NY).

Results

Gene integration and expression analysis

Transgenic T_0 melon plants haboring the *AP3::etr1-1* or *CRC::etr1-1* constructs were produced by *Agrobacterium*-mediated transformation and verified by PCR analysis. Examination of pollen morphology indicated that *AP3::etr1-1* lines had four-sided pollen grains indicative of tetraploidy (Fassuliotus and Nelson, 1992), while *CRC::etr1-1* were diploid. Tetraploidy is a common phenomenon in melons regenerated from tissue culture (Ezura et al., 1992; Yadav et al., 1996) and does not interfere with sex phenotypes as non-transgenic tetraploid segregants show normal sex phenotypes (Papadopoulou et al., 2005). Transgenic T_0 plants were self-pollinated in the greenhouse to produce seed. Presence of the *etr1-1* gene and appropriate *AP3* or *CRC* promoter was verified in T_1 progeny by PCR analysis (Figure 2.3). Segregation ratios of T_1 progeny are consistent with gene insertion at one site, with the exception of AP3-3, which could have one or two transgene insertions depending on when the chromosome doubling event occurred (Table 2.1).

Northern hybridization analysis was conducted to evaluate *etr1-1* transgene expression in leaves and floral tissue (Figure 2.4). In *AP3::etr1-1* lines, *etr-1-1*

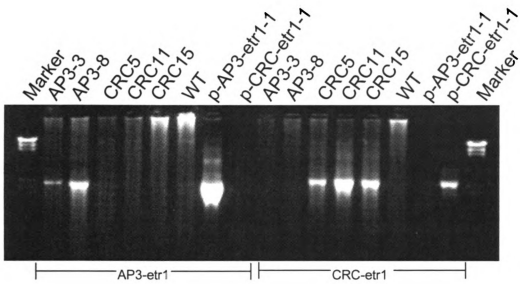


Figure 2.3. PCR amplification of *etr1-1* gene construct from genomic DNA of T_1 transgenic lines with *CRC::etr1-1* and *AP3::etr1-1* specific primers. The picture shows amplification of the same set of plant DNA samples with both *CRC::etr1-1* and *AP3::etr1-1* specific primers. WT=Hale's Best Jumbo wild type plant control; p-*CRC-etr1-1* and p-*AP3-etr1-1* = plasmid controls.

Table 2.1. Segregation analysis of *AP3::etr1-1* and *CRC::etr1-1* self-pollinated transgenic melon progeny.

Line		Segregation Observed	Ploidy	Expected Ratio	χ^2
CRC5		25:14	2n	3:1	1.92ns
CRC11		28:16	2n	3:1	1.98ns
CRC15		37:11	2n	3:1	0.03ns
AP3-3		12:0	4n	15:1	0.61ns
AP3-8	T_1	6:6	4n	3:1	2.77ns
	T_2	19:6		3:1	0.097ns

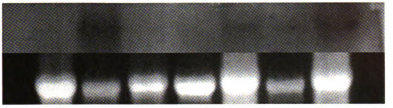
All progeny were T_1 generation except AP3-8, which included T_2. Data are based on the presence of the *AP3::etr1-1* or *CRC::etr1-1* by PCR amplification.

ns- χ^2 value not significantly different at p=0.05

Figure 2.4. Northern hybridization of heterologous *etr1-1* gene expression from greenhouse grown *AP3::etr1-1* and *CRC::etr1-1* melon with a DIG-labeled *etr1-1* probe. (A) Hybridization of total RNA isolated from leaf tissue of non-transgenic (WT), *35S::etr1-1* (35S), *CRC::etr1-1* (CRC11 and CRC15), and *AP3::etr1-1* (AP3-3 and AP3-8) plants. (B) Hybridization of total RNA isolated from young male buds of non-transgenic (WT), *AP3::etr1-1* (AP3-3 and AP3-8), and *CRC::etr1-1* (CRC5 and CRC15) plants and young female buds of non-transgenic (WT) and *CRC::etr1-1* (CRC5 and CRC15) plants. (C) Hybridization of total RNA isolated from mature male buds of non-transgenic (WT), *CRC::etr1-1* (CRC5 and CRC11), and *AP3::etr1-1* (AP3-8) plants. Mature male buds were separated into petal tissue and lower bud, which included sepals, stamens, nectary, and floral cup. The bottom panel in each pair shows rRNA stained with ethidium bromide.

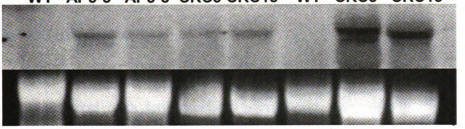
A. Leaves

WT 35S AP3-3 AP3-8 CRC5 CRC11 CRC15



B. Young male buds Young female buds

WT AP3-3 AP3-8 CRC5 CRC15 WT CRC5 CRC15



C. Mature male buds

Petals: Lower-bud:

WT CRC5 CRC11 AP3-8 WT CRC5 CRC11 AP3-8

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expression was absent in leaves and present in male buds as expected based on the petal and stamen specificity of *AP3* in Arabidopsis (Jack et al., 1994). AP3 plants could not be tested for levels of transcript in female buds due to an absence of pistillate buds, which will be discussed later. *CRC::etr1-1* lines showed hybridization in leaves, indicating leaky expression from the promoter in the heterologous melon system. *etr1-1* mRNA was detected in both young male and female buds of *CRC::etr1-1* plants; higher levels were observed in the females buds, consistent with expected carpel expression. Detection of *etr1-1* message in male CRC buds could be the result of expression in the nectaries (Bowman and Symth, 1999; Lee et al., 2005) or leakiness, as is suggested by expression in the petals.

Ethylene Measurement

Expression of *etr1-1* is expected to confer increased ethylene production since the inability to perceive ethylene interferes with the negative feedback inhibition of ethylene production, as has been observed in *35S::etr1-1* petunia and melon (Wilkinson et al., 1997; Papadopoulou 2002). As expected, *35S::etr1-1* plants showed elevated levels of ethylene production from leaves and male buds (1-2 days pre-anthesis) (Table 2.2). Elevated ethylene evolution from leaves and male buds of *CRC::etr1-1* lines (CRC5, CRC11, CRC15), relative to non-transgenic controls (WT, Azygous) was consistent with leaky expression observed by northern hybridization (Table 2.2). Similarly, ethylene production in *AP3::etr1-1* plants was generally consistent with northern analysis. Ethylene production was not elevated in leaves and was slightly elevated in mature male buds.

Table 2.2. Cor

of *AP3::etr1-1*

WT	
4nNEG	
Azygous	
35S	
AP3-3	
AP3-8	
CRC5	
CRC11	
CRC15	

Data shown

Table 2.2. Combined data for all leaf and male bud ethylene measurement experiments of *AP3::etr1-1* and *CRC::etr1-1* transgenic melon lines.

	Experiments (Total number of plants)	Leaf		Male Bud	
		Samples evaluated	C_2H_4 (pmol/g) ± SE	Samples evaluated	C_2H_4 (pmol/g) ± SE
WT	4 (23)	51	53.7 ± 3.1	47	39.9 ± 2.8
4nNEG	2 (11)	24	58.1 ± 5.0	24	63.0 ± 6.2
Azygous	2 (10)	26	66.6 ± 24.9	22	60.0 ± 9.0
35S	1 (5)	12	511.1 ± 109.1	12	188.3 ± 50.5
AP3-3	1 (7)	12	50.1 ± 11.7	12	65.6 ± 9.2
AP3-8	3 (17)	42	82.2 ± 7.6	36	108.0 ± 31.0
CRC5	2 (11)	24	197.8 ± 28.5	23	117.7 ± 16.8
CRC11	2 (11)	24	130.6 ± 15.5	20	169.4 ± 29.6
CRC15	4 (23)	52	190.8 ± 20.7	48	99.6 ± 6.6

Data shown are ± standard error.

Sex expression a

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Sex expression and floral morphology

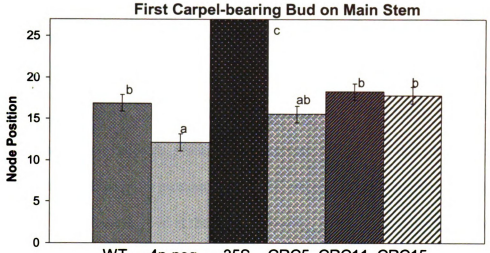
CRC::etr1-1

The typical progression of sex expression along the main stem of andromonoecious melon is 3-4 vegetative nodes, followed by phase of staminate buds, then a mixture of staminate and carpel-bearing buds. As previously observed, *35S::etr1-1* melon plants, which display several phenotypes consistent with the inability to perceive ethylene (Papadopoulou, 2002), failed to produce carpel-bearing buds (Figure 2.5A). In contrast to the prediction that inhibition of ethylene perception in the carpel would also inhibit carpel-bearing buds, *CRC::etr1-1* lines did not fail to produce carpel-bearing buds. Initial evaluation showed no significant difference in the appearance of the first bud bearing a carpel on plants of *CRC::etr1-1* lines CRC5, CRC11, and CRC15 compared to wild type (WT) plants (Figure 2.5A). While the initial experiment was carried out at the end of the growing season, late August to early October, subsequent evaluations were carried out during the spring and summer months, May through August. These evaluations showed significantly earlier appearance of the first bud with a carpel on the main stem of CRC plants (CRC5, CRC11, CRC15) than on non-transgenic controls (WT and AZY) by 6-10 nodes (Figure 2.5B).

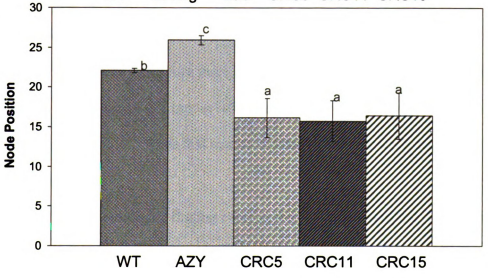
Once the main stem begins producing pistillate buds, both male and female buds are generally produced at the same node for the remainder of the plant's life. Evaluation of the total number of carpel-bearing buds produced in the first 30 nodes on the main stem of CRC plants demonstrated this typical flowering pattern. The increase in number of total buds bearing carpels on CRC plants (CRC5, CRC11, CRC15) compared to

Figure 2.5. Main stem sex expression patterns of melon plants transformed with *CRC::etr1-1*. (A) Node position of first carpel-bearing bud on the main stem of Hale's Best Jumbo 2n and 4n non-transgenic plants (WT and 4n-neg), *35S::etr1-1* plants (35S), and *CRC::etr1-1* plants (lines CRC5, CRC11, and CRC15). The experiment was conducted in a randomized complete block design. Data are the mean ± standard error of seven replicate plants per genotype (except 35S, n=5). (B) Node of first carpel-bearing bud on main stem and (C) Total number of node with carpel-bearing buds on the main stem for Hale's Best Jumbo and azygous non-transgenic plants (WT and AZY), and *CRC::etr1-1* lines CRC5, CRC11, and CRC15. The experiment was repeated three times in a replicated complete block design, with each giving equivalent results. The data were pooled from the three experiments. Data are means ± standard error of 16 plants of each genotype (except n=15 AZY). Bars marked with different letters are significantly different, ANOVA, LSD, P=0.05.

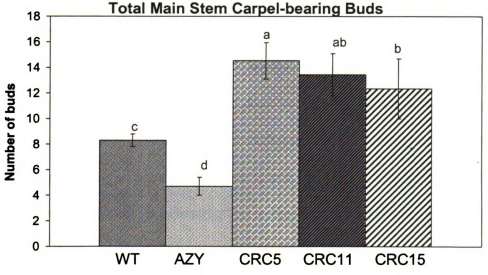
A.


First Carpel-bearing Bud on Main Stem
Node Position
0
5
10
15
20
25
b
a
c
ab
b
b
WT
4n-neg
35S
CRC5
CRC11
CRC15

B.


Node Position
0
5
10
15
20
25
30
b
c
a
a
a
WT
AZY
CRC5
CRC11
CRC15

C.


Total Main Stem Carpel-bearing Buds
Number of buds
0
2
4
6
8
10
12
14
16
18
c
d
a
ab
b
WT
AZY
CRC5
CRC11
CRC15

controls (WT, AZY) reflects the earliness in production of the first pistillate bud (Figure 2.5C). These results indicate that *CRC::etr1-1* plants showed enhanced rather than inhibited femaleness.

Typically, the majority of the buds bearing carpels initiated on the main stem of melon plants abort prior to reaching anthesis (Papadopoulou et al., 2005). Despite the presence of carpel-bearing buds, preliminary observation suggested that premature senescence may be even more pronounced in CRC plants. Measurement of the size of the immature carpel-bearing bud at the time of abortion showed a distinctly different pattern for CRC buds than non-transgenic controls (WT and AZY) (Figure 2.6). The majority (80%) of CRC buds with carpels aborted early in development. Carpel-bearing buds on CRC plants were four times more likely than buds on wild type and azygous control plants to senescence at 4 mm or less, and a similar decrease was observed in proportion of buds on CRC plants that mature beyond 4 mm and reach anthesis relative to controls (Figure 2.6).

An even more surprising finding came in the examination of the few carpel-bearing buds that did reach anthesis. Instead of developing into a bisexual flower, which is typical of the carpel-bearing bud formed on andromonoecious melon, examination of the CRC flowers containing a carpel frequently showed an absence of stamens (Figure 2.7). Thus, the CRC plants exhibited a sex conversion from hermaphrodite to female. Since the pistil of bisexual buds is largely hidden by stamens, the absence of stamens in CRC carpel-bearing flowers was quite obvious (Figure 2.7A). Subsequent evaluation of the rare flowers bearing a carpel produced on lateral branches of *35S::etr1-1* plants also showed an absence of stamens indicating that in the cases where flowers containing a

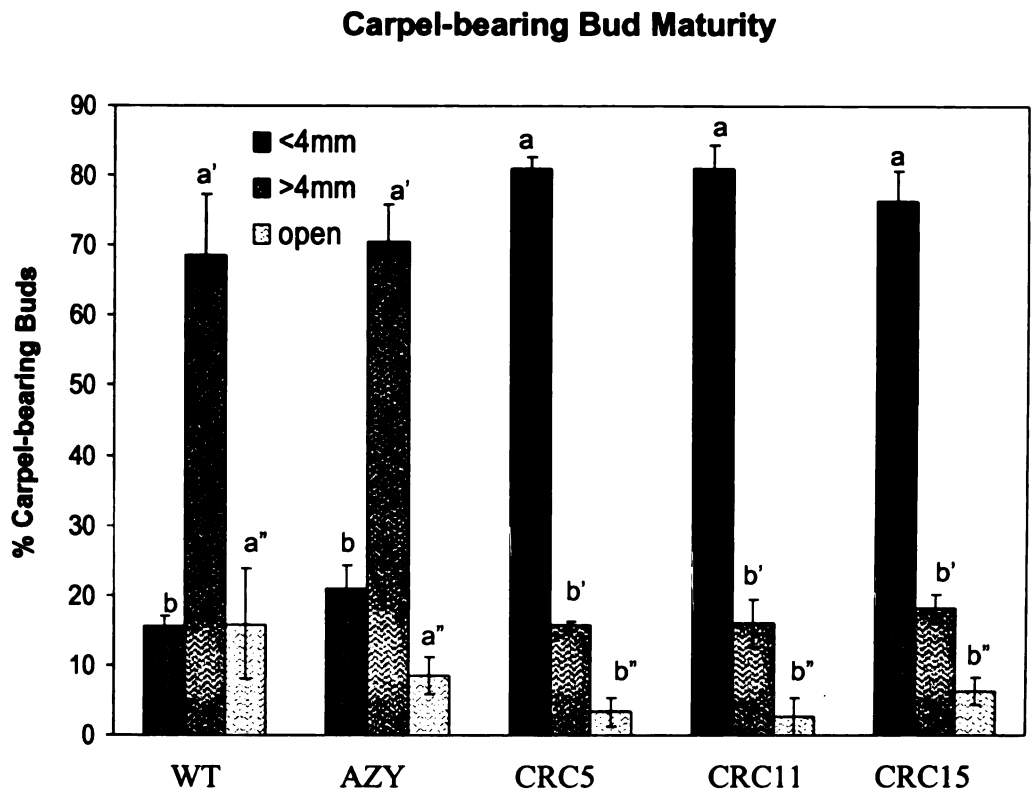
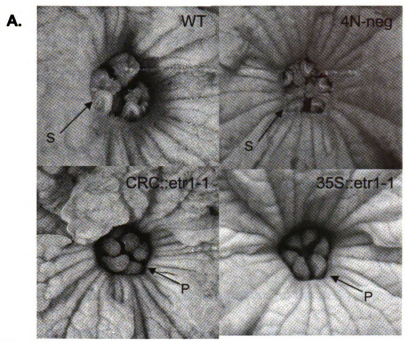
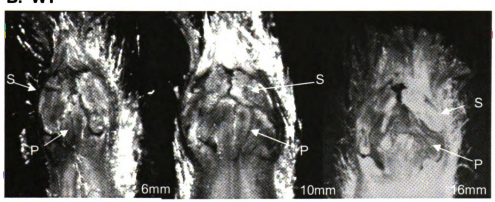
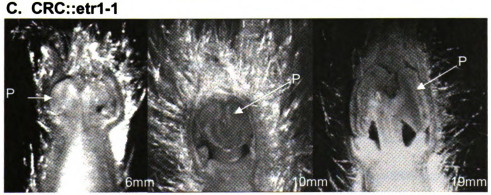


Figure 2.6. Size reached by carpel-bearing buds before senescing for Hale's Best Jumbo and azygous non-transgenic plants (WT and AZY), and *CRC::etr1-1* plants (lines CRC5, CRC11, and CRC15). The experiment was repeated three times in a replicated complete block design with equivalent results. The data were pooled from the three experiments. Data are means ± standard error of 16 plants of each genotype (except n=15 AZY). Bars marked with different letters are significantly different, ANOVA, LSD, P=0.05; with a and b corresponding to <4mm floral buds, a' and b' corresponding to >4mm floral buds, and a" and b" corresponding to open flowers.

