THE EFFECT OF CARPEL AND STAMEN PRIMORDIA-TARGETED ETHYLENE PRODUCTION AND PERCEPTION ON SEX EXPRESSION IN MELON (*CUCUMIS MELO* L.)

By

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ABSTRACT

THE EFFECT OF CARPEL AND STAMEN PRIMORDIA-TARGETED ETHYLENE PRODUCTION AND PERCEPTION ON SEX EXPRESSION IN MELON (*CUCUMIS MELO* L.)

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Commercial melons (*Cucumis melo* L.) are typically andromonoecious, first producing vegetative nodes followed by a male flower-only phase, then a male and bisexual flower phase. Unisexuality arises from differential suppression of sex organ primordia, and ethylene is a key factor modulating sex expression. Thus, a comprehensive model of melon sex determination must include sex organ suppression developmental stages, known sex genes and phenotypes, and ethylene effects. Previous work in our lab using transgenic melons expressing the ethylene receptor mutant *etr1-1* under floral primordia-targeted promoters indicated stamen primordia, not carpel primordia, need to perceive ethylene for carpel development. Previous research reported that the *G* locus, responsible for carpel suppression, encodes a WIP1 transcription factor, and the *A* locus, responsible for stamen suppression in bisexual flowers, encodes an ethylene biosynthetic enzyme gene, 1-aminocyclopropane-1-carboxylic acid synthase (*ACS*). However, it is unknown how molecular ethylene production influences carpel development promotion.

To further examine the roles of floral organ primordia in promoting carpel development, transgenic melons were produced targeting *ACS* expression to either stamen and petal primordia (*AP3::ACS*), or carpel and nectary primordia (*CRC::ACS*). *AP3::ACS* melons showed increased *A* gene expression, and decreased *G* expression. Increased femaleness was observed, manifested as increased carpel-bearing buds, decreased male buds, male-only phase loss, and gain of a bisexual-only phase not seen in wild type. Microscopic analysis of apices showed reduced

progression of floral buds into sex determination stages. In contrast, *CRC::ACS* melons showed no difference in sex expression patterns or sex gene expression. These results, coupled with knowledge of sex gene identities and sex phenotypes, led to an integrated model of melon floral sex determination.

Increased femaleness was also observed in transgenic melons targeting *etr1-1* to carpel and nectary primordia (*CRC::etr1-1*). To investigate if this phenotype is useful for increased and earlier fruit set, *CRC::etr1-1* melons were examined in the field. Transgenic plants had earlier and increased number of carpel-bearing flowers and fruit set. However, *CRC::etr1-1* fruit were smaller, resulting in equivalent kg/plot, and showed either earlier ripening (line M5), or no obvious external ripening (line M15). Externally green M15 fruit had extensive internal ripening with elevated internal ethylene levels, equivalent to wild type orange fruit. Expression of *etr1-1* was higher in M15 exocarp compared to mesocarp, likely leading to external ripening inhibition.

It has been proposed that, in addition to the two major sex loci, one or more modifiers act to stabilize gynoecious and hermaphrodite genotypes. Other members of the *ACS* or *ACC oxidase* (*ACO*; ethylene biosynthetic enzyme) families may modulate sex determination. To evaluate gene expression within different sex genotypes, hermaphrodite (*ggaa*) and monoecious (*G-A-*) lines were produced from a gynoecious (*ggAA*) and andromonoecious (*GGaa*) cross. F_1 progeny were monoecious as predicted and F_2 sex phenotype segregation ratios were consistent with a four gene model. Three new *ACS* gene members were identified using the melon genome; expression of the 7 *ACS and* 3 *ACO* genes was analyzed in apices of different genotypes. All *ACS* and *ACO* members showed higher expression in gynoecious and hermaphrodite apices compared to andromonoecious and monoecious. Together, these studies provide further insight into ethylene perception and production influences in sex expression in melon.

DEDICATION

I dedicate this thesis to my loving family and friends who have given me the strength and encouragement to complete this major milestone in my life. Also to my husband, who has been the most caring and supportive influence in my graduate student career; he's seen me at my worst and best times, and still married me, the crazy glutton for punishment. Love to all of you and know that I couldn't have done it without you.

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CHAPTER 1

Literature Review

Parts of this literature review have been published as Grumet R and Taft J (2011). "Sex Expression in Cucurbits." In: Genetics, Genomics and Breeding in Cucurbits. C Kole, YH Wang, TK Behera (eds). Science Publishers Inc., Enfield, NH and CRC Press (Taylor and Francis) group, Boca Raton, FL. P: 353-375.

Sex expression in angiosperms:

Typical angiosperm species produce bisexual flowers that include both male and female organs (anthers and carpels, respectively) allowing for self fertilization. The promotion of outcrossing and heterogeneity has evolved through different mechanisms such as recognition of 'self' pollen and subsequent termination of fertilization (self incompatibility); changing flower morphology to prevent physical contact of anthers and pistil; or developing unisexual flower phenotypes (Ainsworth, 1998; Barrett, 1998). Two major classifications of sex phenotypes are dioecious, with unisexual flowers separated on two individual plants, and monoecious, unisexual flowers occurring on the same plant (Grant, 1994; Ainsworth, 1998; Barrett, 1998). Dioecy has appeared in ~43% of known plant families and monoecy in ~36% of families (Renner and Ricklefs, 1995).

Cucurbitaceae is an interesting family with ~32% dioecious species and another 36% exhibiting a wide variety of other heritable sex expression types that can produce varying combinations of male, female and bisexual flowers within a single species, i.e. monoecy (Roy and Saran, 1990; Renner and Ricklef, 1995). Some cucurbit species exhibit combinations of sex types that can occur on a single plant, including one species that has one of the rarest forms: androdieocy (separate male and bisexual plants) (Roy and Saran, 1990). Monoecy includes various sex expression types: andromonoecious (male and bisexual), monoecious (male and female), gynoecious (only female), and hermaphrodite (only bisexual). The molecular and physiological aspects of unisexuality are not well understood, but through the use of two model species, *Cucumis melo* (melon) and *Cucumis sativus* (cucumber), these aspects are beginning to come to light (Roy and Saran, 1990; Perl-Treves, 1999).

The typical sex expression pattern of melon begins with vegetative nodes, followed by

similar growth habit, however it has a mix of male and female nodes (monoecious). Depending on the underlying genotype, both species can exhibit other sex types such as gynoecious and hermaphrodite. Cucumber can also be androecious (all male) (Rudich, 1990), although a stable male-only habit has not been documented in melon (Magdum et al., 1982). These expression types can be influenced by the internal (Bai et al., 2004; Papadopoulou et al., 2005b; Little et al., 2007, Boualem et al., 2008; Martin et al., 2009) and external plant environment (Nitsch et al., 1952), and exogenous application of hormones (Rudich, 1990). Taken together, any bud at a single node can be influenced by developmental stage, environment, hormone balance, and genetics leading to the observable sex type.