Figure 2.7. Carpel-bearing flower morphology of non-transgenic (WT and 4nNEG) and transgenic (*35S::etr1-1* and *CRC::etr1-1*) melon plants. (A) Flowers of WT, 4n-NEG, *CRC::etr1-1*, and *35S::etr1-1* at anthesis and at 6, 10 and 16 mm. (B) Developing buds bearing carpels of WT plants. (C) Developing carpel-bearing buds of *CRC::etr1-1* plants at 6, 10, and 19mm. S=stamen; P=pistil.


A.
WT
4N-neg
S
S
CRC::etr1-1
35S::etr1-1
P
P


B. WT
S
S
S
P
P
P
6mm
10mm
16mm


C. CRC::etr1-1
P
P
P
6mm
10mm
19mm

carpel were formed, inhibition of ethylene perception prevented stamen development (Figure 2.7A). Microscopic evaluation of carpel-bearing buds at various stages of development was consistent with the observed inhibition of stamen development in CRC plants (figure 2.7BC); 55% of the CRC flowers/buds evaluated lackied stamens versus 0% of the non-transgenic buds. Stamens can be observed in the region between the enlarging pistil and petals throughout the development of the WT hermaphrodite buds, while in pistillate CRC buds this entire region is occupied by the pistil (Figure 2.7B and 2.7C).

AP3::etr1-1

Evaluation of the sex expression pattern on the main stem of *AP3::etr1-1* plants also provided surprising results. While the inhibition of ethylene perception in the stamens conferred by *AP3::etr1-1* was not predicted to affect sex determination in melon, all AP3-3 plants and the majority of AP3-8 plants (61%) failed to produce any carpel-bearing buds within the first 30 nodes (Figure 2.8A). Those AP3-8 plants, which did produce buds bearing carpels on the main stem, reverted back to staminate buds, after producing only 1-2 buds containing a carpel. The first node of lateral branches from the main stem also was examined for flower sex. Hermaphrodite buds typically form at these positions in wild type andromonoecious melon; however, *AP3::etr1-1* plants also showed staminate buds in these positions (Figure 2.9A). Overall, AP3 plants showed hermaphrodite bud formation on less than 5% of the nodes evaluated while more than 50% of nodes evaluated in non-transgenic plants (WT and AZY) produced buds containing a carpel (Figure 2.8B). As was observed in previous experiments, *CRC::etr1-*

Figure 2.8. Carpel-bearing bud formation on *AP3::etr1-1* trangenic melon plants. (A) Node position of first carpel-bearing bud on main stem and (B) Percentage of nodes bearing buds containing a carpel for Hale's Best Jumbo (WT), azygous (AZY), *AP3::etr1-1* (AP3-8) and *CRC::etr1-1* (CRC15) plants. The experiment was repeated three times (with the exception of AP3-3 which was only only included in one of the experiments), in a randomized complete block design, with a total of n=21 AP3-8, n=18 WT and CRC15 and n=14 AZY plants, and n=7 AP3-3.

All data are combined means ± standard error, and bars marked with different letters are significantly different, ANOVA, LSD, P=0.05.

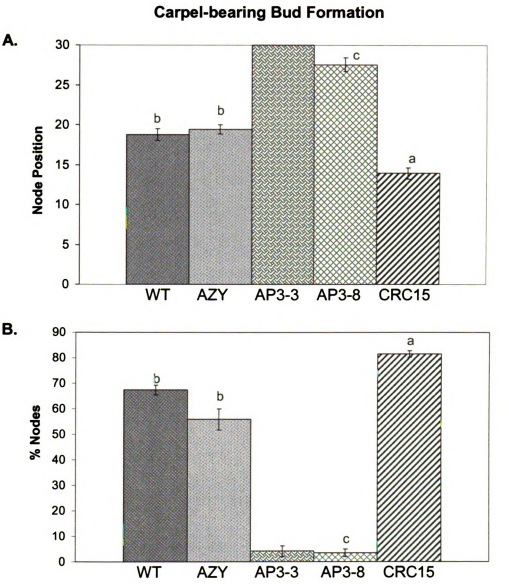
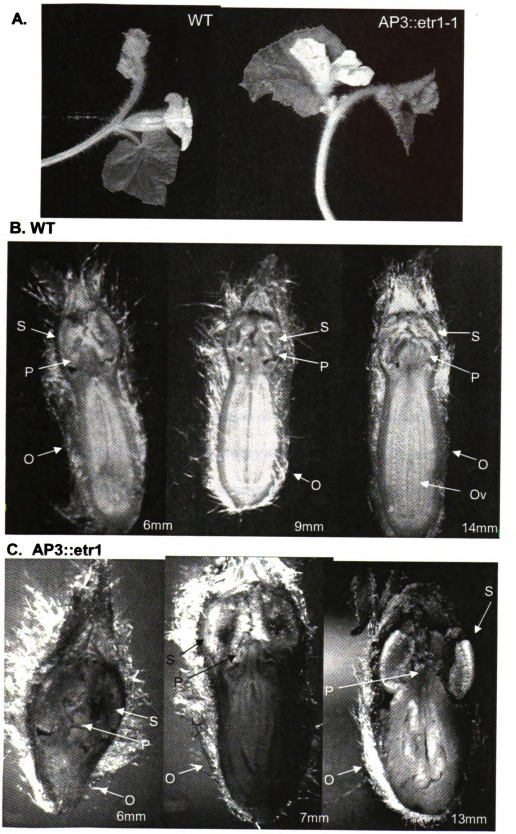

Carpel-bearing Bud Formation
A.
Node Position
30
25
20
15
10
5
0
b
b
c
a
WT
AZY
AP3-3
AP3-8
CRC15
B.
% Nodes
90
80
70
60
50
40
30
20
10
0
b
b
c
a
WT
AZY
AP3-3
AP3-8
CRC15

Figure 2.9. Hermaphrodite flower morphology of non-transgenic (WT) and transgenic (*AP3::etr1-1*) melon plants. (A) First node on lateral of WT and *AP3::etr1-1* at anthesis. (B) Developing hermaphrodite buds of WT plants. (C) Developing hermaphrodite buds of *AP3::etr1-1* plants. S=stamen; P=pistil, O=ovary, Ov=ovule.


A.
WT
AP3::etr1-1
B. WT
S
P
O
6mm
S
P
O
9mm
S
P
O
Ov
14mm
C. AP3::etr1
S
P
O
6mm
S
P
O
7mm
S
P
O
13mm

1 plants that were included for comparison had earlier and increased formation of buds containing carpels (Figure 2.8).

Unlike the *CRC::etr1-1* and *35S::etr1-1* carpel-bearing buds, the occasional buds containing a carpel of *AP3::etr1-1* plants showed well-developed stamens; however, development of carpels was altered relative to wild type (Figure 2.9BC). In the upper region of the wild type hermaphrodite bud, the stigma occupies a large portion of this space while the stamens are tightly nested between the stigma and petals (Figure 2.9B). In hermaphrodite buds of AP3 plants, the stamens occupied most of the upper region of the bud and the stigmas appeared smaller and less developed than those in WT plants at similar stages of development (Figure 2.9C). Evaluation of longitudinal sections of developing wild type hermaphrodite buds also showed distinctive structural development of the ovary and progressive maturation of the ovules. In contrast, the AP3 bisexual buds had a shorter, poorly differentiated ovary and a lack of ovule development, indicating a difference in ethylene perception requirements for the development of ovaries versus stamens. It should also be noted, that consistent with the rare occurrence of hermaphrodite buds and the poorly developed carpels, it was difficult to produce progeny on *AP3::etr1-1* plants.

Discussion

The results presented demonstrate that expression of the dominant mutant ethylene receptor gene, *At-etr1-1*, under the direction of Arabidopsis floral-targeted promoters of *APETALA3* and *CRAB'S CLAW* in andromonoecious melon conferred altered flowering phenotypes strikingly different from each other, from wild type, and

from *35S::etr1-1* melon, with respect to sex expression patterns, and flower maturation and morphology.

As described in the Introduction, the predicted sex expression patterns of *CRC::etr1-1* and *AP3::etr1-1* transgenic melon were based on the changes in sex subsequent to treatment with ethylene. The transition from staminate to female flower production upon ethylene treatment in cucumber (Perl-Treves, 1999) suggested that ethylene promotes carpel development while suppressing stamen development. In melon, an observed shift from staminate to hermaphrodite flower production (Rudich, 1990) suggested that ethylene facilitates carpel development but has no effect on stamens. Therefore, blocking ethylene perception in the stamens of melon plants was not expected to result in a modified flowering phenotype since ethylene was not predicted to play a role in stamen development. In contrast, blocking ethylene perception in the carpels was expected to result in the inhibition of carpel-bearing flower production since ethylene was predicted to be critical for carpel development. Consistent with the suggested requirement of ethylene perception by the carpel, hermaphrodite bud formation in *35S::etr1-1* melon plants was largely abolished (Papadopoulou et al., 2002).

Despite leaky expression, *CRC::etr1-1* plants were phenotypically different from *35S::etr1-1* plants and did not show the expected inhibition of carpel-bearing flowers. Instead, *CRC::etr1-1* plants showed enhanced femaleness as indicated by earlier and increased formation of buds containing carpels and a conversion from hermaphrodite to female bud production. The difference in the sex expression of *35S::etr1-1* and *CRC::etr1-1* plants may result from differences in timing and/or location of *etr1-1* expression at key stages in sex determination

Conversely, and in contradiction to the predicted result that blocked ethylene perception in the stamen would not affect the production of hermaphrodite buds, *AP3::etr1-1* plants almost exclusively produced staminate flowers. In addition, longitudinal sections of limited pistillate buds produced by *AP3::etr1-1* plants, exhibited inhibited carpel development compared to wild type buds of similar size, while stamen development within the pistillate buds appeared normal.

Collectively, the findings of this study were not consistent with expected sexual phenotypes and suggest a revised model for the role of ethylene perception in sex determination. The lack of pistillate buds on *AP3::etr1-1* plants suggests that ethylene perception by stamen primordia is important for development of carpels. On the other hand, the absence of stamens in carpel-bearing buds, as observed on *CRC::etr1-1* plants, may suggest that perception of ethylene by carpel primordia is important for sustained stamen development. Therefore, a revised model would suggest that a reciprocal relationship exists between the stamen and carpel in the development of hermaphrodite buds, such that ethylene perception by the stamen is required for subsequent development of the carpel and vice versa (Figure 2.10). According to this model, andromoneocious melon plants produce a phase of staminate flowers until sufficient levels of ethylene are present to initiate hermaphrodite buds. Within the developing floral bud, stamen primordia must perceive ethylene at a critical time to promote the development of carpels. Similarly, continued stamen development within the hermaphrodite bud requires perception of ethylene by the developing carpel. Based on this model, carpel arrest precedes stamen arrest, which would allow for the occasional female flower observed on

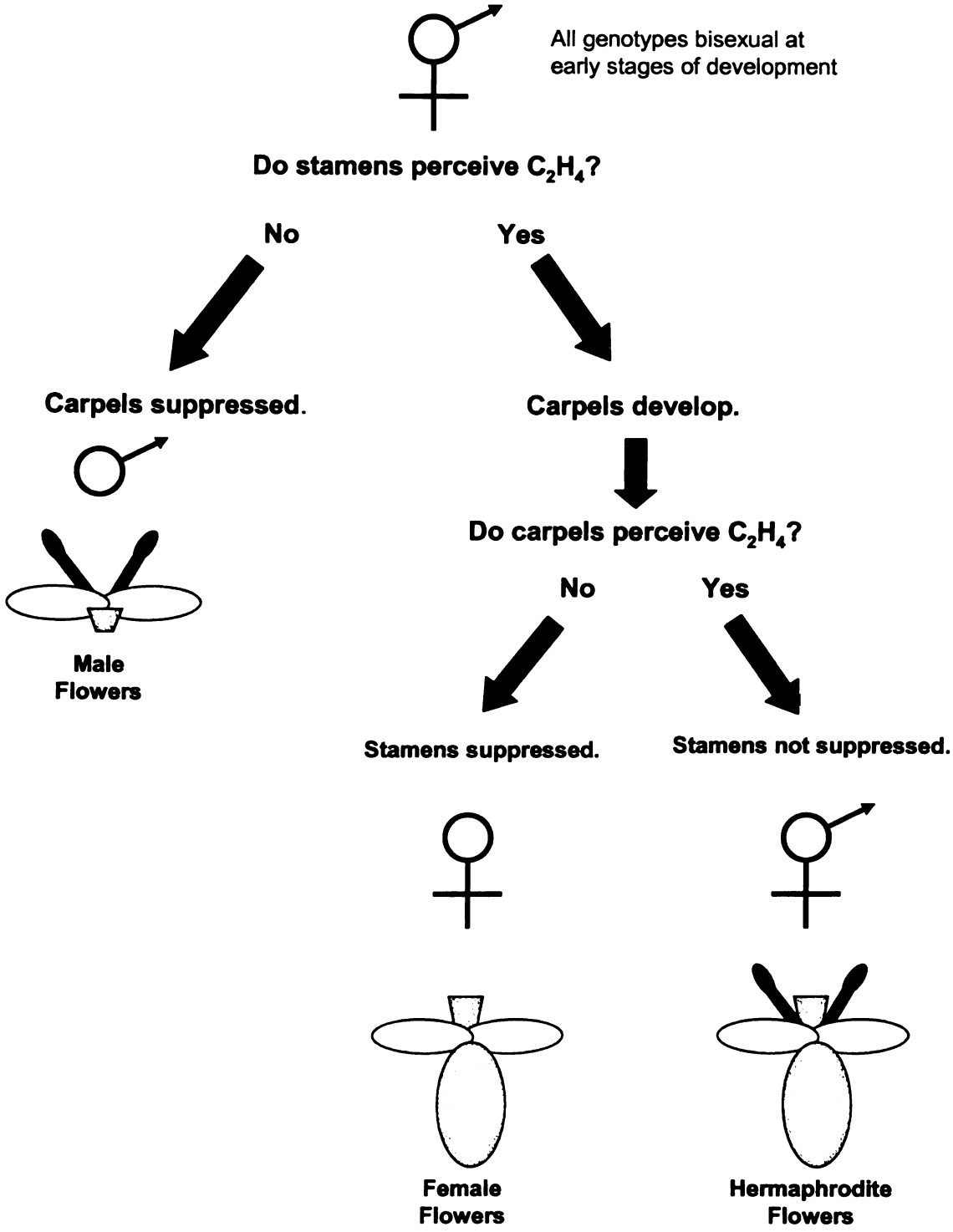


Figure 2.10. Revised model for hypothesized role of ethylene perception in sex determination which suggests a reciprocal relationship between petals and stamens.

35S::etr1-1 plants if the carpel managed to begin developing, but did not perceive enough ethylene at the critical stage to maintain stamen development.

Several lines of evidence from recent studies are consistent with the proposed relationship between stamens and carpel of this revised model. Morphological studies of male and female cucumber bud development have shown that the divergence from a bisexual to unisexual flower begins after the initiation of carpel primordia (Bai et al., 2004) and that the stage of floral development at which sex determination can be modulated by ethylene treatment just preceeds, or immediately follows, differentiation of stamen primordia (Yamasaki et al., 2003). Since both stamens and carpels initiate prior to sexual divergence, it is possible that signaling, stimulated via ethylene perception, between these two adjacent whorls is responsible for the sexual fate of the flower bud. In cucumber, the arrest of carpel development in staminate buds begins to become morphologically apparent at the stage just prior to carpel differentiation into the stigma and ovary (Bai et al., 2004). The arrest of stamen development in female buds becomes apparent just after differentiation between the anther and filament of stamen (Hao et al., 2003; Bai et al., 2004).

In Arabidopsis, ethylene receptors act redundantly to negatively regulate ethylene response such that knock-out of receptors leads to constitutive ethylene response (Hua and Meyerowitz, 1998). The comparison of gynoecious (*F-M-*), monoecious (*ffM-*), and andromonoecious (*ffmm*) cucumber cultivars demonstrated a lack of *ETR1*, *ETR2*, and *ERS* ethylene receptor expression in stamen and carpel primordia of *mm* plants at the time critical for sex determination (Yamasaki et al., 2000, 2001, 2003). Therefore, it is possible that the absence of ethylene receptors in *mm* stamen or carpel primordia

(Yamasaki et al., 2001, 2003) could confer constitutive ethylene response, resulting in the promotion of both carpel and stamen development. This possibility would also explain how carpels could develop in *mm* plants in the absence of ethylene perception by carpel primodia. Similarly, the arrest of the stamen development conferred by the *M* allele could be the result of receptor mediated inhibition of response to ethylene at the time and/or location critical for divergence from bisexual to unisexual flower development. The finding that *CRC::etr1-1* caused a conversion from perfect to female flowers suggests that the inability of the carpel to perceive ethylene at the time critical for sex produced a phenotype similar to observed stamen arrest in *M-* cucumber and *A-* melon. Clearly, a critical factor will be the relative location and timing of expression of both ethylene production and perception components within floral primordia.

Analysis of *CRC::etr1-1* melon plants showed an additional phenotype of interest. Although carpel-bearing buds were able to initiate, virtually all buds containing a carpel on CRC plants aborted prior to anthesis, most before reaching 4 mm in length, suggesting that ethylene plays a role in sustained maturation of the carpel. These results provide support for observations made in transgenic melon constitutively overexpressing *ACC synthase*, *ACS*, a key enzyme in ethylene biosynthesis. The *35S::ACS* melon plants showed earliness and increased production of carpel-bearing buds, and an increase in the proportion reaching anthesis. Thus, it appears that ethylene is important for both sex determination and pistillate bud maturation (Papadopoulou et al., 2005).

In summary, the study of *CRC::etr1-1* and *AP3::etr1-1* transgenic melon provided unexpected results. *CRC::etr1-1* melon plants showed increased femaleness as demonstrated by earlier and increased carpel-bearing bud production and the conversion

of hermaphrodite to female buds. In contrast, increased maleness was observed in *AP3::etr1-1* melon plants as demonstrated by rare production of hermaphrodite buds and poorly developed carpels in many of those which did form. These results suggest that the critical site for ethylene perception in the determination of stamen or pistil development does not reside within the affected organ. Instead, there appears to be a previously unknown reciprocial relationship between stamens and carpels in their requirement for ethylene perception for sexual organ development in hermaphrodite buds, which has led to a revised model of ethylene's role in sex expression of melon. Further study is necessary to the determine the precise timing and localization of *etr1-1* expression at key stages of sex determination and to establish if the effects of altered ethylene perception in cucumber are in line with observations made in melon.

Referenc

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Chapter 3

Evaluation of the effect of modified endogenous ethylene production on sex expression and fruit production in field grown transgenic *Cucumis melo*

Abstract

Sex expression in cucurbit species is affected by environmental, hormonal, developmental, and genetic factors. Ethylene is considered the primary hormone promoting femaleness in cucumber, melon, and squash; exogenous ethylene increases femaleness while inhibition of ethylene perception or production decreases femaleness. Greenhouse experiments with transgenic andromonoecious melon constitutively overexpressing an *ACS* (1-aminocyclopropane-1-carboxylic acid synthase) gene for ethylene biosynthesis showed increased ethylene evolution from leaves and floral buds, increased numbers of hermaphroditic buds, and an increased percentage of hermaphrodite buds that reached full anthesis. In this study I sought to determine whether increased femaleness would be observed in plants grown in field conditions and to determine the effect of increased ethylene and modified sex expression on fruit production. Despite no enhanced earliness in hermaphrodite bud initiation, field grown transgenic *35S:ACS* melon plants exhibited significantly earlier and increased mature hermaphroditic flower production than non-transgenic controls. In addition, ACS plants showed earlier fruit set and increased sequential fruit set on the main stem. These experiments confirmed the importance of endogenous ethylene production for hermaphrodite flower production and maturation, and also suggest a relationship between of ethylene levels and fruit set patterns on the main stem.

Introduction

The Cucurbitaceae family is comprised of species that exhibit a range of heritable patterns of sex expression (reviewed in Perl-Treves, 1999 and Roy and Saran, 1990). For example, cucumber (*Cucumis sativus*) is typically monoecious, in which a phase of male flowers precedes a phase of both male and female flowers, and melon is typically andromonoecious with male flowers followed by male and perfect flowers. Despite differentiation into unisexual flowers, in early stages of development, all initiated floral buds contain the four whorls typical of a hermaphrodite flower, sepals, petals, stamens, and carpels (Goffinet, 1990; Kater et al., 2001; Bai et al., 2004). The subsequent inhibition of carpel primordia results in staminate flower development, while arrest of stamen primordia results in pistillate flower development.

Sexual phenotypes observed in cucumber and melon are subject to the control of various developmental, genetic, hormonal, and environmental factors. Application of auxin, brassinosteroids, ethylene, or gibberellin can influence the sexual fate of developing flower buds (reviewed by Rudich, 1990 and Perl-Treves, 1999; Papadopoulou and Grumet, 2005). Ethylene appears to be the primary hormone affecting sex expression with other hormones acting via ethylene. Application of ethylene or ethylene-releasing compounds has been demonstrated to increase pistillate flower production in cucumber, muskmelon, and squash (Robinson et al., 1969; Rudich et al., 1969; Yin and Quinn, 1995) while treatment with inhibitors of ethylene biosynthesis or action confers an increase in maleness (Rudich, 1990; Atsmon and Tabbak, 1979). In addition, support for the feminizing effect of ethylene is provided by measurements of ethylene evolution, which shown 2-3 fold higher ethylene production from gynoecious compared to

monoecious and andromonoecious cucumber seedlings (Byers et al., 1972; Rudich et al., 1976). While an analogous difference in ethylene production among melon sexual phenotypes has not been observed, the application of hypobaric conditions to melon plants to reduce internal gas concentrations has been shown to reduce femaleness (Byers et al., 1972).

Ethylene is synthesized from S-adenosyl methionine (AdoMet) in a two step process which includes cyclization to 1-aminocyclopropane carboxylate (ACC) mediated by ACC synthase (ACS) followed by an oxidation step mediated by ACC oxidase (ACO) (Adams and Yang, 1979). Although *ACS* and *ACO* are both encoded by multigene families and, as such, are subject to regulation by various inducers, including auxin, ethylene, wounding, fruit ripening, pathogens, and stress, the level of ACS is generally regarded as rate-limiting in ethylene production (Zarembinski and Theologis, 1994; Bleeker and Kende, 2000).

Comparative studies of near-isogenic gynoecious and monoecious cucumber lines has led to the identification and molecular characterization of an additional *ACS* gene (CS-*ACS1G*) present only in gynoecious genotypes and completely co-segregating with the semi-dominant *F* (*Female*) locus (Trebitsh et al., 1997; Mibus and Tatlioglu, 2004). Gynoecious (*F*-) cucumber cultivars provide earlier, more uniform fruit set (Lower and Nienhuis, 1990). An equivalent to the *F* locus allowing stable inheritance of gynoecy has not been identified in melon (Kenigsbuch and Cohen, 1989).

To determine whether a similar feminizing effect could be achieved in melon via increased endogenous ethylene production, transgenic melons were produced haboring an *ACC synthase* from petunia driven by a constitutive (*35S*-CaMV) promoter

(Papadopoulou et al., 2005). Greenhouse studies of the *35S::ACS* melon plants showed phenotypes characteristic of increased endogenous ACS, which included increased ACC content and ethylene evolution from leaves, staminate buds, and hermaphroditic buds. These studies also demonstrated that increased endogenous ethylene production conferred earlier and increased perfect flowering to andromonoecious melon. In addition, greenhouse study showed earlier and increased numbers of hermaphrodite buds reaching anthesis. This observation was a novel finding and suggested a previous unknown role of ethylene in the maturation of carpel-bearing buds.

In this study, I sought to examine whether the sex-related phenotypes also would be observed under field conditions and to test for effects on fruit set and development. The results of this field evaluation further demonstrate the role of ethylene the development of mature hermaphrodite buds and offer insight into resource allocation associated with fruit set.

Materials and Methods

Northern analysis

Fresh young leaf tissue of *35S::ACS* lines 1, 3, and 4, non-transgenic segregants, and wild type andromonoecious Hale's Best Jumbo was collected from T_2 field-grown melon plants to evaluate ACS transgene expression. RNA was isolated from 100 mg fresh leaf tissue frozen in liquid nitrogen using Concert™ Plant RNA Reagent (Invitrogen, Carlsbad, California). RNA quantification and purity was determined by measuring the absorbance of the samples at 260 nm and by gel electrophoresis. Total RNA (20 μg) was electrophoresed on a 1.5% agarose formaldehyde gel and blotted (Sambrook and Russell,

2001). The membrane was hybridized overnight at 55°C with a digoxigenin-UTP labeled RNA probe of ACS (Roche Diagnositics, Germany). Stringency washes and detection via chemiluminescent visualization with CDP-Star was carried out following protocols developed for the DIG system (Roche Diagnostics, Germany).

Field experiments

Field trials were carried out in East Lansing, Michigan during the summer of 2002 using a randomized complete block design with three replications to evaluate flowering and fruit-set of *35S::ACS* lines 3 and 4, non-transgenic segregants, and Hales Best Jumbo. Plants were started in the greenhouse 19 days before hand transplanting, and transgenic plants were selected based on NPTII ELISA (Agia®, Elkhart, Indiana). Each replication contained five plants of each genotype (exception: NT segregants, two plants/rep). Rows of black plastic mulch were spaced three meters apart; plants were spaced at 0.9 meter intervals within rows. Within each row, genotypes were separated by two Hale's Best Jumbo plants. The field was surrounded by two border rows of non-transgenic Hale's Best Jumbo. Plot management included standard fertilizer application, weekly fungicide treatment, and irrigation of 2.5 cm per week.

Each node along the main stem of individual plants was examined to determine the node position of the first visible hermaphrodite bud and first mature hermaphrodite flower, the total number of hermaphrodite buds and mature hermaphrodite flowers, and the position and number of fruit set. For evaluation of ripening, all fruit within a plot were scored for maturity. Fruit were placed in five categories: overripe, ripe (full-slip), not ripe (tan and fully netted but still attached to vine), immature (green netted), and small (green not netted). All fruit was hand harvested ten weeks after field planting.

Fruit within a single plot were pooled by genotype and evaluated for total number, total weight, and size at maturity. Analysis of variance and least square difference comparisons were carried out using SAS software (SAS Institute, Cary, North Carolina).

Results

Northern analysis

Northern hybridization analysis of leaf tissue confirmed the expression of introduced *ACS* gene in field grown transgenic ACS plants (Figure 3.1). The lack of detection of transcript in the Hales wild type and non-transgenic segregant (NT) controls demonstrates the specificity of the probe for the transgene. The levels of expression varied between transgenic families with ACS4 and ACS3 showing higher expression. This northern data is consistent with previous segregation data suggesting two copies of the ACS transgene in ACS3 and ACS4 (Papadopoulou et al., 2005).

Sex expression and hermaphrodite bud development

Field grown transgenic ACS3 and ACS4 were compared to non-transformed Hale's Best Jumbo (WT) and azygous, non-transgenic segregants (AZ) with regard to sex expression patterns and hermaphrodite bud maturation. Hale's Best Jumbo plants are diploid, and *35S::ACS* transgenic lines and their azygous siblings are tetraploid (Papadopoulou, 2002). Tetraploidy is a common occurrence in melons regenerated from tissue culture (Ezura et al., 1992; Yadav et al., 1996) but does not affect sex expression patterns of melon in greenhouse studies (Papadopoulou et al., 2005).

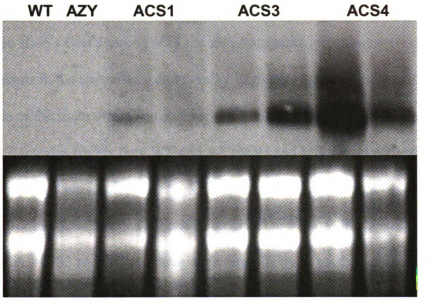


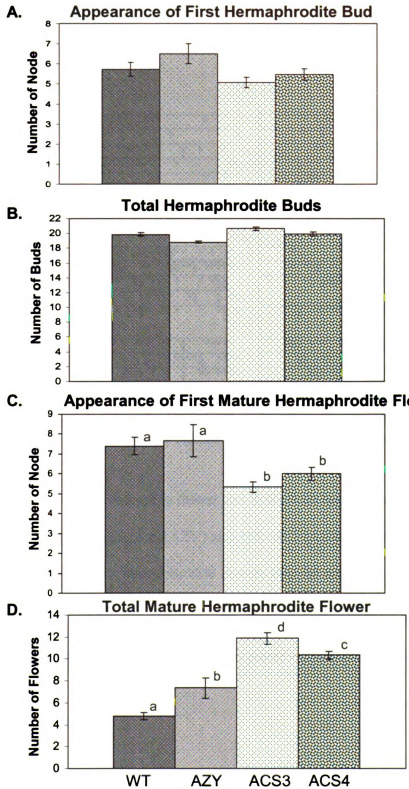
Figure 3.1. Northern hybridization of heterologous *ACS* gene expression in leaf tissue from field grown T_2 transgenic *35S::ACS* lines ACS1, ACS3, and ACS4. The top panel shows hybridization of total RNA from non-transgenic wild-type Hale's Best Jumbo plants (WT), azygous non-transgenic segregants (AZY), and transgenic lines (ACS1, ACS3, and ACS4) with DIG-labeled ACS probe. The bottom panel shows rRNA stained with ethidium bromide.

All genotypes produced hermaphrodite buds earlier in the field than in the greenhouse, node 5-6 in the field versus node 6-9 for 35S::ACS plants and node 10-11 for non-transgenic control plants in the greenhouse (Papadopoulou et al., 2005). While there were no differences in the time of appearance or number of hermaphroditic buds in the field, there was a significant difference between transgenic 35S plants and non-transgenic wild type Hale's Best Jumbo (WT) and non-transgenic tetraploid segregant (azygous, AZY) plants in the appearance and number of mature hermaphroditic flowers on the first 25 nodes of the main stem (Figure 3.2, 3.3). ACS plants produced mature hermaphroditic flowers 2-3 nodes earlier than WT and AZ plants and had higher percentage of the hermaphrodite buds reach maturity. On the non-transgenic plants, only about 30% of the hermaphroditic buds reached anthesis, compared to 55% on transgenic plants (Table 3.1). In addition, ACS3 and ACS4 plants were more likely to produce mature flowers on closely spaced nodes of the main stem than non-transgenic lines (Figure 3.3, Table 3.1).

Fruit development

The field grown melon plants also were observed for fruit set and ripening. Consistent with earlier appearance of mature hermaphrodite flowers on the main stem, ACS plants set fruit significantly earlier along the main stem with fruit appearing on ACS3 and ACS4 at approximately node 8 and 9, respectively, compared to nodes 16 and 21 in azygous and Hale's Best Jumbo controls (Figure 3.4A). In addition, total number of fruit set along the main stem was 2-4 fold higher in ACS plants than on non-transgenic controls (Figure 3.4B). Evaluation of the pattern of fruit on the main stem of ACS3 and ACS4 plants also demonstrated more closely spaced fruit, with 60-70% of the plants

Figure 3.2. Sex expression pattern along the main stem of wild type Hale's Best Best Jumbo (WT), azygous segregants (AZY), and transgenic T_2 *35S::ACS* (ACS3 and ACS4) field grown melon plants. (A) Node position of first hermaphrodite bud. (B) Total number of hermaphrodite buds on first 25 nodes. (C) Node position of the first hermaphrodite flower to reach anthesis. (D) Total number of hermaphrodite flowers that reached anthesis. Experiments were performed using a randomized complete block design with three replicates. (All data are means ± standard error; bars marked with different letters are significantly different, Least square difference, P=0.05.)


A.
Appearance of First Hermaphrodite Bud
Number of Node
B.
Total Hermaphrodite Buds
Number of Buds
C.
Appearance of First Mature Hermaphrodite Flower
Number of Node
a
a
b
b
D.
Total Mature Hermaphrodite Flower
Number of Flowers
a
b
d
c
WT
AZY
ACS3
ACS4

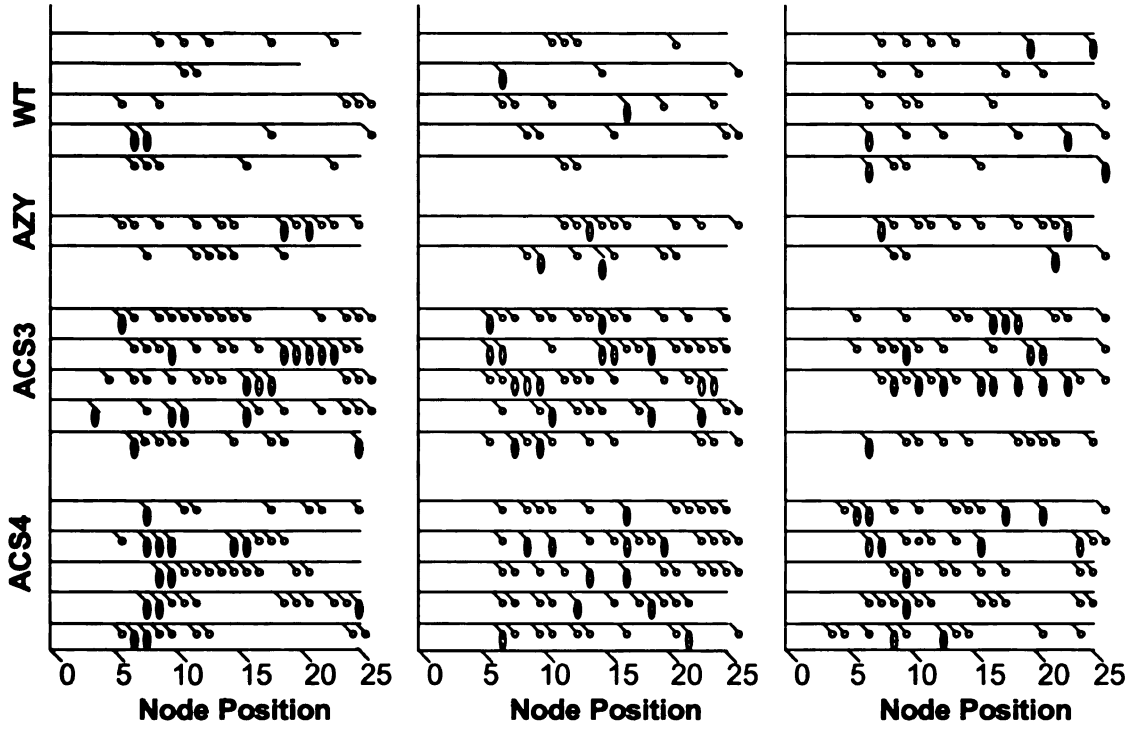


Figure 3.3. Pattern of mature hermaphrodite flower and fruit set on main stem of field grown wild type (WT), azygous segregants (AZY) and *35S::ACS* (ACS3 and ACS4) melon plants. Nodes bearing mature hermaphrodite flowers that did not develop into fruit are depicted by O and nodes bearing fruit by 0. Horizontal lines represent the main stem of individual plants. Each of the three sets of plants represents a replicated plot.