Floral bud development:

Typical floral development follows a standard pattern with initiation of four sequential whorls of organs progressing from the outside to the inside of the flower. The outermost whorl, whorl 1, is where sepals originate; whorl 2, petals; whorl 3, stamens; and whorl 4, carpels (Coen and Meyerowitz, 1991). In unisexuality, all four whorls are initiated, but then arrest of either stamen or carpel primordia development leads to female or male flowers, respectively. In cucumber buds, this arrest occurs at different stages in floral bud development, as described by Bai et al. (2004). Developmental stage 1 is defined as the initiation of inflorescence, while stages 2-5 are sequential initiation of the whorls, beginning with sepals and ending with carpels. This process is completed in the first five days following inflorescence initiation while the floral bud is still microscopic (1-2mm in size) in the apex (Goffinet, 1990). Following initiation, sexual differentiation occurs in buds destined to be male or female unisexual flowers. In male flower buds, carpel arrest occurs at stage 5, when stamen primordia begin to enlarge. Stamen primordial

enlargement continues through to stage 9 when microsporocyte development is initiated, then further maturation occurs throughout the flower ending at stage 12 (anthesis). In female flower buds, the carpel primordia begin to elongate at stage 6 and continues until stage 9 (macrosporocyte initiation). Stamen arrest in female buds occurs at stage 8 although DNA damage is seen in the anther at stage 7 (Hao et al., 2003). Similar stages have also been observed in melon floral buds (Boualem et al., 2008).

Studies using a series of homeobox mutants in the model plant *Arabidopsis thaliana* and *Antirrhinum* (snapdragon), Coen and Meyerowitz (1991) and Bowman (1997) have elucidated the ABC model of floral development. Floral development is controlled by a set of MADS-box transcription factors A-E, as diagramed in Figure 1.1. Development of sepals is controlled by A genes (*APETALA1*), petal; A and B genes (*APETALA3*), stamen; B and C genes (*AGAMOUS*), and carpels require C genes. Loss of the A genes causes C genes to infiltrate the expression domain of A and vice versa, influencing sex organ development and showing their antagonistic nature. Further studies established the roles of the E genes, which are involved in the development of all four whorls (Pelaz et al., 2000; Honma and Goto, 2001), and D genes, which are specific for ovule development (Figure 1.1) (Colombo et al., 1995; Favaro et al., 2003; Pinoyopich et al., 2003). Yeast two-hybrid studies using A, B or C proteins as bait found that they can form homo or heterodimers (Pelaz et al., 2000), and quartet structures to control organ development (Honma and Goto, 2001; Theiben and Saedler, 2001).

Some cucumber homeobox genes have been cloned showing similar expression profiles as observed in the homologous Arabidopsis genes (Perl-Treves et al., 1998), and mutants demonstrated the importance of whorl position in floral development (Kater et al., 2001). A typical B gene mutant would show a phenotype where the petals and stamens are replaced by

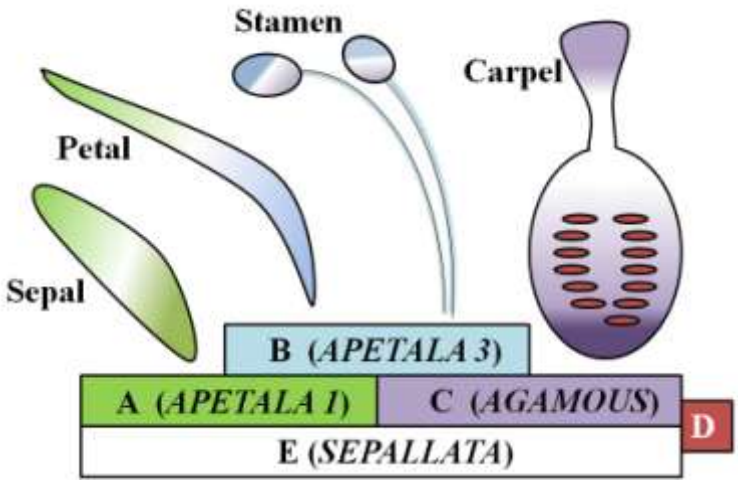


Figure 1.1. Diagram of the ABCDE model of floral development based on research performed by Coen and Meyerowitz (1991), Pelaz et al. (2000), Honma and Goto (2001), Colombo et al. (1995), Favaro et al. (2003), and Pinyopich et al. (2003). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis (or dissertation).

sepals and carpels, however the equivalent mutant in cucumber, *green-petals*, showed a different floral phenotype depending on the sexual fate of the bud. Female buds did have the predicted sepals in the first two whorls, with an undeveloped third whorl and carpels in the fourth. Buds destined to be male had sepals-sepals-carpels and the fourth whorl failed to develop. These phenotypes showed that whorl position, not sexual identity, was the main determinant for developmental arrest leading to unisexuality. A C-class gene of cucumber *ERAF17* was cloned and expression correlated with female flower development. After the initiation of female buds, *ERAF17* expression was found in the apex and continued in female tissues throughout development (Ando et al., 2001). The induction of female buds and *ERAF17* expression also seemed to be correlated with higher concentrations of the phytohormone ethylene. Recent transcriptomic analysis of apices from monoecious and gynoecious lines identified a set of differentially expressed genes with higher expression in gynoecious versus monoecious, such as ACC synthase, putative MYB transcription factor, auxin-related, and ABA-related genes, providing evidence for the role of hormones in sex determination (Gu et al., 2010; Wu et al., 2010).

Hormone involvement in sex expression:

Multiple factors influence the decision in floral buds to arrest the preferred primordia or allow for their continued development. Use of hormone mimics or inhibitors have been used in the field to promote male or bisexual flowers in gynoecious cucumber and melon lines, respectively, to allow for self pollination in order to maintain these lines (Den Nijs and Visser, 1980; Owens, 1980). Early studies in melon and cucumber investigated the effect of auxin and gibberellins. Increases in auxin level from exogenous application studies demonstrated that it had

a feminizing effect, as manifested by increased number of carpel- bearing buds and flowers, or the conversion of male flowers into bisexual/female flowers (Galun 1959; Galun et al., 1963; Rudich et al., 1969). This finding was supported by comparisons of endogenous auxin levels within different cucumber sex phenotypes (Galun et al., 1965; Trebitsh et al., 1987).

High levels of gibberellins (GAs) have been associated with increased maleness as manifested as an increase in male buds and/or flowers, or the conversion of female flowers into bisexual (Atsmon et al., 1968; Perl-Treves et al., 1998). Exogenous application of GA promoted male buds/flowers and prevented maturation of female buds. Likewise, applications of GA inhibitors promoted femaleness (Yin and Quinn, 1995). Observations of endogenous GA concentrations in a monoecious cucumber line versus a gynoecious line showed that higher levels of GA were present in monoecious plants (Atsmon et al., 1968).

A later study looked at the increased femaleness phenotype observed after brassinosteriod (BR) application in melon, cucumber and zucchini (Papadopoulou and Grumet, 2005a). Increasing concentrations of BR applied to monoecious cucumber showed earlier onset and increased number of female buds, however there was no effect on gynoecious cucumber, monoecious zucchini, or andromonoecious melon. Ethylene evolution measurements taken from apices of cucumber and zucchini after application of BR showed an increase in ethylene levels in both monoecious and gynoecious lines.

Ethylene's role in sex expression:

Application of ethylene using ethephon or ethrel has a feminizing effect on melon and cucumber plants (McMurray and Miller, 1968; Robinson et al., 1969; Rudich et al., 1969; Karchi, 1970; Augustine et al., 1973; Owens et al., 1980). Application at different developmental time

points, especially early on (i.e. at the one or two-leaf stage) can cause earlier and increased carpel-bearing buds in monoecious and andromonoecious melon plants (Rudich et al., 1969; Karchi, 1970). Likewise, 2-3 fold higher endogenous ethylene levels were found in gynoecious cucumber apices compared to monoecious, however this was not observed in melon (Byers et al., 1972a; Rudich et al., 1972; Makus et al., 1975; Rudich et al., 1976; Trebitsh et al., 1987; Yamasaki et al., 2001, 2003). In melon, studies using hypobaric gas chambers demonstrated the importance of endogenous ethylene levels (Byers et al., 1972b). After plants were subjected to a reduction of internal ethylene gas within the chambers, they exhibited increased maleness that could be reversed by application of ethylene.