Table 3.1. Flowering, fruit set, and yield characteristics of field grown *35S::ACS* and control melon plants.

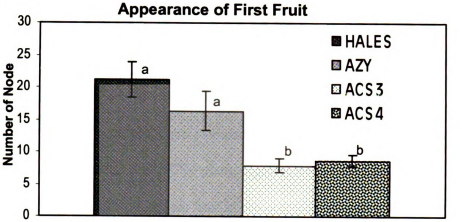
Characteristic (ploidy)	**WT** (2n)	**AZY** (4n)	**ACS3** (4n)	**ACS4** (4n)
% mature perfect flowers (main stem)	24.0±2.0 a	39.1±1.1 b	57.4±1.1 c	52.0±3.4 c
Instances of adjacent mature perfect flowers (main stem)	0.9± 0.3 a	3.2± 0.7 b	6.2± 0.6 c	5.7± 0.6 c
% mature perfect flowers setting fruit (main stem)	15.0±4.0 a	20.8± 8.1 a	27.8±1.8 a	22.22±1.6 a
% plants with 2 fruit within 4 nodes (main stem)	6.7±3.8 a	16.7±9.6 a	60.0±6.7 b	71.2±3.5 b
Average fruit size (kg)	0.91± 0.17 a	0.47±0.01 b	0.45±0.02 b	0.43±0.01 b
Total number of fruit set per plant	9.6±1.0 a	6.8±2.7 a	11.6±1.0 a	9.9±1.5 a

Data are mean of three replicate plots ±standard error.

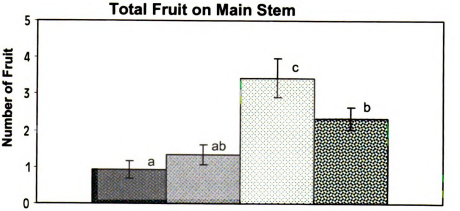
Values within a row followed by a different letter are significantly different by LSD, P=0.05.

Figure 3.4. Fruit set and ripening pattern of field grown wild type Hale's Best Jumbo (WT), azygous segregants (AZY), and T_2 *35S::ACS* melon plants (ACS3 and ACS4). (A) Node position of first fruit on the main stem. (B) Total fruit set on first 25 nodes of main stem. (C) Profile of fruit development. All data are means of three replicate plots ± standard error. Bars marked with different letters are significantly different, Least square difference, P=0.05. Ripening data were collected on the fruit within the plot (adjusted to per plant basis) at 8 ½ weeks after planting. Individual fruit were scored as: OR, over-ripe; Ripe, full-slip; Not Ripe, tan and fully netted but still attached to plant; Immature, green netted; or Small, green not netted.

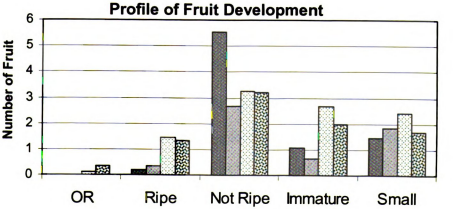
A.


Appearance of First Fruit
Number of Node
30
25
20
15
10
5
0
HALES
AZY
ACS3
ACS4
a
a
b
b

B.


Total Fruit on Main Stem
Number of Fruit
5
4
3
2
1
0
a
ab
c
b

C.


Profile of Fruit Development
Number of Fruit
6
5
4
3
2
1
0
OR
Ripe
Not Ripe
Immature
Small

setting fruit within four nodes versus 7-17% in non-transgenic controls (Figure 3.3, Table 3.1). In several cases, fruit set on three consecutive nodes and in one extreme case on five consecutive nodes (Figure 3.3, Figure 3.5). All lines showed similar proportions of mature hermaphrodite buds producing fruit on the main stem (Table 3.1).

The fruits within each plot were evaluated for stage of ripening at 8 ½ weeks post-transplanting. Fruit on ACS3 and ACS4 plants ripened sooner than on non-transgenic plants as depicted by no non-transgenic fruit in the over-ripe category and almost exclusively ACS fruit in the ripe category at the time of observation (Figure 3.4C). The profile also shows a more uniform distribution among stages of ripening of ACS3 and ACS4, while the majority of fruit from the non-transgenic plants cluster in the not-ripe category, suggesting different temporal patterns of fruit set (Figure 3.4C).

At harvest, all fruit from the plants were collected. Despite higher numbers of fruit on the main stem of ACS plants, the genotypes did not differ in the total number of fruit set per plant (Table 3.1), indicating a difference in fruit distribution on the plant, such that more fruit was set on lateral branches of non-transgenic than transgenic plants. Fruit from wild type Hale's Best Jumbo plants were larger than fruit from the azygous and transgenic plants (Table 3.1). The smaller fruit size from azygous and transgenic plants can be attributed to the effects of tetraploidy, as has previously been observed (Nugent, 1994a,b).

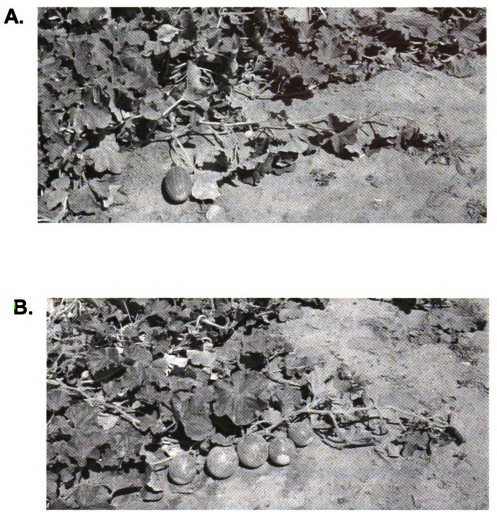


Figure 3.5. Consecutive node fruit set on main stem of field grown Hale's Best Jumbo wild type (WT) melon and *35S::ACS* transgenic melon. (A) Typical main stem fruit set of Hale's Best Jumbo WT. (B) Extreme fruit set on consecutive nodes of main stem in ACS3.

Discussion

Treatment with ethylene or ethylene-releasing compounds, such as ethephon, has a feminizing effect on a range of cucurbits, including *Cucumis melo* (Robinson et al., 1969; Rudich, 1969; Owens et al., 1980; Sadhu and Das, 1978), and so it was predicted that increased endogenous ethylene would similarly enhance femaleness, potentially allowing for earlier hermaphrodite flower appearance and fruit set, and a shorter growing season. Our earlier greenhouse studies with *35S::ACS* melon plants verified that the ACS transgene conferred increased endogenous ethylene production and earlier appearance of the first hermaphroditic bud on the main stem (Papadopoulou et al., 2005). In addition to an effect on sex determination, the transgenic *35S::ACS* plants showed an increase in the earliness and proportion of hermaphrodite buds that matured to anthesis suggesting an additional role of ethylene in carpel-bearing bud maturation.

In the field, *35S::ACS* melon plants produced mature hermaphrodite flowers sooner and a higher percentage of hermaphrodite buds reached anthesis on the main stem. The increase and earliness of mature hermaphrodite buds supports greenhouse results indicating that ethylene is needed for hermaphrodite bud to reach anthesis development, and rules out the possibly that failure of buds to mature in the greenhouse was an artifact of the greenhouse environment. A possible role of ethylene in carpel development is not unprecedented. In orchid, ethylene biosynthesis stimulated by pollination is critical for subsequent ovary maturation (Bui and O'Neill, 1998; Zhang and O'Neill, 1993), and in tobacco, down-regulation of a pistil-specific *ACC oxidase* gene resulted in the arrest of ovule development that could be reversed with exogenous ethylene (DeMartinis and

Mariani, 1999). Whether ethylene is needed for ovule development or other aspects of hermaphrodite bud maturation in melon remains to be determined.

The *35S::ACS* melon plants also showed earlier and greater fruit set on the main stem and exhibited fruit set on closely spaced nodes. While earlier appearance of mature hermaphroditic flowers is a prerequisite for earlier fruit set, the plants must also be developmentally ready to bear fruit. They must, therefore, have the photosynthetic capacity to establish and maintain the sink a developing fruit creates. The earlier fruit set on the main stem of ACS plants seen in the field trial demonstrated that the transgenic plants had the resource capacity to set fruit earlier.

The ACS melon plants showed a strikingly different pattern of fruit set on the main stem. Multiple ACS plants set four or more fruit on the main stem. In one extreme case, five fruit were set on consecutive nodes, and in another case, eight fruit were on the main stem with no more than two nodes separating any of the fruit. This is a sharp contrast to what was observed on the main stems of non-transgenic plants where typically only one fruit was set and in only two instances were two fruit set within four nodes. Previous work in the area of plant reproductive ecology has suggested that in some monoecious plant species, sex expression is a reflection of resource availability, such that a developing fruit results in a reduction in pistillate flower production while the lack of fruit results in increased pistillate flower production (Stephenson, 1981; Schapendonk and Brouwer, 1984; Krupnick and Weis, 1998; Krupnick et al., 1999). A developing fruit in squash and gourd was shown to lead to reduced subsequent pistillate flower production and increased fruit abortions (Stephenson et al., 1988). The relationship of developing fruits to endogenous ethylene levels has been studied in monoecious *Cucurbita texana*

(Krupnick et al., 1999). These studies show that internal levels of ethylene were highest adjacent to tips of branches bearing fruit nearing maturity and lowest when branches carried two or more fruit, suggesting that the number of developing fruit and their stages of development regulate endogenous ethylene levels (Krupnick et al., 1999). If an analogous relationship between ethylene production and fruit exists in *Cucumis melo*, then the increased production of endogenous ethylene in the transgenic lines may interfere with the signaling which would normally result in lower ethylene and decreased pistillate flowering on branches carrying fruit.

The fruit on ACS plants also showed a different trend of maturation as fruit ripened sooner on ACS3 and ACS4 plants than on non-transgenic controls. Confounding factors may play a role in the earlier ripening observed in the fruit on ACS plants. The earlier fruit set on transgenic plants relative to non-transgenic would confer earlier ripening regardless of any effect on the time required for maturation. Secondly, in climacteric fruit, a concomitant burst in respiration and ethylene initiates the ripening of mature fruit (Giovannoni, 2004); thus, the increased levels of endogenous ethylene in ACS plants may hasten the climacteric and speed up the ripening process.

In summary, evaluation in a field setting made possible further phenotypic analysis of transgenic melon over-expressing an ACC synthase gene. Field grown *35S::ACS* melon plants produced earlier and greater numbers of mature hermaphrodite flowers and showed increased fruit set on the main stem. These experiments demonstrate the importance of endogenous ethylene production for hermaphrodite flower development in andromonoecious melon and suggest a role of ethylene in signaling associated with fruit set patterns. *35S::ACS* melon plants also exhibited earlier fruit set, a

characteristic which may be horticulturally advantageous in areas, such as Michigan, with a short growing season. Engineering of melon with *ACS* gene constructs conferring localization of *ACS* expression to regions of the developing floral organ at the times critical for sex determination and maturation may help to achieve the benefits of increased earlier fruit set without potentially agronomically underdesirable effects of constitutively elevated ethylene production.

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Chapter 4

Risk assessment of transgenic plants with altered signaling pathways: A case study of modified ethylene

Abstract

The past decade has seen a tremendous increase in the types of genes and variety of species used for crop improvement via genetic engineering. Many current efforts include transformation with genes that encode transcription factors, members of signal transduction networks, or key enzymes in metabolic pathways. Since these genes can affect the expression of additional (often many) genes and pathways, their introduction may have secondary effects on plant phenotypes, which may be important to consider from an environmental risk assessment standpoint. The goal of this case study is to focus on the effects associated with genetic engineering to modify traits regulated by ethylene. Ethylene production and perception have been modified to alter plant physiological characteristics including ripening, senescence, and flowering. These alterations may in turn result in secondary effects such as altered stress and disease response, as well as changes in floral characteristics and growth habit. In addition, pleiotropic effects may alter the likelihood of gene flow into neighboring non-transgenic populations and/or alter reproductive fitness of emerging hybrids in the event of gene flow. These possibilities will be discussed within the context of modification of the ethylene signaling pathway.

Introduction

With 67.7 million hectare of biotech crops grown worldwide in 2003, the commercialization and widespread cultivation of genetically engineered crops is a reality (ISAAA; http://www.isaaa.org). The majority of transgenics in cultivation are engineered for two traits, herbicide resistance and insect resistance, with a small amount of acreage for virus resistant plants. In this initial wave of transgenic crops, the genes transformed into the plant encode a protein product which itself confers the trait of interest. For example, the widely cultivated Roundup Ready™ soybean harbors a single transgene isolated from a soil bacterium, which renders the plant resistant to the biodegradable glyphosate herbicide Roundup® (Padgette et al., 1995). Another example is *Bt*-corn, which is engineered to express a *cry* gene that encodes a protein toxic to certain species of insects, therefore, conferring insect resistance to the plant (Peferoen, 1997). In these cases, even though the protein is present throughout the plant, it does not play a role in the plant's own growth and development. Changes in the plant phenotype other than the direct effects of the introduced trait are likely to be a result of a gene position effect and would be selected against in the early stages of evaluations of the transgenic plant material.

A much broader range of traits and variety of genes are under current research and development in plant biotechnology laboratories, including genes for increased stress tolerance, enhanced disease resistance, modified metabolism, and altered growth and development (Wolfenberger and Grumet, 2003). Such modifications could result in the ability to cultivate crops in regions previously not possible, reduce chemical input, or tailor the content of the plants for nutritional or industrial needs. Unlike the genes used

to engineer traits of the initial wave of transgenics, these newer traits often utilize genes which affect regulatory, metabolic, or signaling pathways of the plant. For example, engineering increased tolerance to dehydration stress by overexpression of the *C-repeat/dehydration-responsive element binding factor* (*At-CBF*, *-DREB*), a transcription factor, stimulates the expression of a regulon of genes involved in the acclimation to environmental stresses related to dehydration (ie freezing, drought, salinity) (Jaglo-Ottosen et al., 1998; Thomashow, 1999; Fowler and Thomashow, 2002). Another example is the modification of the signaling pathway of the plant hormone ethylene, through the expression of a mutant ethylene receptor or antisense biosynthetic gene in an effort to delay ripening or flower senescence (Shaw et al., 2002).

In these cases, the introduced transgene, as a component of a transcription or signaling pathway, will affect the activities of many additional gene products and may, therefore, have a greater probably for unanticipated secondary effects on the engineered plant (Wolfenbarger and Grumet, 2003). For example, a detailed microarray analysis of gene expression in response to constitutive expression of *At-CBF* in Arabidopsis showed that approximately 300 genes of the 8000 evaluated were affected (Fowler and Thomashow, 2002). Such changes in gene expression could in turn cause ecologically relevant changes in phenotype. As conscientious scientists, it is important to continually assess the risk associated with the introduction of new plant varieties, in an effort to determine if they pose new or additional risks, which might affect their impact on the environment. Relevant changes include those which might affect gene flow as well as those affecting the fitness of the resulting hybrid plant in the event of gene escape (Snow 2002b; Hancock, 2003).