The biosynthesis of ethylene begins with *S*-Adenosylmethionine (*S*-Adomet) being converted to 1-aminocyclopropane-1-carboxylate (ACC) by the rate-limiting enzyme ACC synthase (ACS). ACC is then oxidized to ethylene by ACC oxidase (ACO) (Johnson and Ecker, 1998; Argueso et al., 2007). Transgenic melons constitutively expressing *ACS* under the 35S promoter from the Cauliflower Mosaic virus (CaMV) showed increased endogenous ethylene levels and an increase in femaleness as exhibited by increased and earlier onset of carpel-bearing flowers (Papadopoulou et al., 2005b). Expression of *AP3::CsACO2* in Arabidopsis led to defective stamen development, potentially linking *ACO2* to stamen inhibition (Duan et al., 2008).

Inhibition of ethylene perception via silver nitrate/thiosulfate or ethylene production by aminoethoxyvinylglycine (AVG) application leads to increased maleness in cucumber and melon (Byers et al., 1972a; Den Nijs and Visser, 1980; Owens et al., 1980). Yamasaki and Manabe (2011) observed that functional bisexual and male flowers were obtained from application of silver nitrate on gynoecious cucumber, however AVG only induced male flowers. AVG converted female flowers to males independent of concentration, while higher concentrations of

silver nitrate were required to bring about the same conversion. This demonstrated that inhibition of ethylene production had a stronger effect on sex expression compared to blocking ethylene perception in cucumber (Yamasaki and Manabe, 2011).

Although GA, auxin, and BR had an effect on sex expression in cucumber and, to a lesser extent, melon, the effect of these hormones was not as strong as exogenous ethylene producers and inhibitors, especially for melon and gynoecious cucumbers (Kubicki et al., 1969a; Byers et al., 1972a; Tolla and Peterson, 1979; Trebitsh et al., 1987; Yin and Quinn, 1995). Inhibitors of GA increased maleness in cucumber, however ethylene inhibitors were more effective (Kubicki et al., 1969b; Tolla and Peterson, 1979). The increase in femaleness observed in monoecious cucumbers following auxin and BR application could be a product of these hormones' induction of ethylene production (Trebitsh et al., 1987; Papadopoulou et al., 2005a). Application of GA and BR was unable to influence sex expression in melon, however exogenous ethylene increased femaleness while inhibitors increased maleness (Byers et al., 1972a; Den Nijs and Visser, 1980; Owens et al., 1980). Based on this evidence, ethylene has been proposed as the predominant sex hormone in melon and cucumber (Rudich, 1990).

Ethylene is involved in many different processes within plants including senescence, abscission, pathogen responses, and responses to abiotic stresses (Abeles et al., 1992; Roman et al., 1995; O'Donnell et al., 1996). It is also involved in developmental processes such as fruit ripening, apical hook formation, and flower development (Abeles et al., 1992; Barry and Giovannoni, 2007; Grumet and Taft, 2011). The ethylene perception pathway has been extensively studied in Arabidopsis using mutants that show different responses to ethylene (Bleeker and Kende, 2000; Steptanova and Ecker, 2000; Wang et al., 2002). Ethylene receptors are encoded by a multigene family that resembles bacterial two-component signal transduction

proteins including kinase and receiver domains (Bleeker et al., 1988; Chen et al., 2005). Perception of ethylene produces a response by inhibiting phosphorylation of the immediate downstream component, CTR1 protein, which blocks the response pathway in the absence of perceived ethylene (Chen et al., 2005; Kendrick and Chang, 2008). Homologs of genes involved in the signaling pathway have been cloned in both cucumber and melon, and expression examined in cucumber in relation to sex expression (Yamasaki et al., 2000).

Increased expression of the receptor genes *CsETR1*, *CsETR2* and *CsERS* was observed in gynoecious cucumber apices compared to monoecious, and following ethylene application (Yamasaki et al., 2000, 2001). *CsETR2* and *CsERS* showed stronger expression upon onset of femaleness and in situ hybridization studies localized *CsETR2* to the carpel region of female buds, providing evidence for receptors in sex determination (Yamasaki et al., 2003). Results from heterologous expression in Arabidopsis of the antisense cucumber homologue of *ETR1*, under the control of the *AP3* promoter resulted in inhibition of stamen development (Wang et al., 2010).

Homologs of Arabidopsis ethylene receptor genes have been identified in melon (*CmETR1*, *CmETR2* and *CmERS1*) fruit (Sato-Nara et al., 1999; Takahashi et al., 2002; Owino et al., 2007). A link between gene expression and sex types has yet to be determined, however work with transgenics has provided evidence for ethylene's role in melon sex expression. Constitutive expression of an Arabidopsis dominant negative ethylene receptor mutant, *etr1-1*, in transgenic melons showed an almost complete loss of carpel-bearing buds, demonstrating that ethylene perception is critical for carpel development (Little et al., 2007). In order to further elucidate where perception is critical for sex determination, promoters that direct *etr1-1* expression to the stamen and petals (*APETALA3* or *AP3*) (Irish and Yamamoto, 1995), and the carpel and nectaries

(*CRABS CLAW* or *CRC*) (Bowman and Smyth, 1999) were used. If perception in the carpels was required for carpel development, then inhibiting it would show a loss of carpel-bearing buds while stamen inhibition of perception would show no change in expression. However, *CRC::etr1-1* melons did not support this hypothesis; instead an increase in femaleness was observed. Conversely, *AP3::etr1-1* melons showed an almost complete loss of carpel-bearing buds. These results provide evidence that ethylene perception in the stamen primordia is required for carpel development, indicated that stamens are a regulator of carpels (Little et al., 2007).

Genetics of sex expression:

While ethylene is a key regulator of melon sex expression, genetics is also has a significant role in sex determination. Cucumber sex expression involves two major loci, *Female* (*F*) and *Monoecious* (*M*), along with a few minor loci (Perl- Treves, 1999). The *M* locus acts to inhibit stamens in carpel-bearing flowers and was found to be the ethylene biosynthetic enzyme ACC synthase, namely *CsACS2*, which localizes to the ovary in carpel-bearing buds (Kamachi et al., 1997; Yamasaki et al., 2003; Saito et al., 2007; Boualem et al., 2009, Li et al., 2009). Sequence analysis of *CsACS2* in monoecious (*MMffAA*), gynoecious (*MMFFAA*), androecious (*MMffaa*), and hermaphrodite (*mmFFAA*) lines pointed to a single nucleotide polymorphism (SNP) causing an amino acid change linked to the *M* locus (Boualem et al., 2009). Further analysis of 28 cucumber accessions revealed a second SNP associated with the *M* locus in hermaphrodite and andromonoecious lines. In a separate study, a third SNP causing a reduction in enzymatic activity was identified within *CsACS2* that also showed a link to the *M* locus (Li et al., 2009). Protein structure analysis revealed that all three mutations occurred in or near the catalytic site where SAM helps with positioning the pyridoxal 5'- phosphate (PLP) cofactor,

causing a reduction or loss of enzymatic activity (Huai et al., 2001; Rottman et al., 1991). This reduction in ethylene production results in failure to inhibit stamen primordia development in bisexual flowers.