In terms of gene flow, it can be generally assumed that if crops are grown within close proximity to wild relatives, hybridization events will occur (Ellstrand, 2001; Hancock, 2003). This concept has been a reality in conventional agriculture and is, therefore, not unique to genetically modified crop plants. The ability of most cultivated crops to mate with wild relatives is well documented (Snow, 2002a; Ellstrand et al., 1999). Many factors influence gene flow, such as mating system, mode of pollination, mode of dispersal of seed or vegetative propagules, and environmental characteristics; therefore, phenotypic changes to multiple plant structures need to be carefully evaluated when characterizing gene flow (Snow, 2002b; Messeguar, 2003). Since secondary effects resulting from the introduced transgene may cause one or more of these factors to deviate from what is observed in their non-transgenic source, careful evaluation of attributes affecting gene flow should be considered in the establishment of confinement criteria for field grown transgenic plants. For example, dispersal of pollen alone can be affected by many components, including number of flowers per plant, amount of pollen per flower, number of anthers per flower, and longevity of pollen, as well as changes in floral structure that alter the attractiveness and/or accessibility to flowers by pollinators (some which may not otherwise visit). Moreover, since pollen is not the only mechanism of gene flow, phenotypic alterations affecting the movement of seed and vegetative propagules are also relevant from a risk assessment perspective and may include changes in seed dormancy, timing of ripening, seed shedding or shattering, and dispersal by animals and environmental elements (Snow, 2002b).

In addition to understanding how secondary effects impact gene flow, knowledge regarding how the transgene may impact fitness of the recipient organism and the

ecosystem is crucial to risk assessment. Two of the most important questions to address when considering risk assessment of transgenic crops are: 1. Will the transgene contribute to the evolution of increased invasiveness in natural populations?; and 2. How will the transgene impact non-target species? (Snow and Palma, 1997; Hails, 2000; Ellstrand, 2001). If the transgene has the potential to increase the invasiveness of a natural population, high levels of successful introgression could result in reduced natural genetic diversity and even contribute to extinction (Wolfenbarger and Phifer, 2000). Whether or not the introgression of the transgene poses an ecological risk depends on if it confers a selective fitness advantage (Hails, 2000). The fitness impact may be detrimental, neutral, or positive and may depend on the environment in which the crop is introduced (Hancock, 2003). For example, the introgression of an herbicide resistance gene into a wild population is likely to confer a positive fitness advantage only in areas where that herbicide is used for weed control, but in areas where the herbicide is not routinely applied, the gene is likely to be neutral and, therefore, have no impact on fitness. When the transgene alters the environmental tolerance or patterns of plant growth and development (which is often the goal of modifying a signaling pathway), significant shifts in adaptability could result, having major impacts on plant fitness (Hancock, 2003). Assessments of the risks associated with transgenes may become even more complex if the trait of interest confers a selective advantage while the secondary effects have a detrimental fitness effect or vise versa.

For this discussion of genetically engineered plants, the concept of non-target effects encompasses a range of potential ecological impacts that can be a direct or indirect result of the transgenic plant's introduction. Possible effects include, but are not

limited to: toxicity to non-target organisms, shift in the food supplies available to organisms, and impact on natural habitats (Poppy, 2000; Dale et al., 2002). It is important to note that these risks can be quite variable, difficult to predict, and must take into account the locale of plant introduction on a case by case basis (Snow and Palma, 1997). For example, the direct effects of ingestion of pollen from *Bt* plants by the non-target monarch butterfly in the United States has been intensely studied with field and laboratory evaluations giving contrasting results (Barton and Dracup, 2000; Poppy, 2000). These studies have demonstrated the importance of conducting realistic risk assessment analysis to determine the likelihood of non-target species exposure and susceptibility (Poppy, 2000).

How can we begin to assess these secondary effects that may influence gene flow, invasiveness, or non-target impacts?

As research in plant molecular biology progresses at a rapid rate, information regarding the complexity of signaling pathways is becoming available that may help to anticipate how genetically engineered modification will affect the plant. One example is the research that has expanded the understanding of the molecular biology of the plant hormone ethylene. Ethylene has a range of diverse roles throughout plant growth and development. These roles include but are not limited to seed germination, root and shoot growth, xylem differentiation, flower development, senescence, stress response, and fruit ripening (as reviewed by: Arshad and Frankenberger, 2002; Abeles et al., 1992; Mattoo and Suttle, 1991). Because of its diverse array of functions there has been interest in the manipulation of ethylene production or action in order to modify plant phenotypes.

However, altering ethylene signaling for a characteristic such as increased flower longevity may in turn result in secondary changes in seed dormancy, stress response, or disease resistance. Therefore, the goal of this paper is to conduct a case study focusing on ethylene as a signaling molecule to examine the complexity of its signal transduction and interconnectedness with other pathways within the context of pleiotropic effects that may have ecological significance. Ethylene is a good candidate for this case study as there is an extensive body of literature examining the effects of ethylene through mutant or transgenic approaches. Pleiotropic effects on plant growth and development, which have been observed as secondary to the initial desired goal of genetic modification, also will be reviewed. In addition, how these secondary effects may be relevant to risk assessment of plants engineered to modify signaling pathways will be discussed.

Why modify ethylene?

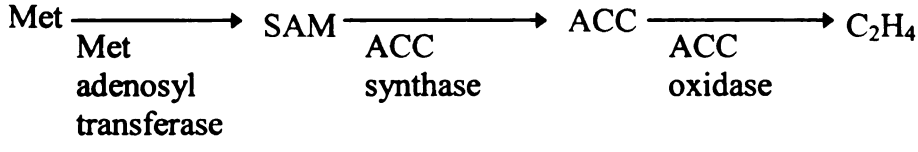
Modification of ethylene to manipulate plant growth and development is not a new concept. Ethylene-releasing compounds as well as inhibitors of ethylene synthesis and perception are tools that have been used broadly in agriculture to control multiple facets of crop production. Ethephon (2-chloroethylphosphonic acid) is an ethylene-releasing compound that has a range of applications including the treatment of wheat and barley to retard growth and prevent lodging, initiate uniform flowering in pineapple, induce female sex expression in cucurbits, increase bud hardiness and bloom delay in sweet cherry and plum, reduce curing time in tobacco, promote fruit abscission in cotton, and promotedefoliation in tree species (reviewed in: Arshad and Frankenberger, 2002; Abeles et al., 1992). Alternatively, ethylene production can be inhibited through

treatment with aminoethyloxyvinylglycine (AVG) or aminooxyacetic acid (AOA) (Abeles et al., 1992), and inhibition of ethylene response at the level of perception is also possible through chemical treatment with compounds such as silver thiosulfate, 2,5-norbornidiene, and 1-methylcyclopropene (1-MCP) (Abeles et al., 1992; Sisler and Serek, 1997). Applications for these compounds include the delay of fruit ripening, the prevention of flower senescence or fruit abscission, and increasing the shelf life of leafy vegetables (Arshad and Frankenberger, 2002.).

Thus, there are many reasons for modifying ethylene, and it is not difficult to see why the possibility of genetically engineering plants with altered ethylene would be desirable.

What are the genetic components of ethylene biosynthesis and signaling?

Much effort has been devoted to elucidation of the genetics and molecular mechanisms underlying ethylene biosynthesis and signaling and has led to the cloning of key gene components in multiple species (Kende, 1993; Zarembinski and Theologis, 1994). Methionine is the amino acid precursor to ethylene. Methionine is converted to S-adenosyl-L-methionine (S-AdoMet), mediated by methionine adenosyltransferase (Adams and Yang, 1979; Kende, 1993; Bleeker and Kende, 2000).



The synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) from S-Ado-Met is catalyzed by ACC synthase (ACS) (Adams and Yang, 1979; Kende, 1993; Bleeker and Kende, 2000). 5'-Methyl-thioadenosine is also produced, which is used to synthesize a

new methionine (Kende, 1993). The reaction of ACC with oxygen, catalyzed by ACC oxidase (ACO), produces ethylene. ACS is generally regarded to be rate-limiting in ethylene biosynthesis and is encoded by a multigene family in which genes are differentially regulated by developmental, environmental, and hormonal inducers (Kende, 1993; Bleecker and Kende, 2000; Zarembinski and Theologis, 1994; Johnson and Ecker, 1998).

The path of ethylene perception and signaling also has been well characterized, primarily in Arabidopsis, through the evaluation of mutants displaying either an ethylene insensitive or a constitutive response phenotype (Wang et al., 2002). A family of receptors (in Arabidopsis: ETR1, ETR2, ERS1, ERS2, and EIN4) homologous to bacterial two-component histidine kinases are responsible for binding ethylene (Johnson and Ecker, 1998; Bleeker and Kende, 2000). Mutations that eliminate ethylene binding of at least one receptor result in a dominant, ethylene-insensitive phenotype, indicating that unbound receptors function to negatively regulate downstream ethylene responses and are, therefore, inactivated by binding (Zarembinski and Theologis, 1994; Johnson and Ecker, 1998; Bleeker and Kende, 2000). The signaling component directly downstream of the receptors is CTR1, which was isolated as a loss-of-function mutant displaying a constitutive ethylene response and is a member of the Raf family Ser/Thr protein kinases. Since loss-of-function mutations in CTR1 result in the plant responding as if it were exposed to ethylene even in ethylene's absence, CTR1 functions as a negative regulator of downstream signaling events (Bleeker and Kende, 2000; Wang et al., 2002). Further downstream is EIN2, which upon knock out, results in a loss of ethylene responsiveness, suggesting that it functions as a positive regulator of ethylene

signaling (Johnson and Ecker, 1998; Wang et al., 2002). Transcription factors involved in changes in the expression of ethylene response genes lie downstream of EIN2 and include the EIN3 family and ERF1 (Wang et al., 2002; Bleeker and Kende, 2000).

How are these genes used to modify ethylene?

The availability of cloned genes involved in endogenous ethylene production and signaling have created opportunities for evaluating the potential of genetic engineering to modify ethylene-controlled processes in plants. Although the primary goals of modifying endogenous ethylene have been for post-harvest characteristics such as delayed ripening and flower senescence in an effort to extend shelf-life and reduce spoilage (reviewed in: Yang and Oetiker, 1998; Bleeker and Kende, 2000), increasing ethylene biosynthesis in order to prevent lodging in barley, increase latex flow in rubber tress, increase pistillate flower production in melon, and promote leaf abscission prior to tree harvest have been suggested as possible goals as well (Hedden and Phillips, 2000; Papadopoulou et al., 2005).

Transgenic plants evaluated for characteristics involving reduced ethylene production or activity have included apple, melon, tomato, broccoli, carnation, and petunia (reviewed by Stearns and Glick, 2003). Approaches to attain this end have included down-regulation of genes encoding ethylene biosynthetic enzymes ACS and ACO (via antisense or cosuppression); introduction of genes encoding enzymes to reduce ethylene production, ACC degrading enzyme ACC deaminase, SAM inactivating SAM hydrolase; and introduction of mutant ethylene receptors (Yang and Oetiker, 1998; Dandekar et al., 2002; Stearns and Glick, 2003). For example, ethylene induced ripening

and senescence was inhibited in fruits of tomato plants engineered with an antisense copy of an endogenous ACS gene which reduced fruit ethylene levels by 95.5%; however, treatment with exogenous ethylene could reverse the inhibition and lead to normal ripening (Oeller et al., 1991). Similarly, but through the heterologous expression of the *Arabidopsis etr1-1* mutant ethylene receptor, senescence of carnations flowers was delayed three-fold relative to controls (Bovy et al., 1999). A review of many of the plants and genes used to transgenically modify ethylene biosynthesis or perception has recently been compiled (Stearns and Glick, 2003).

Four plants engineered for modified ethylene have already been deregulated, and therefore approved for environmental release. A carnation has been approved for environmental release in Australia and the European Union, which has been transformed for reduced ethylene content to provide a longer life to cut flowers through the introduction of a truncated *ACS* gene (htpp://www.agbios.com). Similarly, a tomato engineered for delayed ripening by introducing a truncated *ACS* gene has been approved in the United States. Two other tomato lines genetically engineered for delayed ripening have been approved for release in the United States. In these cases, ethylene biosynthesis is thwarted through the introduction of a SAM hydrolase or ACC deaminase gene (htpp://www.agbios.com).

While these plant materials have undergone extensive field testing and have been shown to be safe for environmental release, one of the ways to maintain safe environmental release of these and new plant introductions is to have as much knowledge about the introduced transgene as possible. As current research continually demonstrates the complexity of ethylene's role as a signaling molecule, highlighting some of the recent

findings on ethylene's role in plant growth and development may help to stimulate thoughts regarding what to look for in terms of secondary effects when assessing the risk of genetically engineered plants.

What phenotypic effects are associated with modified ethylene signaling?

Plant growth and development

Transgenic and mutant lines have implicated ethylene to play a role in virtually all stages of plant growth and development, e.g. germination, vegetative growth, reproduction development, and ripening and senescence, (Smalle and Van Der Straeten, 1997). Evidence for a role of endogenous ethylene perception in germination has been established in evaluations of ethylene insensitive *etr1* mutant Arabidopsis and transgenic *etr1* petunia showing decreased seed germination (Bleeker et al., 1988; Clevenger et al., 2004). However, there appears to be considerable variation in the requirement of ethylene for germination among species (Smalle and Van Der Straeten, 1997).

The role of ethylene on further plant growth and establishment has also been studied. In fact, the characteristic 'triple response' phenotype of dark grown seedlings under continuous exposure to ethylene, which includes thick and short roots and hypocotyls, and an exaggerated apical hook, provided a key bioassay for screening mutant populations in the initial efforts to identify primary genes in ethylene signaling (Stepanova and Ecker, 2000). While results have been mixed, several studies also suggest that ethylene affects leaf expansion by suppressing cell enlargement (Kieber et al., 1993; Hua et al., 1995; Grbic and Bleeker, 1995; Tholen et al., 2004). Characterization of growth of ethylene insensitive Arabidopsis mutants based on rosette

leaf area has shown an increase in growth relative to wild type plants (Grbic and Bleecker, 1995) while the constitutive ethylene response mutant *ctr1* exhibits reduction in size (Kieber et al., 1993). However, further study on growth in Arabidopsis, tobacco, and petunia showed no difference in leaf area between ethylene-sensitive and –insensitive plants (Tholen et al., 2004).

The effects of ethylene on root formation have also been studied. The development of adventitious roots was greatly inhibited in vegetative cuttings of transgenic ethylene-insensitive tomato, petunia, and melon (Clark et al., 1999; Papadopoulou et al., 2002). Secondary effects associated with adventitious rooting may become particularly relevant in plant species which depend on vegetative propagation. In addition, reduced root hair length in tomato and Arabidopsis and impairment in root penetration in less than optimal soil medium, such as sand, in tomato was reported for ethylene-insensitive genotypes (Clark et al., 1999). The presence of longer and more root hairs in the Arabidopsis *ctr1* provide further support for ethylene as a regulator of root hair development (Tanimoto et al., 1995). Such effects on rooting may affect the plant's ability to become established and thrive in certain soils or environmental conditions.

A function of ethylene in reproductive development has been demonstrated in several species. Ethylene is considered the primary hormone influencing female floral development in cucumber, melon, and squash, with cucumber being the most studied. The strong correlation between ethylene and female flower development in the moneocious species cucumber is now supported with molecular evidence including the cosegretion of an ACS gene with a locus used for breeding gynoecious (female) cucumber as well as correlation of ACS mRNA with female flowering (Trebitsh et al.,

1997; Kamachi et al., 1997; Kamachi et al., 2000; Yamasaki et al., 2003; Mibus and Tatlioglu, 2004). In addition, increasing endogenous ethylene production via heterologous expression of an *ACS* transgene in andromonoecious melon conferred earlier and increased production of hermaphrodite flowers (Papadopoulou et al., 2005) while ethylene insensitivity as a result of heterologous expression of ethylene receptor mutant, *etr1-1*, modified sex expression, with effects dependent on the location of gene expression (Chapter 2). In another study, transgenic tobacco expressing an antisense ACO under the control of a pistil-specific promoter demonstrated the importance of ethylene for ovule development in tobacco. Transgenic plants with reduced ethylene production displayed female sterility as a result of arrested ovule development; however, the phenotype could be overcome with treatment with ethylene (DeMartinis and Mariani, 1999). In addition, studies suggest that ethylene production that is triggered upon pollination in orchid flowers initiates signaling events important for ovary development, and several ACS genes that respond to pollination signals in the orchid flower have been identified (Bui and O'Neill, 1998).

Based on these studies, reduced ethylene production or sensitivity is likely to be detrimental to a plant's reproductive capacity, and from an ecological risk assessment perspective would not result in increased fitness. However, the observation of earlier and increased numbers of hermaphrodite buds reaching anthesis in melon overexpressing *ACS* (Papadopoulou et al., 2005), may be relevant to ecological risk assessment. Evaluation of these melon plants in a field setting also showed earlier fruit maturity and modified patterns of fruit set on the main stem. Such effects may warrant close

evaluation as a reproductive characteristic is being altered in a way that might confer a fitness advantage in the event of gene flow.

As might be assumed by the types of traits in the deregulated transgenic plants described above, a primary function of horticultural importance is the role of ethylene in senescence and ripening. Floral and leaf senescence is a complex biochemical process of catabolism, mobilization of metabolites, and physical disintegration leading to programmed cell death that is under the control of many hormonal factors and developmental cues (Abeles et al., 1992, Bovy et al., 1999). An increase in sensitivity and autocatalytic production of ethylene in an aging tissue typically precedes the onset of senescence, and ethylene is regarded as the strongest promoter of senescence among the plant hormones (Bovy et al., 1999). Ripening is characterized by changes in biochemical and physical attributes that take place at fruit maturity including changes in color, texture, flavor, aroma, and nutritional content in preparation to attract seed-dispersing organisms (Gray et al., 1994; Adams-Philips et al., 2004; Giovannoni, 2004). In climacteric fruit such as tomato, banana, apple, and stone fruit, ripening is initiated by a sharp increase in respiration that is accompanied by a burst in ethylene production (Giovannoni, 2004). The role of ethylene in fruit ripening is exemplified by isolation of the *Never ripe* (*Nr*) locus of the tomato mutant, which was characterized by a non-ripening phenotype. Cloning of the *NR* gene established its identity as an ETR1 ethylene receptor homologue impaired in the ability to bind ethylene (Wilkinson et al., 1995). Since positive feedback regulation of ethylene biosynthesis is an important component of senescence and ripening programs, controlling production and perception of ethylene has been an area of economic and agricultural interest.