The *F* locus, a promoter of carpel development, was mapped to an additional copy of *CsACS1*, namely *ACS1G* which is the result of a gene duplication and a recombination event involving a branched- chain amino acid transaminase gene (Trebitsh et al., 1997; Knopf and Trebitsh, 2006). This extra *ACS* can account for the 2-3 fold higher ethylene levels observed in gynoecious cucumbers (Rudich et al., 1972; Makus et al., 1975; Rudich et al., 1976; Trebitsh et al., 1987; Yamasaki et al., 2001, 2003). Studies of *ACO* gene expression following ethrel application showed increased expression of *ACO3* in apices (Kahana et al., 1999). *ACO3* expression was stronger in the developing stamen and carpel primordia of female buds, however response to application was dependant on whether the plant was gynoecious or androecious (only male flowers).

A minor locus, *Androecious* (*A*), has been linked to increased maleness in cucumbers as *aaff* plants fail to make carpel-bearing buds resulting in an androecious phenotype (Rudich et al., 1976). A series of other minor loci have been identified including *In-F*, *gy*, *m-2* and *Tr* (Kubicki 1969b; Malepszy and Niemirowicz-Szczytt, 1991; Robinson and Decker-Walters, 1997). The *m-2* (*andromonoecious-2*) gene is linked to development of bisexual flowers with normal ovary formation, while to the co-dominant *Tr* (*Trimonoecious*) allele allows carpel formation in male buds (becoming bisexual) with irregular ovaries (Malepszy and Niemirowicz-Szcytt, 1991; Robinson and Decker-Walters, 1997). *In-F* (*Intensifier-Female*) and *gy* (*gynoecious*) are both involved in enhanced femaleness in monoecious/gynoecious cucumber (Kubicki 1969b; Malepszy and Niemirowicz-Szcytt, 1991). Two new modifiers of femaleness, *Mod-F1* and *mod-*

Table 1.1. Melon sex expression patterns with corresponding genotypes and phenotypes.

Sex expression pattern	Genotype	Phenotype
Andromonoecious	*Ga*	♂⚥ - Male and bisexual flowers
Monoecious	*GA*	♂ ♀ - Male and female flowers
Gynoecious	*gA*	♀ - Female flowers only
Hermaphrodite	*ga*	⚥ - Bisexual flowers only

F2 were reported by Chen et al. (2011) to increase gynoecy independently of F in monoecious and hermaphrodite lines.

Analogous to cucumber, melon sex expression has two major loci controlling sex determination, *Andromonoecious* (*A*) and *Gynoecious* (*G*), along with one to two minor loci (Perl-Treves, 1999; Grumet and Taft, 2011). Table 1.1 outlines the different sex expression patterns in melon along with their corresponding genotype and flower phenotype. The *A* locus is similar to the *M* locus in cucumber which is involved in stamen inhibition, and found to be a new member of the ACS family, *CmACS7* (Boualem et al., 2008). Expression of *CmACS7* is localized to the carpel primordia of carpel-bearing buds at stage 7. Dominant *A* plants with a fully functional ACS7 enzyme inhibits stamens resulting in female flowers. In contrast, the recessive *a* allele has a missense mutation leading to a 50% reduction in enzymatic function from analysis of a segregating F_2 population from a cross between a monoecious (*AAGG*) and andromonoecious (*aaGG*) parents. Similar to the *M* locus SNPs, this missense mutation was found within the active site of ACS7, leading to the observed reduction in ethylene production and prevention of stamen inhibition in bisexual flowers.

The *G* locus, which is involved in carpel inhibition, was identified as the Wound Inducible Protein1 (WIP1) transcription factor by positional cloning from a segregating F_2 population from a monoecious (*GGAA*) and gynoecious (*ggAA*) cross (Martin et al., 2009). Plants with recessive *g* were found to have a transposon from the hAT family (Gyno-hAT) inserted in the 1.4kb non-coding region upstream of *CmWIP1*. The transposon insertion leads to the downstream methylation of *CmWIP1* inhibiting expression, resulting in the loss of carpel inhibition. Dominant *G* plants lack this insertion and have separate staminate flowers. *CmWIP1* localizes to the carpels of buds destined to be male, thus expression causes inhibition of carpels

in developing male buds. The identity of the *G* locus as neither an ethylene biosynthetic nor a perception gene, corresponds to earlier observations for the lack of a difference in ethylene levels in apices of gynoecious melon compared to monoecious (Byers et al., 1972a).

Interestingly, when localization of *CmACS7* was studied, it was found to reside in carpel primordia at Stages 4 and 7, and yet it influences stamen development (Boualem et al., 2008). This suggests that crosstalk between the stamen and carpel primordia is occurs during development. The *AP3::etr1-1* results show stamen-mediated inhibition of carpel development, also suggest a role for inter-whorl regulation during sex determination (Little et al., 2007). An epigenetic change of *CmWIP1* in melon leads to carpel inhibition, however a link to ethylene has yet to be discovered as yeast one-hybrid studies did not show an interaction between the *CmACS7* promoter and CmWIP1 protein (Martin et al., 2009). The idea of ethylene influencing the *G* locus is valid, as studies that blocked ethylene perception or biosynthesis with AVG and silver nitrate/thiosulfate caused increased maleness to occur in dominant *G* genotypes (Owens et al., 1980). Gene expression changes that occur within the stamen primordia after ethylene perception is unknown.

Transcriptomic gene expression studies of female and bisexual flowers in cucumber have provided information on candidates involved in carpel-bearing flower development, including known ethylene and other hormone genes, and transcription factor genes (Guo et al., 2010; Wu et al., 2010). While this information is a valuable resource in studying flower development, only later developmental stages, i.e. stage 8 and beyond, were analyzed, leaving gene expression changes at differentiation still unknown.

Proposed melon sex expression model:

A proposed model for sex determination in melon based on current knowledge is shown in Figure 1.2, and begins with the typical bisexual floral bud before developmental stage 5. The first decision point, whether to inhibit carpel development, occurs between developmental stages 5 and 6 in the floral bud (Bai et al., 2004). Previous studies suggest that if ethylene is not perceived in the stamen primordia, carpel development is inhibited (Little et al., 2007). Upon ethylene perception in the stamen primordia, the carpel primordia will continue developing. Based on this proposed model, increasing endogenous ethylene concentrations perceived by the stamen primordia potentially leads to increased femaleness. The *G* locus acts between stages 5 and 6 (Martin et al., 2009), however there is no current evidence for a link between ethylene and *G* locus expression.

The second decision point in melon sex expression, whether to inhibit stamens, occurs between developmental stages 6 and 7 (Bai et al., 2004). The *A* locus (or *M* locus in cucumber) acts between stages 6 and 7 where increased ethylene produced in the carpels by CmACS7 (or CsACS2) action leads to the inhibition of stamens in female flowers (Boualem et al., 2008, 2009; Li et al., 2009). The presence of a mobile signaling molecule is potentially acting to cause this regulation, which has yet to be discovered. It is unknown whether there is further involvement of endogenous ethylene biosynthesis, perception and related genes in the melon sex determination process.