Plant-microbe interactions

Symbiotic associations between the plant species, such as legumes, clover and alfalfa, and soil borne nitrogen-fixing microbes enable the plant to access nitrogen in a useable form (Crawford et al., 2000). Root nodules are the sites of plant-rhizobia symbiosis, and studies suggest that ethylene is involved in nodule formation (Wang et al., 2002). Studies of hyper-nodulation mutant, *sickle* (*skl*), plants of *Medicago truncatula* (alfalfa) show that *skl* plants also have ethylene insensitive phenotypes, including delayed senescence, decreased abscission, and insensitivity of seedling to ACC treatment (Penmetsa and Cook, 1997). Nodule formation has also been studied in legume, *Lotus japonicus*, engineered to express a mutant ethylene receptor conferring ethylene insensitivity (Nukui et al., 2004). Upon inoculation with *Mesorhizobium loti*, transgenic plants demonstrated enhanced formation of infection threads and nodule promordia (Nukui et al., 2004). These studies suggest that ethylene is involved in the regulation of rhizobial infection to balance symbiosis and control the number of nodulation events (Penmetsa and Cook, 1997; Nukui et al., 2004). The implications of effects on these symbiotic interactions may be of interest from a risk assessment perspective on multiple levels. Enhancement of interactions, which allows the plant to develop more vigorously, may contribute to increased fitness and invasiveness in events of gene flow. In addition, nontarget effects on microbe populations may result from changes in plant-rhizobia associations.

Disease response

While accelerated ethylene production is a typical response to a pathogen attack by fungi, bacteria, viruses, and nematodes (Arshad and Frankenberger, 2002), its role in disease signaling and, subsequent, susceptibility or resistance has been more difficult to elucidate (Hoffman et al., 1999; Thomma et al., 1999; Diaz et al., 2002). Research to decipher the role of ethylene in disease response has incorporated the use of plants with altered ethylene sensitivity and, depending on the type of pathogen and plant species, reduced sensitivity has resulted in increased susceptibility or increased resistance (Wang et al., 2002). For example, study of mutant soybean lines with reduced ethylene sensitivity showed that the mutants had reduced or no difference in disease severity in response to virulent bacterial *Psuedomonas syringae* and fungal *Phytophthora sojae* compared to wild type lines (Hoffman et al., 1999). In contrast, observation of more severe symptoms in response to fungal *Septoria glycines* and *Rhizoctonia solani* seemed to suggest that ethylene-insensitivity increased disease susceptibility to other pathogens in soybean (Hoffman et al., 1999).

Results showing contrasting effects of ethylene involvement in disease response depending on the pathogen type have also been reported in studies of ethylene-insensitive *ein2-1* mutants of Arabidopsis. Mutants showed enhanced susceptibility to strains of the gray mold fungus *Botrytis cinerea*; however, the mutation caused no change in the plant's response to other fungal pathogens, *Peronospora parasitica* or *Alternaria brassicola* (Thomma et al., 1999). In yet another study, evaluation of ethylene-insensitive transgenic *etr1* tobacco and mutant *ein2-1* Arabidopsis demonstrated increased disease susceptibility to soilborne pathogens, including several *Pythium* strains, which had been

previously isolated from diseased ethylene-insensitive plants grown in nonautoclaved potting soil (Geraats et al., 2002). Further investigation of the altered disease resistance of ethylene-insensitive tobacco and Arabidopsis included a range of root and leaf pathogens and concluded that the role of ethylene signaling in disease development is dependent on the type of host-pathogen interaction rather than site of infection or pathogen species (Geraats et al., 2003).

Despite a lack of clarity in the role of ethylene in response to infection, studies have demonstrated ethylene's function as an effector molecule in the signaling process of disease response and resistance. For example, study of mutant *ein2-1* Arabidopsis (Thomma et al., 1999; Penninckx et al., 1996) and transgenic *etr1* tobacco (Knoester et al., 1998) have demonstrated that these ethylene insensitive lines lack pathogen-inducible expression of a subset of PR (pathogen-related) genes, indicating dependence on ethylene for some host responses. In contrast to studies of ethylene insensitivity, Ciardi et al. (2001) evaluated antisense *LeETR4* tomato plants, which exhibited a phenotype of increased sensitivity to ethylene. Upon infection with *Xanthomonas campestris*, transgenic plants gave a more rapid disease response and exhibited accelerated necrosis; however, pathogen levels decreased more quickly and to a lower level in transgenic plants (Ciardi et al., 2001). In addition, transgenic plants showed stronger and more rapid increases in ethylene synthesis and PR gene expression following inoculation, providing support for ethylene's role in regulating the defense response to *X. campestris* in tomato.

Clearly, the function of ethylene in plant defense against disease is complex, and studies suggest that manipulating ethylene production or perception may increase tolerance to some diseases while increasing susceptibility to others. From a risk

assessment point of view, an evaluation of how an engineered trait modifying ethylene may affect plant fitness, with regard to disease resistance, will likely be variable, and the effect might be detrimental, positive, or neutral. Secondary effects on disease resistance will be dependent on the pathogen pressures on a specific species in specific locations and should, therefore, be assessed on a case by case basis. For example, hypothetically, if a transgenic plant having a secondary effect of increased tolerance to *Phytophora* is introduced into a region where disease pressure by *Phytophora* controls populations of related species of the introduced plant, hybridization between the two species may result in a fitness advantage and lead to increased invasiveness in the natural populations. Conversely, if an engineered plant that has the secondary effect of increased susceptibility to *Phytophora* is introduced into the same region, hybrids may have reduced fitness and be, subsequently, rogued out of natural populations, as the disease pressure would inhibit their establishment.

Abiotic stress

There are many examples of increased ethylene production following an array of abiotic disturbances including temperature extremes, drought, ozone, hypoxia, wounding, and crowding (Abeles et al., 1992). As has been shown in response to biotic stress, understanding of the function of such increases is not complete. In many cases, the stimulation of ethylene biosynthesis in response to environmental stresses is reported to involve reactive oxygen species (ROS), which act as preliminary signaling molecules (Wang et al., 2002). In addition, studies to evaluate the mechanisms underlying plant responses to abiotic stressors suggest similarities to pathogen response pathways.

In a study of heat-induced oxidative stress, the Arabidopsis ethylene-insensitive *etr1* mutant showed increased damage and reduced survival following heat treatment, while pretreatment of wild type plants with ACC reduced damage and enhanced survival (Larkindale and Knight, 2002). This study provides support for a role of ethylene in the protection against or repair of heat-induced oxidation stress, in Arabidopsis. However, somewhat conflicting results were reported in studies of transgenic tobacco and potato with suppressed expression of a ozone(O_3)-inducible *ACS* (Nakajima et al. 2002; Sinn et al., 2004). Transgenic lines showed increased ozone tolerance, and the extent of O_3-induced damage was positively correlated with the level of O_3-induced ethylene production, suggesting that ethylene increases sensitivity to ozone.

The role of ethylene in the acclimation of wetland and flood-tolerant species to the oxygen-deprived status of submergence has been well studied. As observed in species, such as maize and rice, ethylene production promotes the formation of aerenchyma in root tissue as well as adventitious roots, which allows transport of oxygen to the hypoxic roots to facilitate root growth and minimize damage resulting from hypoxic conditions (Bray et al., 2000). Such a relationship between ethylene and the response to flooding was exemplified in a study which reported better performance under waterlogged conditions of wild type tobacco over transgenic ethylene-insensitive (*etr1-1*) tobacco, as measured by adventitious root formation (MacDonald and Visser, 2003). In addition, ethylene production has been correlated with the activities of hydrolytic enzymes and enhanced formation of aerenchyma and adventitious roots in response to root submersion of maize seedlings (Bragina et al., 2001, 2003).

Ethylene also has been shown to be involved in the rapid elongation response of deepwater rice to submergence (Kende et al., 1998) as well as the hyponastic leaf growth in semiaquatic species such as *Rumex palustis* (Cox et al., 2004). Studies to characterize the rapid increase in ethylene biosynthesis in deepwater rice triggered by flooding have identified that the induction and sustained expression levels of a particular ACS gene family member is correlated with the growth response (Van der Straeten et al., 2001; Zhou et al., 2001; Zhou et al., 2002). The role of ethylene in the induction of petiole elongation in *R. palustris* is supported by molecular studies showing up-regulation of *ACS* and *ACO* genes as well as increases in their enzymatic activities concomitant with submergence (Voesenek et al., 2002).

In contrast, in flood-sensitive species, such as tomato, ethylene induced upon flooding is associated with deleterious consequences, including epinasty, leaf chlorosis, necrosis, and reduced fruit yield, often referred to as 'ethylene syndrome' (Grichko and Glick, 2001a). An evaluation of flood tolerance in transgenic tomato expressing a root-specific *ACC deaminase* gene to confer reduced ethylene production demonstrated increased tolerance as measured by shoot growth, leaf chlorophyll content, and epinastic curvature, further substantiating the adverse effects of stress ethylene on sensitive species.

In addition, recent work has studied the ability of root-associated bacteria with ACC deaminase (ACD) activity to confer resistance to otherwise sensitive crop plants to the effects of flooding, salt, and drought stress by reducing levels of ACC available for oxidation to ethylene within the plant, therefore alleviating effects associated with ethylene syndrome (Grichkco and Glick, 2001b; Mayak et al., 2004a; Mayak et al.,

2004b). The soil bacterium, *Achromebacter piechaudii*, was evaluated for its effect on tomato and pepper under drought stress (Mayak et al., 2004a) and tomato under salt stress (Mayak et al., 2004b), and results, based on measurements of fresh and dry weight as well as recovery, indicated that treatment with the bacteria allowed continued growth throughout the duration of the stress and a faster recovery upon removal of the stress. A related study of the changes in genes expressed in canola roots treated with rhizobacteria, *Enterbacter cloacae*, which also has ACC deaminase (ACD) activity, in comparison to plants treated with an ACD knockout mutant, suggests that the effect of the bacteria on the plants is primarily the result of lowering ethylene levels (Hontzeas et al., 2004). A down-regulation of stress genes and up-regulation of genes involved in cell division and proliferation was observed in roots treated bacteria with ACD activity.

Research also has demonstrated an ethylene-mediated decrease in leaf expansion and shoot growth in response to soil compaction by evaluating transgenic tomato harboring an antisense *ACO* (Hussain et al., 1999). Reduced ethylene producing tomato plants were able to maintain uncompacted control growth rates while wild type plant exhibited greatly reduced shoot growth (Hussain et al., 1999). Recent studies have also evaluated the shade avoidance response, which includes enhanced shoot elongation and elevated leaf angles enabling a plant to optimize light capture in crowded growth conditions, using transgenic ethylene-insensitive tobacco (Tetr) (Pierek et al., 2003). When grown within a crowded canopy along with wild type plants, Tetr plant growth was severely suppressed as a result of delayed shade avoidance response, suggesting that ethylene is required for plants to optimally respond to neighbors and successfully compete for light (Pierek et al., 2003; Pierek et al., 2004). Collectively, these studies

demonstrate that the effects of ethylene on growth can be variable and are dependent on environmental conditions.

While an increase in ethylene production concurrent with abiotic stress cannot be argued, a dichotomy exists in the nature of the downstream responses triggered by such an increase and appears to be dependent on properties of individual species. As will be addressed in the next section, much of the difficulty in assessing the function of ethylene in stress response is related to its interactions with other hormones in synergistic and antagonistic ways. Nonetheless, efforts characterizing the acclimation of tolerant species to particular abiotic stresses, such as flooding, suggest that ethylene is involved in the signaling of particular mechanisms that have evolved to allow species to cope with specific stresses (Peeters et al., 2002). With regard to risk assessment, as was discussed with disease response, the impact of modified ethylene on a plant's ability to respond to abiotic stress is likely to be variable and evaluation should, therefore, be considered on a case-by-case basis. Difficulty arises in the lack of resolution of the signal transduction pathways accounting for the mechanisms of adaption, resulting in the inability to assess with certainty how gene flow may impact the invasiveness of a related species.

Cross talk with other hormones

The study of hormone-response mutants has repeatedly demonstrated that the path of hormone perception or response is not a distinct linear one. Instead hormone signaling pathways intersect at many levels to create a complex network of overlapping signals (Gazzarrini and McCourt, 2003). Research has demonstrated interaction of ethylene

synthesis, signaling and response with that of auxin, abscissic acid (ABA), cytokinin (CK), gibberellin (GA), jasmonic acid (JA), and salicylic acid (SA).

Cross talk between ethylene and auxin is apparent in the regulation of ethylene biosynthesis and response. While low concentrations of auxin reduce ethylene formation and promote growth, high concentrations induce ethylene production and ethylene-associated responses (Grossman and Hansen, 2001). Characterization of regulatory regions of *ACS* genes has resulted in the identification of auxin response elements (ARES) responsible for the upregulation of ethylene synthesis by auxin (Ishiki et al., 2000). Induction of *ACO* gene expression and post-translation modification in response to auxin treatment provides further support and additional mechanisms for modulation of auxin-induced ethylene biosynthesis (Chae et al., 2000). Interaction between auxin and ethylene signaling pathways has become apparent through an evaluation of ethylene-insensitive mutant *ein2* and an auxin influx mutant, which showed that responsiveness to ethylene is important for auxin-induced root hair elongation (Rahman et al., 2002). In addition, studies of *R. palustris* suggest an interaction between ethylene and auxin signals in flood-induced adventitious root formation (Voesenek et al., 2003).

Ethylene and ABA interactions have been shown to affect germination, rooting, and shoot growth (Gazzarrini and McCourt, 2003; LeNoble et al., 2004; Hussain et al., 2000). Characterization of the shoot growth in Arabidopsis ABA-deficient mutant, *aba2-1*, showed that the mutant had increased ethylene production and sensitivity, and demonstrated that ABA plays a role in the maintenance of shoot growth by suppression of ethylene synthesis (and, perhaps, sensitivity) (LeNoble et al., 2004). An antagonistic interaction between ethylene and ABA has also been observed in studies of germination

as ethylene-response mutants, such as Arabidopsis *etr1-1*, increase the sensitivity of the seed to ABA (Gazzarini and McCourt, 2003). In contrast, ethylene-response mutants have shown reduced sensitivity to ABA in root tissue, demonstrating an additive interaction between the two hormones with respect to root growth (Beaudoin et al., 2000).

Interactions between ethylene and cytokinin (CK) pathways have been identified through study of factors affecting ethylene production, mutants, and transgenics. Studies of mutants overproducing ethylene have provided support for the post-transcriptional regulation of ACC synthase levels by cytokinin (Woeste et al., 1999). In addition, altered development including smaller leaves with reduced color and modified shape in transgenic Arabidopsis CK overproducing lines was partially attributed to increased ethylene levels (van der Graaff et al., 2001). However, an antagonistic relationship between ethylene and CK during senescence is suggested by the phenotype of petunia transformed to produce increased levels of CK at senescence (Chang et al., 2003). In these transgenics, higher levels of CK at flower maturity, resulted in lower levels of ethylene production, reduced ethylene sensitivity, and delayed senescence.

Communication between signaling pathways of ethylene and gibberellin have been demonstrated in the release of seed dormancy as well as growth processes including shade avoidance and response to flooding (Kende et al., 1998; Brady and McCourt, 2003; Cox et al., 2004; Rao et al., 2004). While the molecular mechanism of the interplay between ethylene and GA in the breaking of seed dormancy is not completely understood, the ability of ethylene to rescue the Arabidopsis GA-deficient mutant *ga1* at germination suggests that there is cross talk between their signaling pathways at this

stage of development (Brady and McCourt, 2003). Similarly, the ability of gibberellin to enhance the germination of *etr1* Arabidopsis suggests a reciprocal relationship between GA and ethylene (Bleeker et al., 1988). In addition, it was recently shown in tobacco that the growth-promoting effects of ethylene involved in phytochrome-mediated shade avoidance response are dependent on the action of GA (Pierik et al., 2004). An analogous dependence on GA in the mediation of an ethylene-induced growth response has been demonstrated in the studies of semiaquatic plants such as *R. palustris* and deepwater rice. Studies suggest that upon flooding ethylene mediates the rapid growth response in semiaquatic plants by increasing the plant's sensitivity to GA and, also, by increasing the ratio of GA to ABA by reducing ABA levels (Kende et al., 1998).