Thesis objectives:

The overall objective for this study was to develop a comprehensive melon sex expression model that encompasses known phenotypes, genotypes, developmental stages,

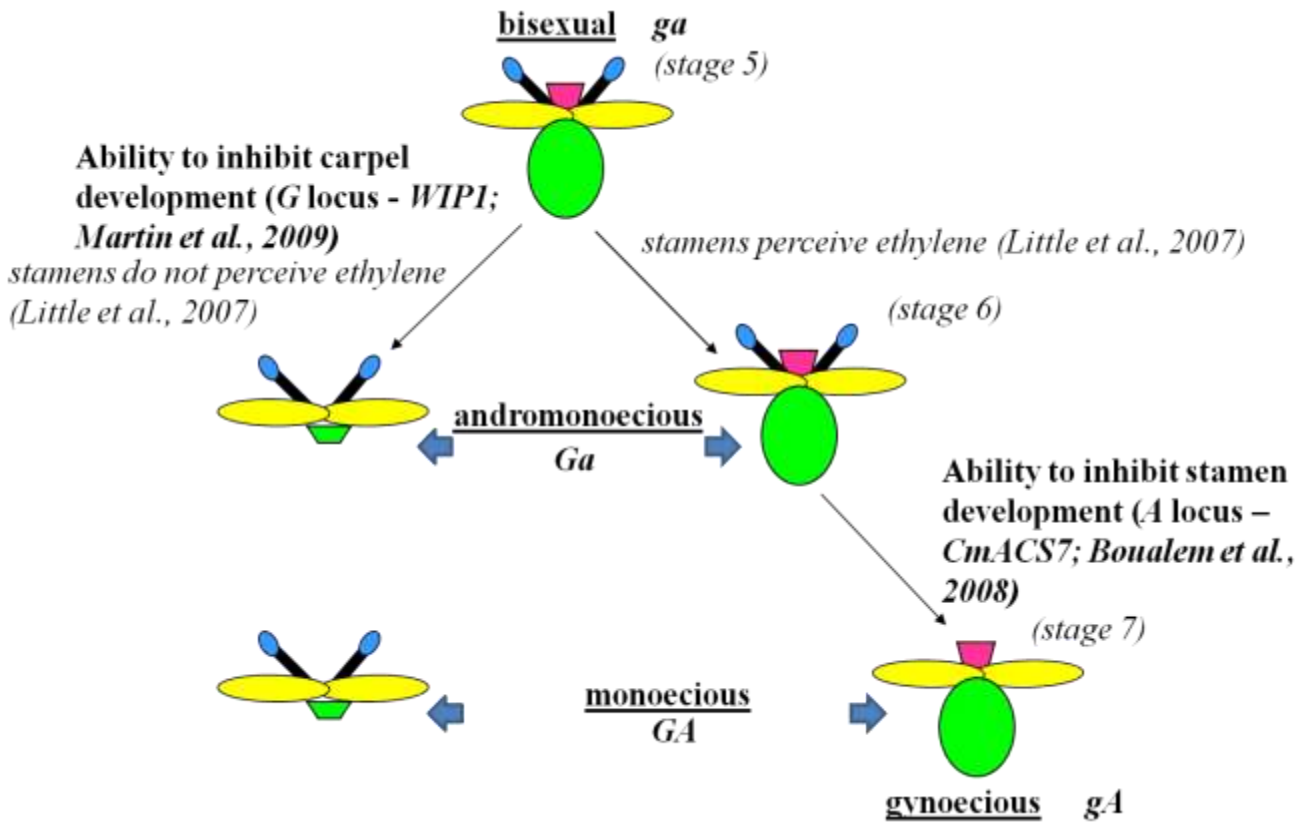


Figure 1.2. Progression of melon floral bud development including developmental stages (Bai et al., 2004), sex phenotypes (Goffinet, 1990), and native sex genes (Boualem et al., 2008; Martin et al., 2009).

ethylene influences, and gene expression. The previous *etr1-1* work provided evidence for the importance of ethylene perception in the stamens for carpel development (Little et al., 2007). However, the location of ethylene production that is perceived by the stamens is unknown. Utilization of transgenic melons targeting ethylene production to the stamen or carpel primordia will provide insight into the influence on sex expression. Based on the perception data, increased ethylene production in the stamens should show an increased femaleness phenotype if the stamens are major players in carpel development.

Increased femaleness observed in transgenic melons with ethylene perception inhibition targeted to the carpel primordia has been observed under greenhouse conditions (Little et al., 2007). This increased femaleness was manifested by increased and earlier carpel-bearing buds and flowers, and a ~50% conversion of bisexual-to-female flowers. Earlier and increased carpel-bearing flowers have a possible application in the field leading to earlier and increased fruit set. Two lines were observed in the field to determine the effect of carpel primordia-targeted ethylene perception on sex expression, and fruit set, development and ripening.

The two major sex loci of melon have been identified, however gene models exist where one or two minor loci are involved in bisexual development (Kubicki, 1969b; Kenigsbuch and Cohen, 1990). It is possible that one of these minor loci could be another member of the ethylene biosynthetic pathway or serve as a link to the interaction between ethylene and *WIP1*, and could be mediated by another ethylene biosynthetic enzyme. Melon has three *ACO* and four *ACS* members that have been published (Miki et al., 1995; Lasserre et al., 1996, 1997; Ishiki et al., 2000; Boualem et al., 2008), however only the major sex loci *ACS7* and *WIP1* have had their expression thoroughly studied (Boualem et al., 2008; Martin et al., 2009). The other members have been characterized in vegetative or fruit tissue, but not apices, buds or flowers where sex

determination occurs (Miki et al., 1995; Lasserre et al., 1997; Ishiki et al., 2000). Characterization of all of the members of *ACO* and *ACS* gene expression in andromonoecious, monoecious, gynoecious and hermaphrodite melon genotypes may elucidate one of these minor loci, and with the newly sequenced melon genome (Garcia-Mas et al., 2012), new members can be identified.

LITERATURE CITED

LITERATURE CITED

Abeles FB, Morgan PW, Saltveit MEJ (1992). Ethylene in plant biology. Academic Press Inc., New York.

Ainsworth C, Parker J, Buchanan-Wollaston V (1998). Sex determination in plants. In Curr Top Dev Biol 38: 167-223.

Ando S, Sato Y, Kamachi S, Sakai S (2001). Isolation of a MADS-box gene (*ERA17*) and correlation of its expression with the induction of formation of female flowers by ethylene in cucumber plants (*Cucumis sativus* L.). Planta 213: 943-952.

Argueso CT, Hansen M, Kieber JJ (2007). Regulation of ethylene biosynthesis. J Plant Growth Regul 26: 92-105.

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

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

Bai SL, Peng YB, Cui JX, Gu HT, Xu LY, Li YQ, Xu ZH, Bai SN (2004). Developmental analyses reveal early arrest of the spore-bearing parts of reproductive organs in unisexual flowers of cucumber (*Cucumis sativus* L.). Planta 220: 230-240.

Barrett S (1998). The evolution of plant sexual diversity. Nat Rev 3: 274-284.

Barry CS and Giovannoni JJ (2007). Ethylene and fruit ripening. J Plant Growth Regul 26: 143-159.

Bleeker AB, Estelle MA, Somerville C, Kende H (1988). Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science 241: 1086-1089.

Bleeker AB and Kende H (2000). Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Bi 16: 1-18.

Boualem A, Fergany M, Fernandez R, Troadec C, Martin A, Morin H, Sari M, Collin F, Flowers J, Pitrat M et al. (2008). A conserved mutation in an ethylene biosynthesis enzyme leads to andromonoecy in melons. Science 321: 836-838.

Boualem A, Troadec C, Kovalski I, Sari M, Perl-Treves R, Bendhamane A (2009). A conserved ethylene biosynthesis enzyme leads to andromonoecy in two *Cucumis* species. PLoS ONE 4: e6144.

Bowman JL (1997). Evolutionary conservation of angiosperm flower development at the molecular and genetic levels. J Bioscience 22: 515-527.

Bowman JL and Smyth DR (1999). *CRABS CLAW*, a gene that regulates carpel and nectary development in *Arabidopsis*, encodes a novel protein with zinc finger and helix-loop-helix domains. Development 126: 2387-2396.

Byers RE, Baker LR, Dilley DR, Sell HM (1972a). Chemical induction of perfect flowers on a gynoecious line of muskmelon, *Cucumis melo* L. HortScience 9: 321-331.