Cross talk between ethylene and jasmonic acid is a key component in the regulation of various plant processes including response to pathogen attack, wounding, stress and apical hook formation (Lorenzo et al., 2003). The negative interaction that exists between ethylene and jasmonic acid in a plant's response to the stress of ozone has been characterized through the study of JA- and ethylene-insensitive mutants of Arabidopsis. JA and ethylene appear to modulate each other's activity in ozone-induced cell death, whereby ethylene stimulates cell death while JA protects tissues (Tuominen et al., 2004). An analogous antagonistic relationship is apparent in the formation of an apical hook by emerging seedlings, as has been shown in studies in which the typically exaggerated apical hook of the Arabidopsis constitutive ethylene response mutant (*ctr1*) of Arabidopsis can prevented by jasmonate treatment (Ellis and Turner, 2001).

A plant's defense response to pathogen attack involves a complex network of signaling that includes salicylic acid (SA), jasmonic acid (JA), and ethylene. The

signaling pathways of ethylene and jasmonic acid interact synergistically to respond to some pathogens, and recent study of Arabidopsis JA and ethylene response mutants demonstrated that sensitivity to both is required for the expression of transcription factor ERF1, which in turn is responsible for the activation of defense-related genes (Lorenzo et al., 2003). In addition, characterization of Arabidopsis mutant *cev1* revealed its constitutively active JA and ethylene signaling pathways as well as enhanced resistance to pathogens, providing further support for cooperative regulation of JA/ethylene signal pathways (Ellis and Turner, 2001).

Although contradictory, research has provided evidence for cross-talk between SA and ethylene (Kunkel and Brooks, 2002). In a study utilizing tomato lines that were ethylene-insensitive, ethylene-deficient, or SA-deficient, ethylene was shown is be required for the accumulation of SA in plants infected with *Xanthomonas campestris* (O'Donnell et al., 2001). In addition, results of evaluation of the interaction of Tobacco mosaic virus and tobacco in transgenic ethylene-insensitive lines demonstrates that ethylene perception is necessary to initiate the signaling cascade, which triggers SA accumulation, pathogen-related gene expression, and systemic acquired resistance (SAR) (Verberne et al., 2003). However, in contrast, based on a series of experiments investigating SAR in ethylene-insensitive Arabidopsis, SA-mediated responses leading to the development of acquired resistance were found to not require ethylene sensitivity and, therefore, suggested to be independent of ethylene (Lawton et al., 1994). Another level of interaction between ethylene and SA signaling pathways during disease response has been identified by studying the expression of ERFs (ethylene response factors) from tomato in Arabidopsis. This work suggested that the depending on the type of pathogen

interaction, the ERF transcription factor, Pti1, may be positively regulated either by JA and ethylene or by SA (Gu et al., 2002). In addition, Gu et al. proposed that SA can repress the activity of JA/ethylene-induced Pti4, giving rise to an addition level of pathway interaction.

As research continues to reveal cross-talk between hormones, it is becoming increasingly difficult to deduce independent roles of any one hormone. Interactions between pathways can be antagonistic, synergistic, or cooperative, and may depend of the plant species or the developmental or physiological status of the plant (Rojo et al., 2003; Chow and McCourt, 2004). When considering the secondary effects associated with modifying ethylene pathways, it is very likely that the signaling pathways of additional hormones could be altered in some way as a result of disruption of their interactions with ethylene and may result in pleitropic effects, reflected developmentally or in responses to environmental stimuli. Consideration of cross talk between hormone signaling pathways is relevant from a risk assessment perspective as phenotypic changes in the engineered plant may be unexpected and subtle enough to not be identified in routine characterization.

What pleiotropic and secondary effects have been observed?

This wide-reaching range of developmental, disease, and stress responses and cross talk with other hormones suggests that alteration of ethylene for one purpose may have secondary effects on other phenotypes. Observations of effects secondary to the phenotype of interest sometimes are included in published work and may be relevant from a risk assessment perspective. In addition, expression profiling of ethylene related

genes by microarray analysis further demonstrates the high degree of transcriptional regulation that is affected by ethylene signaling.

As already indicated, flower longevity and delayed ripening have been primary targets of modified ethylene. Although not surprising from the preceding description of ethylene-related phenomena, evaluation of transgenic petunia, tomato, melon, and potato has revealed a range of unintended consequences of modified endogenous ethylene production or perception including effects on seed germination, rooting, fertility, and disease resistance, some of which were unexpected. For example, upon trying to vegetatively propagate ethylene-insensitive lines of tomato, petunia, and melon through cuttings, the requirement of ethylene for adventitious root formation became apparent (Clark et al., 1999; Klee, 2002; Papodopoulou, 2002). In addition, difficulty of the roots of ethylene-insensitive lines penetrating heavy soils has been observed (Klee, 2002). Other unexpected effects noted in characterization of ethylene-insensitive petunia and melons include the formation of larger flowers (Shaw et al., 2002), production of a greater number of flowers (Klee, 2002), and the conversion of hermaphrodite to female flowers (Chapter 2). An unexpected phenotype observed in melon engineered to overexpress an *ACS* gene to increase femaleness was the earlier and increased production of mature hermaphrodite flowers providing support for a previously unknown role of ethylene in pistillate bud development (Papadopoulou et al, 2005). In addition, earlier and increased sequential fruit set on the main stem was also observed, suggesting that ethylene signaling may be associated with fruit set.

Clevenger et al. (2004) recently investigated factors affecting seed production in transgenic ethylene-insensitive petunia, including pollen viability, pollen tube growth,

seed yield, seed germination, and whole plant vigor. Ethylene-insensitive pollen showed reduced viability; however, pollen tube growth was not affected. In addition, seeds produced on an ethylene-insensitive maternal parent showed reduced seed weight, germination, and seedling vigor.

On a slightly different note, potato that was engineered to express the Arabidopsis *ETR1* ethylene receptor gene in an effort to better understand ethylene's role in tuber dormancy gave rise to a range of abnormalities in growth and development (Haines et al., 2003). Preliminary phenotypic characterization of transformants revealed changes in leaf size and shape, plant growth habit, and tuber size and shape that were attributed to the altered sensitivity to ethylene.

Additional pleitropic effects associated with modified ethylene that have been reported relate to the plant's response to biotic and abiotic stress. Increased disease susceptibility has been observed in reduced ethylene-producing tomato as well as ethylene-insensitive petunia (Klee, 2002; Shaw et al., 2002; Shibuya et al., 2004). However, as already mentioned modified ethylene can also result in enhanced tolerance to certain pathogens. Melon engineered for delayed ripening by inhibiting ethylene biosynthesis (Ayub et al., 1996) was subsequently found to have increased resistance to the abiotic stress of chilling (Ben-Amor et al., 1999).

Microarray analysis has provided a valuable tool for investigating gene activity by allowing examination of the expression levels of thousands of genes simultaneously (Van Zhong and Burns, 2003). Comparison of expression profiles in Arabidopsis of ethylene-insensitive mutant *etr1-1*, constitutive response mutant *ctr1*, ethylene-treated wild type, and untreated wild type plants has provided insight into the extent of gene transcriptional

regulation that is affected by ethylene (Van Zhong and Burns, 2003). These studies revealed that 7% of the approximately 6000 unique genes included in the microarray were regulated by ethylene, which if extrapolated to the whole Arabidopsis genome, would suggest the presence of ca. 1800 ethylene-responsive genes. A wide variety of genes were found to be differentially regulated by ethylene, including those encoding its own biosynthetic and signaling components, transcription factors, components of other hormone pathways, primary metabolic proteins, and defense-related proteins, as well as many of unknown function.

Additional microarray studies have provided information about genes regulated by ethylene in response to environmental stresses (Schenk et al., 2000; Chen et al., 2002). Results from both of these studies strongly support the presence of a complex signaling network of positive and negative interactions between multiple signaling pathways to achieve an appropriate defense response. The inclusion of hormone response or biosynthetic mutants in evaluations provides evidence for the effects of ethylene on the expression of genes involved in the plant's response to pathogen attack (Chen et al., 2002).

Despite a tremendous volume of literature supporting ethylene's involvement in virtually all facets of a plant's life cycle, there has been limited report of secondary effects associated with engineered ethylene pathways in publications describing plants transformed for potential commercial applications. This may be a result of research and publication focus on the intended goals of the genetically engineered product. Another factor may be the subtleness of the pleitropic effects such that characterization of these

secondary phenotypes is not a part of the research which is presented in the published results.

Nonetheless, the pleitropic effects associated with modified ethylene pathways pose interesting possible secondary plant phenotypes, which may be relevant in the context of risk assessment. For example, a change in the size or number of flowers on the transgenic plant may impact pollinator behavior resulting in increased gene flow. However, the difficulty arises in predicting how the transgene will impact fitness of resulting hybrids or surrounding ecosystem in the event of gene flow into adjacent related populations as effects will largely be dependent on the stresses imposed in that environment. This calls for scrutiny in the assessment of plant phenotypes and careful consideration of environmental effects.

Conclusions: implications for risk assessment

This case study of ethylene has provided an analysis of the dynamic nature of signaling pathways throughout a plant's life. As efforts in plant biotechnology continue to expand to include genes encoding transcription factors and signaling or metabolic components, the likelihood of impacting the expression of many genes with the transgene's introduction is quite high. As has been reviewed in the case of ethylene, genetic engineering these pathways has the potential to impact plant development, stress response, and interactions with other pathways. In addition, advances in plant molecular biology continue to reveal the complexity of plant signaling.

As a result of the interconnectedness of regulatory, metabolic and signaling pathways, modification of individual components may have pleiotropic effects, some of

which are unexpected and unpredicted. In addition, phenotypes secondary to the trait of interest which may be relevant from an ecological risk perspective, such as changes in attractiveness to pollinators or changes in pollen morphology, amount, or viability, may go unnoticed in laboratory, greenhouse, and field trials (Hokanson, 2002). Therefore, such effects could go unrecognized until large scale commercial production. Due to the difficulty in predicting and identifying some secondary effects, one recommendation for the genetic engineering of signaling pathways is the use of tissue-targeted or temporally regulated promoters rather than constitutive promoters for transgene expression. This strategy could confer localization or timing of transgene expression to meet the specific requirements of the trait of interest in an effort to minimize pleiotropic effects of the expressed transgene. Another recommendation is the establishment and enforcement of a set of guidelines to collect baseline data for many of the plant life cycle characteristics of both transgenic and non-transgenic plants at various stages of crop development from laboratory to large scale field evaluation. The implementation of such data into a publically accessible database may help to identify common secondary phenomenon associated certain engineered traits or transgene types and assist in the development of appropriate confinement and risk assessment protocols. In conclusion, the preceding information suggests that careful and thorough characterization of the effects of transgenes on crop biology is imperative for adequate assessment of the ecological impact of genetically engineered plants destined for commercial production.

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CONCLUSIONS AND FUTURE RESEARCH

The objectives of this research included tissue targeted modification of ethylene perception of melon plants and study of the effects of increased endogenous ethylene production of sex determination and fruit set in field grown *35S::ACS* melon. Another objective was to evaluate secondary effects associated with genetic engineering to modify traits regulated by ethylene within the context of ecological risk assessment.

For the first objective, andromonoecious melon was transformed with gene constructs of the Arabidopsis *etr1-1* mutant driven by heterologous floral targeted promoters. The *Crab's Claw* promoter from Arabidopsis was used to target carpels and nectaries, and the *Apetala3* promoter, also from Arabidopsis was used to target stamens and petals. Greenhouse characterization of sex expression and floral morphology of *AP3::etr1-1* and *CRC::etr1-1* transgenic melon plants provided unexpected results. *AP3::etr1-1* plants showed increased maleness as demonstrated by largely abolished pistillate bud formation and, of the few pistillate buds produced, many showed poorly developed carpels. In contrast, *CRC::etr1-1* plants produced the opposite phenotype, with increased femaleness as demonstrated by earlier and increased pistillate bud production of the main stem and the observation of many female buds, in place of the expected hermaphrodite bud. Collectively, these observations suggest that a reciprocal relationship exists between stamens and carpels in the hermaphrodite bud development such that stamens must perceive ethylene to promote carpel formation and vice versa. While pistillate buds initiated on *CRC::etr1-1*, most aborted early in development, providing support for a role of ethylene in sustained carpel maturation. In order to further

establish the proposed role of ethylene perception by the stamens and carpels, it will be necessary to determine the timing and localization of *etr1-1* expression in the developing buds. Support for the model could be provided by expression of the *etr1-1* transgene at the time critical for sex determination. *In situ* hybridization of apices containing floral buds at various ages would allow a detailed analysis of *etr1-1* expression. Interest for future research also includes the evaluation of transgenic monoecious cucumber harboring *AP3::etr1-1* and *CRC::etr1-1* to allow further insight into the role of ethylene in sex determination.

Therefore, future research on the role of ethylene perception in sex determination should focus on trying to fill in the gaps of the proposed or other established models. Questions that remain unsolved include: 1. Is the expression of the *etr1-1* in transgenic *AP3::etr1-1* and *CRC::etr1-1* within the developing floral primordia consistent with the proposed role of ethylene in the development of carpels and stamens? 2. Is the proposed model of ethylene role in sex determination for melon consistent with its role in cucumber? 3. Is the *M* locus in cucumber analogous to the *A* locus in melon? 4. Is the inhibition of stamens in pistillate buds of *mm* cucumber the result of a constitutive response to ethylene or of a reduced ability to respond to ethylene? 5. Is a more specific carpel-targeted promoter necessary to confidently support the proposed model?

For the second goal, a field trial was conducted to study sex expression and fruit set on transgenic andromonoecious melon constitutively over-expressing an *ACC synthase* (*ACS*) gene to confer increased endogenous ethylene production. Consistent with greenhouse observations, field grown *35S::ACS* melon plants showed earliness and increase of mature hermaphrodite flower production indicating ethylene is important for

full hermaphrodite development. *35S::ACS* melon plants also showed earlier and increased fruit set on the main stem. The observation of increased sequential fruit set on the main stem suggests that ethylene may play a role in the signaling associated with fruit set patterns. The earliness of fruit set could have horticultural significance in areas, such as Michigan, with a short growing season. However, pleiotropic effects, which may be associated with constitutively elevated ethylene such as accelerated ripening or altered stress response, would be agronomically undesirable.

Future research could focus on achieving temporal and tissue localized *ACS* expression to enhance femaleness produce earliness in fruit set without deleterious secondary effects. As described in Appendix A, *CRC::ACS* and *AP3::ACS* constructs have already been prepared. Unfortunately, as was demonstrated in *CRC::etr1-1* melon plants and in preliminary evaluation of *CRC::ACS* melon, the *CRC* promoter in this heterologous system lacked tissue specificity. The effect of *AP3::ACS* on sex expression is yet to be determined as the construct has not yet been transformed into melon. Additional *ACS* constructs to target floral tissue include the promoter region of a melon homologue of *CRC*, although this would require its cloning. Additional options might be the promoter of the Arabidopsis stamen and carpel targeted AGAMOUS MADS box gene (Yanofsky et al., 1990), or the cucumber carpel restricted AGAMOUS-Like gene (CAG2) (Perl-Treves et al., 1998), if its promoter was available.

For the final objective, a case study was conducted to describe many of the possible pleitropic effects associated with altered ethylene signaling to demonstrate how modification of signaling pathways can affect the activities of many genes and result in unexpected phenotypes. Secondary effects which could alter gene flow or fitness in the

event of gene flow were emphasized with regard to their relevance from a perspective ecological risk assessment. Continuation of this work might include description of possible methodology to implement in ecological risk assessment strategies to identify relevant pleiotropic effects. In addition, genetic engineering strategies to minimize unexpected phenotypes could also be discussed.

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Appendix A:

Floral targeted expression of a heterologous *ACS* in andromonoecious melon

Introduction

Analysis of *35S::ACS* transgenic melon plants allowed the evaluation of increased endogenous ethylene on sex expression in melon (Papadopoulou et al., 2005). However, constitutive expression of a transgene with the potential to affect many processes throughout plant growth and development, such as *ACS*, is undesirable from a practical and regulatory perspective; therefore, from an applied standpoint, constitutive *ACS* expression should be avoided to minimize secondary effects on other plant characteristics. In addition, tissue specific or temporal expression of the *ACS* transgene may permit further eludication of the critical timing or site of ethylene synthesis required to impact sex expression. Therefore, constructs were prepared to evaluate increased *ACS* expression targeted to developing petals and stamens via the promoter of the *Arabidopsis* class B floral identity gene *Apetala3* (Irish and Yamamoto, 1995) and to developing carpels and nectaries via the promoter of *Arabidopsis* carpel and nectary development regulator *Crab's Claw* (Bowman and Smyth, 1999).

Materials and Methods

Plasmid Construction

CRC::ACS: The petunia *ACS* cDNA (kindly provided by Dr R. Woodson, Purdue University) was PCR amplified from pBS-*ACS* to introduce a *BamHI* site at the 5' and 3' ends using specific primers 5'-CCCGGATCCCTCTCTCTCTCTCCTCGTGCC (RG280)

for the 5' end and 5'GGGGGATCCGGGCGAATTGGGTACCGGGCC (RG281) for the 3' end, and the resulting PCR fragment cloned with pGEM®-T Easy Vector System (Promega Corporation, Madison, Wisconsin). The *etr1-1* from the previously prepared pCambia2300-*CRC::etr1-1* (Chapter 2) was removed as a *BamHI* fragment and replaced with the *ACS BamHI* fragment.