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

Chen YF, Etheridge N, Schaller GE (2005). Ethylene signal transduction. Ann Bot 95: 901-915.

Chen H, Tian Y, Lu XY, Lu XH (2011). The inheritance of two novel subgynoecious genes in cucumber (*Cucumis sativus* L.). Sci Hortic-Amsterdam 127: 464-467.

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

Colombo L, Franken J, Koetje E, Vanwent J, Dons HJM, Angenent GC, Vantunen AJ (1995). The petunia MADS box gene FBP11 determines ovule identity. Plant Cell 7: 1859-1868.

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

Duan QH, Wang DH, Xu ZH, Bai SN (2008). Stamen development in Arabidopsis is arrested by organ-specific overexpression of a cucumber ethylene synthesis gene *CsACO2*. Planta 228: 537-543.

Favaro R, Pinyopich A, Battaglia R, Kooiler M, Borghi L, Ditta G, Yanofsky MF, Kater MM, Colombo L (2003). MADS-box protein complexes control carpel and ovule development in Arabidopsis. Plant Cell 15: 2603-2611.

Galun E (1959). The role of auxin in the sex expression of cucumber. Physiol Plant 12: 48-61.

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

Galun E, Ishar S, Atsmon D (1965). Determination of relative auxin content in hermaphrodite and andromonoecious *Cucumis sativus* L. Plant Physiol 40: 321-326.

Garcia-Mas J, Benjak A, Sanseverino W, Bourgeois M, Mir G, Gonzalez VM, Henaff E, Camara F, Cozzuto L et al. (2012). The genome of melon (*Cucumis melo* L.). P Natl Acad Sci 109: 11872-11877.

Goffinet MC (1990). Comparative ontogeny of male and female flowers in *Cucumis sativus*. In: Biology and Utilization of the *Cucurbitaceae* (eds. DM Bates, RW Robinson, C Jeffery). Cornell University Press, New York: 288-304.

Grant S, Houben A, Vyskot B, Siroky J, Pan WH, Macas J, Saedler H (1994). Genetics of sex determination in flowering plants. Develop Genet 15: 214-230.

Grumet R and Taft J (2011). Sex expression in cucurbits. In: Genetics, Genomics and Breeding in Cucurbits. (eds) C Kole, YH Wang, TK Behera. Science Publishers Inc., Enfield and CRC Press (Taylor and Francis) group, Boca Raton: 353-375.

Guo S, Zheng Y, Joung JG, Liu S, Zhang Z, Crasta OR, Sobral BW, Xu Y, Huang S, Fei Z (2010). Transcriptome sequencing and comparative analysis of cucumber flowers with different sex types. BMC Genomics 11: 384-397.

Hao Y, Wang D, Peng Y, Bai S, Xu L, Li Y, Xu Z, Bai S (2003). DNA damage in the early primordial anther is closely correlated with stamen arrest in the female flower of cucumber (*Cucumis sativus* L.). Planta 217: 888-895.

Honma T and Goto K (2001). Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature 409: 525-529.

Huai Q, Xia Y, Chen Y, Callahan B, Li N, Ke H (2001). Crystal structure of 1-aminocyclopropane-1-carboxylate (ACC) synthase in complex with aminoethoxyvinylglycine and pyridoxal-5'-phosphate provide new insight into catalytic mechanisms. J Biol Chem 276: 38210-38216.

Huang S, Li R, Zhang Z, Li L, Gu X, Fan W, Lucas WJ, Wang X, Xie B, Ni P et al. (2009). The genome of cucumber, *Cucumis sativus* L. Nat Genet 41: 1275-U29.

Irish VF and Yamamoto YT (1995). Conservation of floral homeotic gene function between *Arabidopsis* and *Antirrhinum*. Plant Cell Reprod 9: 160-164.

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

Ishiki Y, Oda A, Yaegashi Y, Orihara Y, Arai T, Hirabayashi T, Nakagawa H, Sato T (2000). Cloning of an auxin-responsive 1-aminocyclopropane-1-carboxylate synthase gene (CMe-ACS2) from melon and the expression of ACS genes in etiolated melon seedlings and melon fruits. Plant Sci 159: 173-181.

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

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

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

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

Kendrick MD and Chang C (2008). Ethylene signaling: new levels of complexity and regulation. Curr Opin Plant Biol 11: 479-485.

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

Knopf R and Trebitsh R (2006). The female-specific *CsACS1G* gene of cucumber. A case of gene duplication and recombination between the non-sex specific 1-aminocyclopropane-1-carboxylate synthase gene and a branched chain amino acid transaminase gene. Plant Cell Physiol 47: 1217-1228.

Kubicki B (1969a). Sex determination in muskmelon (*Cucumis melo* L.). Genet Pol 10: 145-163.

Kubicki B (1969b). Investigations on sex determination in cucumber (*Cucumis sativus* L.). VI Androecium. Genet Pol 10: 87-99.

Lasserre E, Bouquin T, Hernandez JA, Bull J, Pech JC, Balague C (1996). Structure and expression of three genes encoding ACC oxidase homologs from melon (*Cucumis melo* L.). Mol Genet Genomics 251: 81-90.

Lasserre E, Godard F, Bouquin T, Hernandez JA, Pech JC, Rody D, Balague C (1997). Differential activation of two ACC oxidase gene promoters from melon during plant development and in response to pathogen attack. Mol Genet Genomics 256: 211-222.

Li Z, Huang S, Liu S, Pan J, Zhang Z, Tao Q, Shi Q, Jia Z, Zhang W, Chen H et al., (2009). Molecular isolation of the *M* gene suggests that a conserved-residue conversion induces the formation of bisexual flowers in cucumber plants. Genetics 182: 1381-1385.

Little HA, Papadopoulou E, Hammar SA, Grumet R (2007). The influence of ethylene perception on sex expression in transgenic melon as assessed by expression of the mutant ethylene receptor gene, *At-etr1-1,* under control of constitutive and floral-targeted promoters. Sex Plant Reprod 20: 123-136.

Magdum MB, Shinde NN, Seshadri VS (1982). Androecious sex form in muskmelon. Cucurbit Genet Coop 5: 24-25.

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

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

Martin A, Troadec C, Boualem A, Mazen R, Fernandez R, Morin H, Pitrat M, Dogimont C, Bendahamane A (2009). A transposon-induced epigenetic change leads to sex determination in melon. Nature 461: 1135-1139.

McMurray AL and Miller CH (1968). Cucumber sex expression modified by 2-chloroethane phosphonic acid. Science 162: 1397-1398.

Miki T, Yamamoto M, Nakagawa H, Ogura N, Mori H, Imaseki H, Sato T (1995). Nucleotide-sequence of a cDNA for 1-aminocyclopropane-1-carboxylate synthase from melon fruits. Plant Physiol 107: 297-298.

Nitsch JP, Kurtz EB, Liverman JL, Went FW (1952). The development of sex expression in cucurbit flowers. Am J Bot 39: 32-43.

O'Donnell PJ, Calvert C, Atzorn R, Wasternack C, Leyser HMO, Bowles D (1996). Ethylene as a signal mediating the wound response of tomato plants. Science 274: 1914-1917.

Owens KW, Peterson CE, Tolla GE (1980). Production of hermaphrodite flowers on gynoecious muskmelon by silver nitrate and aminoethyoxyvinylglycine. HortScience 15: 654-655.

Owino WO, Shoji T, Ezura H (2007). Isolation and characterization of an ethylene receptor homolog gene, Cm-ETR2, from melon fruit. Acta Hortic 731: 427-431.

Papadopoulou E and Grumet R (2005a). Brassinosteriod-induced femaleness in cucumber and relationship to ethylene production. HortScience 40: 1763-1767.