AP3::ACS: Construct preparation was analogous to *CRC::ACS*, with the exception that *ACS* was transferred as a *BamHI* fragment into the previously prepared pCambia2300-*AP3::etr1-1* (Chapter 2), with *etr1-1* dropped out as a *BamHI* fragment.

Plant Transformation

The pCambia-*CRC-ACS* and –*AP3-ACS* constructs were introduced into *Agrobacterium tumefaciens* strain C58 (Deblaere et al.,1985) for subsequent transformation into melon. Andromonoecious muskmelon, Hale's Best Jumbo (Hollar Seed, Rocky Ford, Colorado), was transformed via *Agrobacterium*-mediated transformation of cotyledon tissue via the method of Fang and Grumet (1990) with revisions based on the cucumber transformation method of Tabei et al. (1998) (Chapter 2). Regenerated shoots (T_0) were selected on kanamycin (200 mg/L) and evaluated for the presence of neomycin phosphotransferase by NPTII ELISA (Agia®, Elkhart, Indiana) as well as presence of the *ACS* gene by polymerase chain reaction (PCR) (described below). PCR-Positive T_0 plants were transferred to the greenhouse for self-pollination and production of T_1 progeny for further evaluation.

PCR analysis

The Wizard Genomic Plant DNA Purification Kit (Promega Corporation, Madison, WI) was used to extract genomic DNA from leaf tissue of melon. PCR was

used to amplify the inserted transgene using 5' primer RG280 and 3' primer RG281 for amplification of *ACS*, and 5' primer 5'-CTTGCAATCCCTAGCCAG (RG 299) and 3' primer 5'-CCAGCGTTACTCTTCAATGC (RG310) for amplification of *CRC-ACS*. The amplification reactions of *ACS* were carried out with Taq DNA polymerase (Invitrogen, Carlsbad, California) in 3.0 mM $MgCl_2$ for 1 cycle of 2 minutes at 95°C followed by 40 cycles of 95°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute, then 1 cycle of 5 minutes at 72°C (Sambrook and Russell, 2001). Amplication reactions of *CRC-ACS* were carried out as with *ACS* but with the following program: 1 cycle of 2 minutes at 95°C followed by 40 cycles of 95°C for 50 seconds, 58°C for 50 seconds, and 72°C for 2:20 minutes, then 1 cycle of 5 minutes at 72°C.

Northern Analysis

Leaf, male bud, and female bud tissue of greenhouse grown T_1 *CRC::ACS* lines 168 and 169, *35S::ACS* line 4, and wild type Hale's Best Jumbo were sampled for RNA isolation. Petals were excised from the male buds with a razor blades and designated as "petals" while the remaining portion of the male bud containing stamens, nectaries, and floral cup was designated "lower bud." Female buds were cut with a razor blade such that the top portion of contained stamen, stigma, and petal tissue (designated "sti, pet, sta") and the bottom portion contained the tissue of the ovary (designated "ovary"). RNA was isolated and hybridization carried out as described in Chapter 3.

Ethylene Experiments

Young leaves (approximately 4-7 cm wide) were removed from lateral branches of greenhouse grown plants with a razor blade, weighed, and placed in a 50 ml syringe containing ¼ of a Whatman #2 filter paper (90 mm) moistened with 200 µL distilled

water (dH_2O). The syringe plunger was adjusted to 30 mL, and the end of the syringe was fitted with a rubber septum and sealed with parafilm. Gas samples were removed via an airtight syringe (1mL) inserted through the rubber septa into the airspace of the tissue containing 50mL syringe. Samples were analyzed by gas chromatography (Hach Carle series 100GC, Linear 1200 recorder) with an activated alumina column and flame ionization detector. Samples were collected three times from 4 randomly sampled plants per genotype.

Results

While both *AP3::ACS* and *CRC::ACS* transformation constructs were prepared. Only two families of T_1 transgenic melon plants harboring *CRC::ACS* were available in time for evaluation. T1 seed has been produced for seven additional *CRC::ACS* melon lines; however, no *AP3::ACS* melon lines were produced. Preliminary greenhouse characterization of two T_1 *CRC::ACS* lines included PCR, Northern blot hybridization, and measurement of ethylene production, but no evaluation of sex expression was conducted. PCR verified the presence of the *CRC::ACS* construct. Segregation ratios are based on PCR results and are consistent with one transgene insertion event in CRC168 and two in CRC169 (Table 1).

Northern analyses were done to evaluate expression in the leaf, male bud, and female bud tissue of *CRC::ACS* lines 168 and 169 (CRC168 and CRC169). While the *CRC* promoter was expected to confer transgene expression to floral regions of the carpel and nectary, northerns of leaf tissue showed *ACS* transgene expression in the leaf of CRC168 and CRC169 melon comparable to that of the 35S line ACS4 (Figure 1A).

Table A.1. Segregation analysis of *CRC::ACS* T_1 transgenic melon.

Line	Segregation Observed	Ploidy	Expected Ratio	χ^2
CRC168	9:4	2n	3:1	1.12 ns
CRC169	16:1	2n	15:1	0.02 ns

Progeny were evaluated for the presence of the *ACS* transgene by PCR amplification.

ns- χ^2 value not significantly different at p=0.05

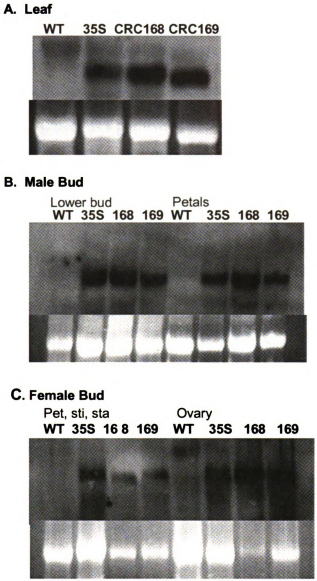

Figure A.1. Northern hybridization of heterologous *ACS* gene expression in tissue from greenhouse grown T_1 transgenic *CRC::ACS* melon. The top panel of each pair shows hybridization of total RNA from non-transgenic plants (WT), *35S::ACS* plants (35S), and *CRC::ACS* plants (lines 168 and 169) with DIG-labeled *ACS* probe. The bottom panel shows rRNA stained with ethidium bromide. (A) leaf tissue. (B) Male buds (upper bud, including stamen, nectary, and floral cup, and petal region). (C) Female Buds (petal/stigma/stamen region and ovary region)

Northern blots of male bud tissue showed similar lack of tissue specificity from the *CRC* promoter in this heterologous system (Figure 1B); however, hybridization to RNA isolated from female buds suggested higher expression levels in *CRC::ACS* lines than the *35S::ACS* line (ACS4) used as a positive control, taking into account the higher loading of RNA from ACS4 than CRC168 and CRC169 (Figure 1C).

Gas chromatography was used to measure ethylene production from leaves of CRC168 and CRC169 in comparison to ACS4 and WT. Both CRC168 and CRC169 showed the elevated ethylene evolution relative to wild type Hale's Best Jumbo, consistent with leaky expression from *CRC* promoter (Figure 2).

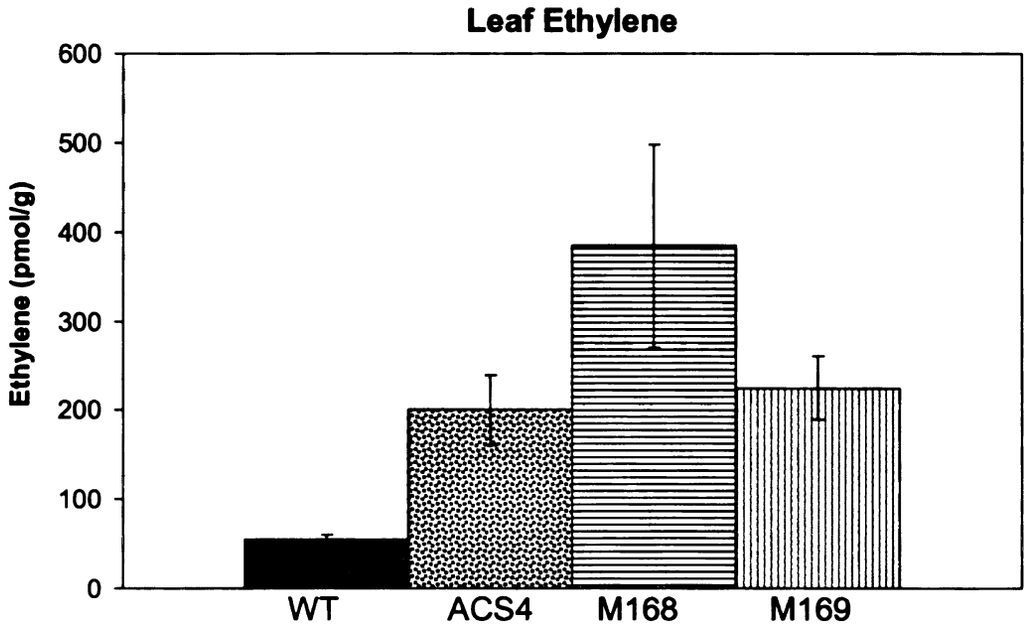

Figure A.2. Ethylene production from leaves of wild type (WT) Hale's Best Jumbo, transgenic *35S::ACS* (ACS4), and *CRC::ACS* (M168 and M169) Hale's melon. Samples were collected three times from randomly sampled plants, 4 times per genotype. (All data are means ± standard error.)

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Appendix B:

Cloning of a partial genomic sequence of *CS-ACS3*

Introduction:

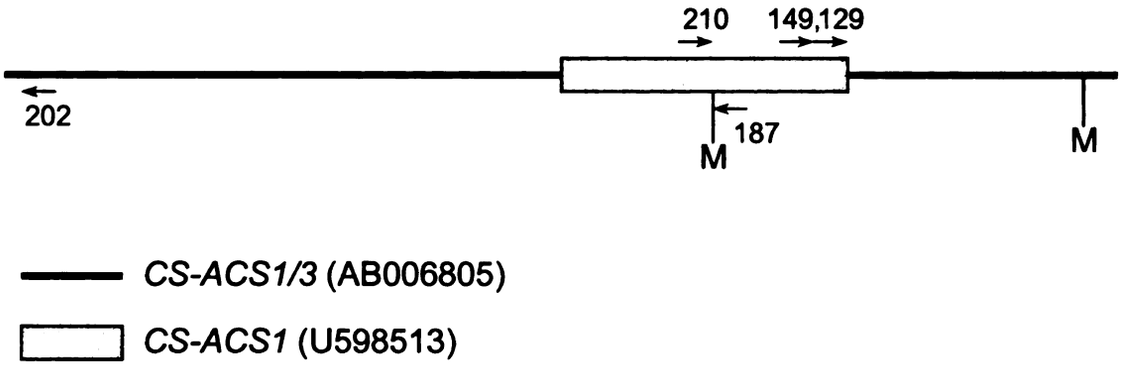
The objective this project was to gain a better understanding of the feminizing effect of ethylene and its relationship to the *F*-locus (*Female*) conferring gynoecy in cucumber. Analysis of the near isogenic monoecious (*ff*) and gynoecious (*FF*) cucumber lines demonstrated complete co-segregation of the *F* allele with an additional copy of the CS-*ACS1* gene, designated as *ACS1G* (Trebitsh et al., 1997). Southern hybridization analysis suggested that *ACS1* and *ACS1G* were closed linked, residing on a 19.4 kb *BamHI* fragment. I sought to clone genomic sequences of *CS-ACS1* and *CS-ACS1G* to compare coding sequences as well as non-coding regulatory regions to look for sequence elements that may confer expression specificities (e.g. hormone response elements, tissue or developmental targeting).

The strategy for this cloning project was to use inverse PCR to amplify the desired unknown regions flanking the partial *ACS1* sequence reported by Trebitsh et al. (1997) in GenBank (accession number U59813). Inverse PCR utilizes the polymerase chain reaction to amplify unknown flanking regions of a known template that is within a restriction fragment that has been self-ligated to form a monomer circle using primers oriented in opposing directions. Restriction site information concerning the two genes of interest was available from Trebitsh et al. (1997).

Materials and methods

High molecular weight nuclear DNA was isolated from gynoecious (GP-14) and monoecious cucumber using Bio101 Floraclean nuclear DNA extraction kit. DNA was fully digested with *XbaI*, *HindIII*, or *MspI*. Southern analysis of Trebitsh et al. (1997) indicated that digestion with *XbaI* should result in the presence of 10 kb and 12 kb fragments, which contained the *CS-ACS1* and *CS-ACS1G* genes, respectively. Similarly, Southern analysis indicated that digestion with *HindIII* should result in the presence of two 5kb fragments, each corresponding to one of *ACS1* genes and that *MspI* digestion should result in 0.75 and 2.5 kb fragments corresponding to *CS-ACS1* and the a 3.25 kb fragment corresponding to *CS-ACS1G* (Trebitsh et al., 1997).

The fully digested genomic DNA (verified by gel electrophoresis) was subjected to conditions favoring intramolecular ligation (ie larger volume, overnight at 16°C). The precipitated and resuspended ligation product was used as template for inverse PCR using primers designed from sequence information for *CS-ACS1* (GenBank accession number U59813) and homologous *CS-ACS3* (GenBank accession number AB006805, Shiomi et al., 1998) (Figure 1). The resulting PCR products were analyzed by gel electrophoresis; bands corresponding to the desired fragment size based on the above restriction information were isolated from the gel and cloned for sequence analysis.



Inverse PCR Primers:

RG149: 5'-GGCTCCCAGGTTTTCGAGTTGGTGC-3'

RG129: 5'-GGTCGTGGCGGCTGCTACTAAAATG-3'

RG187: 5'-GGAAGCCGTTGGAGCTAGTGCAATG-3'

RG201: 5'-GCCTAGGGATGGATGGAAGAAG-3'

RG202: 5'-CAGCTGGCGCTACATCAGCC-3'

Figure B.1. Layout of primers designed for inverse PCR based on *CS-ACS1* and *CS-ACS3* sequences. Numbered arrows indicate relative location of primer sequence. M denotes the relative location *MspI* restriction sites.

Results and Discussion

Initial efforts on this project focused on amplification of circularized *XbaI* and *HindIII* fragment by inverse PCR. However, many attempts at optimizing the PCR system which included nested primers, varying concentrations of template, and various polymerases failed to warrant amplification of DNA fragments near the desired size. Therefore, efforts were shifted to the *MspI* digest in order to obtain to smaller self-ligated product for PCR amplification.

Sequential PCR amplification with primers 3' of the *MspI* site in the Trebitsh et al. (1997) sequence (RG187/RG149 followed by RG187/RG129) led to identification of a 600 bp fragment. Subsequent cloning and sequence analysis indicated perfect homology to *CS-ACS3* (GenBank accession number AB006805, Shiomi et al., 1998). The *CS-ACS3* cDNA, which was found to be identical to *ACS1* (this work; Mibus and Tatlioglu, 2004) cloned in a study which characterized differential expression of *ACC synthase* and *ACC oxidase* genes in cucumber fruit in response to auxin and wounding.

A 3' primer (RG201) was designed to a region at the 5' end of *ACS1/3* coding sequence to amplify non-coding sequence further upstream by inverse PCR. This 3' primer in combination with a 5' primer just before the *MspI* site (RG202) in the Trebitsh sequence was added to the ligated *MspI* digestion for PCR. A 2.0 kb fragment was amplified and cloned. Sequence evaluation showed perfect homology to the 5' and 3' ends *ACS3* sequence as well as new non-coding 5' sequence.

Non-coding sequence was evaluated for auxin response element (ARE) sequence TGTC and ethylene response element sequence (ERE) GCCGCC. The search for AREs yielded eight matches; six are upstream of the putative TATA box, and three are the

sequence complement GACA (Figure 2). However, no putative EREs were identified. Therefore, this sequence analysis provides support for the modulation of ethylene levels by auxin.

References

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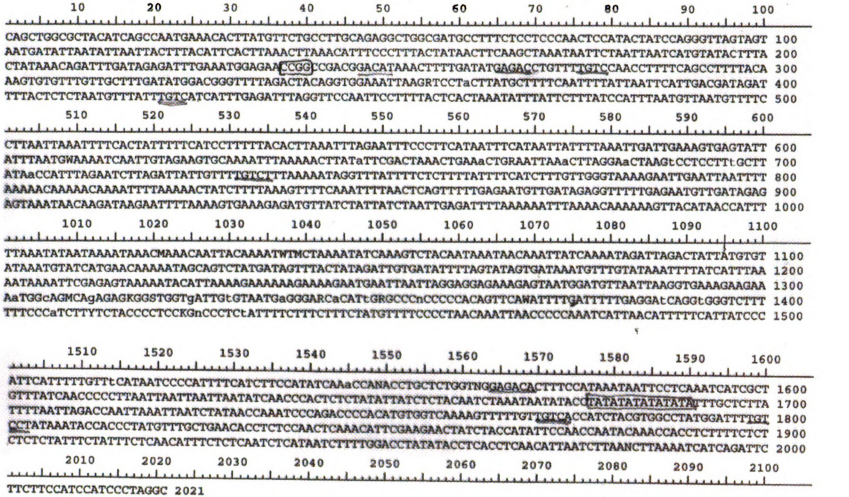

Figure B.2. 2.0 kb 5' noncoding sequence of *CS-ACS1* (*CS-ACS3*). AREs are underlined. TATA box and MspI site are in boxes.