Papadopoulou E, Little HA, Hammar SA, Grumet R (2005b). Effect of modified endogenous ethylene production on hermaphrodite flower development and fruit production in melon (*Cucumis melo* L.). Sex Plant Reprod 18: 131-142.

Pelaz S, Ditta GS, Baumann E, Wisman E, Yanofsky MF (2001). B and C floral organ identity functions require *SEPALLATA* MADS-box genes. Nature 405: 200-203.

Perl-Treves R, Kahana A, Roseman N, Yu X, Silberstein L (1998). Cloning of three cDNAs encoding 1-aminocyclopropane-1-carboxylate oxidases from cucumber floral buds. Plant Physiol 116: 1192.

Perl-Treves R (1999). Male to female conversion along the cucumber shoot: approaches to study sex genes and floral development in *Cucumis sativus*. In: Sex Determination in Plants. CC Ainsworth (eds). BIOS Scientific, Oxford: 189-215.

Pinoyopich A, Ditta GS, Savidge B, Liljegren SJ, Baumann E, Wisman E, Yanofsky MF (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature 424: 85-88.

Renner SS and Ricklefs RE (1995). Dioecy and its correlates in the flowering plants. Am J Bot 82: 596-606.

Robinson RW, Shannon S, DeLaGuardia MD (1969). Regulation of sex expression in the cucumber. BioScience 19: 141-142.

Robinson RW and Decker-Walters DS (eds). (1997). Cucurbits. CAB International, New York: 9-10, 17-19, 195-198.

Roman G, Lubarsky B, Kieber JJ, Rothenberg M, Ecker JR (1995). Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana* – 5 mutant loci integrated into a stress response pathway. Genetics 139: 1393-1409.

Rottman W, Peter G, Oeller P, Keller J, Shen N, Nagy B, Taylor L, Campbell A, Theologis A (1991). 1-aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. J Mol Biol 222: 937-961.

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

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

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

Rudich J, Baker LR, Scott JW, Sell HM (1976). Phenotypic stability and ethylene evolution in androecious cucumber. J Am Soc Hort Sci 181: 48-51.

Rudich J (1990). Biochemical aspects of hormonal regulation of sex expression in cucurbits. In: Biology and utilization of the *Cucurbitaceae*. DM Bates, RW Robinson, C Jeffrey (eds). Cornell University Press, Ithaca: 288-304.

Saito S, Fujii N, Miyazawa Y, Yamasaki S, Matsuura S, Mizusawa H, Fuijta Y, Takahashi H (2007). Correlation between development of female flower buds and expression of the *CS-ACS2* gene in cucumber plants. J Exp Bot 58: 2897-2907.

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

Steptanova AN and Ecker JR (2000). Ethylene signaling: from mutants to molecules. Curr Opin Plant Biol 3: 353-360.

Takahashi H, Kobayashi T, Sato-Nara K, Tomita K, Ezura H (2002). Detection of ethylene receptor protein Cm-ERS1 during fruit development in melon (*Cucumis melo* L.). J Exp Bot 53: 415-422.

Theiben G and Saedler H (2001). Floral quartets. Nature 409: 469-471.

Tolla GE and Peterson CE (1979). Comparison of gibberellins A4/A7 and silver nitrate for induction of staminate flowers in a gynoecious cucumber line. HortScience 14: S61-S71.

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

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

Wang KLC, Li H, Ecker JR (2002). Ethylene biosynthesis and signaling networks. Plant Cell S131-S151.

Wang DH, Li F, Duan QH, Han T, Xu ZH, Bai SN (2010). Ethylene perception is involved in female cucumber flower development. Plant J 61: 862-872.

Wu T, Qin Z, Zhou X, Feng Z, Du Y (2010). Transcriptome profile analysis of floral sex determination in cucumber. J Plant Physiol 167: 905-913.

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

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

Yamasaki S, Fujii N, Takahashi H (2003). Characterization of ethylene effects on sex determination in cucumber plants. Sex Plant Reprod 16: 103-111.

Yamasaki S and Manabe K (2011). Application of silver nitrate induces functional bisexual flowers in gynoecious cucumber plants (*Cucumis sativus* L.). J Japan Soc Hort Sci 80: 66-75.

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

CHAPTER 2

Effect of floral primordia-targeted expression of the ethylene biosynthetic enzyme gene, *ACS*, on sex expression and native sex genes in melon (*Cucumis melo* L.)

Introduction:

Typical angiosperm species produce bisexual flowers that include both male and female organs (anthers and carpels, respectively). The Cucurbitaceae family, including melon (*Cucumis melon* L.) and cucumber (*Cucumis sativus*), is exceptional with a wide variety of heritable sex expression types that can produce varying combinations of male, female and/or bisexual flowers within a single species (Roy and Saran, 1990; Perl-Treves, 1999; Grumet and Taft, 2011). Melon plants are typically andromonoecious, starting with vegetative nodes followed by a male flower-only phase, then a male and bisexual flower phase. Many factors influence the sex expression of a developing floral bud, including developmental stage, hormones, genetics and environment. Developing cucumber floral buds go through a series of stages including carpel or stamen primordia arrest in male and female buds, respectively (Bai et al., 2004). Inflorescence initiation and sequential initiation of the four floral organ whorls (sepals, petals, stamens, and carpels) occurs during stages 1-5. Carpels are arrested in males at bud stage 6. In females, DNA degradation was observed in anther primordia at bud stage 7 and complete arrest of stamen development was seen at bud stage 8 (Hao et al., 2003; Bai et al., 2004).

Hormones are key factors in melon sex determination, with ethylene playing the predominant role (Kenigsbuch and Cohen, 1989; Rudich, 1990). The biosynthesis of ethylene begins with the conversion of *S*-Adenosylmethionine (*S*-Adomet) to 1-aminocyclopropane-1-carboxylate (ACC) by the rate-limiting enzyme ACC synthase (ACS). ACC is then oxidized to ethylene by ACC oxidase (ACO) (Johnson and Ecker, 1998). The *ACS* and *ACO* gene families have several members, with at least three members of each in melon (Miki et al., 1995; Lasserre et al., 1996; Ishiki et al., 2000; Boualem et al., 2008). Application of ethylene via ethephon has a feminizing effect on melon and cucumber plants that is observed as an increase in total number

and/or an earlier onset of carpel-bearing buds/flowers (i.e. conversion of male nodes to carpel-bearing nodes) and/or a conversion of bisexual to female buds (McMurray and Miller, 1968; Robinson et al., 1969; Rudich et al., 1969; Karchi, 1970; Augustine et al., 1973; Owens et al., 1980). Likewise, 2-3 fold higher endogenous ethylene levels were found in gynoecious cucumber apices compared to monoecious (Byers et al., 1972; Rudich et al., 1972; Makus et al., 1975; Rudich et al., 1976; Trebitsh et al., 1987; Yamasaki et al., 2001, 2003). Although high ethylene evolution was not observed in gynoecious melon apices, hypobaric studies in melon showed that reduced internal ethylene caused increased maleness that was reversible by ethylene application (Byers et al., 1972).

Inhibition of ethylene biosynthesis or perception via aminoethoxyvinylglycine (AVG) or silver nitrate/thiosulfate application leads to increased maleness in cucumber, melon, and certain genotypes of zucchini (*Cucurbita pepo*) (Byers et al., 1972; Den Nijs and Visser, 1980; Owens et al., 1980; Manzano et al., 2011). Our previous work showed that transgenic melons expressing the dominant negative *Arabidopsis thaliana* ethylene receptor mutant *etr1-1* under the 35S promoter exhibited an almost complete loss of carpel-bearing buds, demonstrating that ethylene perception is a requirement for carpel development (Little et al., 2007). To further elucidate the critical location of ethylene perception involved in sex determination, transgenic melon plants were produced to target *etr1-1* expression to stamen and petal primordia under the Arabidopsis *APETALA3* (*AP3*) promoter, or carpel and nectary primordia under the *CRABS CLAW (CRC*) promoter (Little et al., 2007). If perception in the carpels is required for carpel development, then inhibition of perception would show reduced production of carpel-bearing buds while stamen inhibition of perception would show no change in expression. *CRC::etr1-1* melon plants continued to make carpels, however *AP3::etr1-1* plants showed an almost complete loss of

carpel-bearing buds (Little et al. 2007). These results indicated that the stamen primordia, not carpel primordia, need to perceive ethylene in order for the carpel primordia to develop and implicates cross talk between the sex organ whorls.

Genetic analyses show that two major loci control sex determination in melon: the *A* locus which inhibits stamens in carpel-bearing flowers, and the *G* locus which inhibits carpels in male flowers (Kenigsbuch and Cohen, 1989; Perl-Treves, 1999; Boualem et al., 2008; Martin et al., 2009). The identity of the two major sex loci has recently been identified. The *G* locus encodes a transcription factor named Wound Inducible Protein1 (CmWIP1) (Martin et al., 2009). The mutant form, *g*, is silenced by a methylated transposon sequence inserted upstream of its promoter, resulting in plants for which all flowers are carpel-bearing, i.e. are either gynoecious (only female), or hermaphrodite (only bisexual) (Martin et al., 2009). Expression of the *WIP1* gene is localized to the carpels of buds destined to be male, causing inhibition of carpels in developing male buds (Martin et al., 2009). Regulation of *WIP1* expression has not been determined. The *A* locus encodes the ethylene biosynthetic gene, ACC synthase 7 (*CmACS7*) that is expressed in carpel primordia (Boualem et al., 2008). A fully functional ACS7 can inhibit stamen primordia to form a female flower, however the mutant ACS7, which has reduced enzymatic activity, cannot suppress stamens resulting in bisexual flowers (Boualem et al., 2008). Interestingly, although *CmACS7* inhibits stamen development, expression occurs in carpel primordia during sex determination stages (Boualem et al., 2008). This complements the *AP3::etr1-1* results indicating inter-whorl regulation of sex determination (Little et al., 2007).

The stages involved in melon sex determination based on our knowledge to date, are presented in a model in Figure 2.1. Early melon floral bud development includes initiation of all four floral organ whorls, thus providing the ability to produce both stamens and carpels (Goffinet,

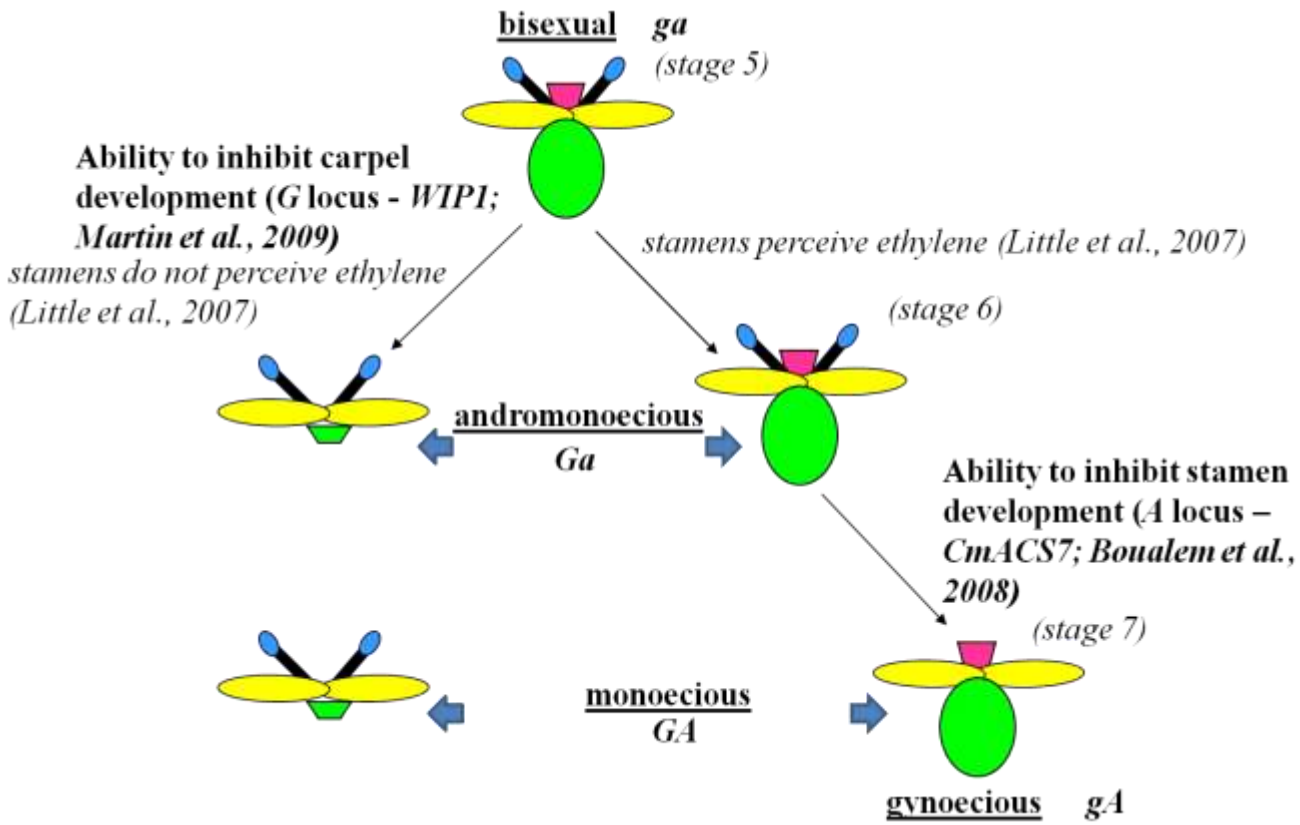


Figure 2.1. Progression of melon floral bud development including developmental stages (Goffinet, 1990; Bai et al., 2004), native sex genes (Boualem et al., 2008; Martin et al., 2009), and sex phenotypes (Kenigsbuch and Cohen, 1990).

1990; Bai et al., 2004). The first critical step in sex differentiation occurs between stages 5 and 6; the choice to inhibit the carpel to become male, or allow the carpels to develop. In buds destined to be male, expression of the *WIP1* transcription factor in the carpel primordia (*G* locus), and/or the failure of the stamen primordia to perceive ethylene, leads to carpel primordia arrest (Little et al., 2007; Martin et al., 2009). The second critical step of sex differentiation occurs between stages 6 and 7 for carpel-bearing buds; whether to inhibit the stamens to become female, or remain bisexual. For both developmental paths, the carpel primordia initiate at stage 5 and the stamen primordia continue to differentiate. In female flowers, increased ethylene produced by a functional ACS7 (*A* locus) in the carpel primordia leads to stamen primordia arrest at stage 8 (Boualem et al., 2008).

At the first decision point during melon sex determination, ethylene perception by the stamen primordia is critical for carpel primordia development. However, the location, timing, and level of ethylene production required to promote carpel development and inhibit stamen development is not well defined. To better understand the role of ethylene production, transgenic melons were used to study the effect of ethylene produced by either the stamen or carpel primordia. Sex organ primordia-targeted ethylene production was also investigated to determine if there is an effect on native sex gene expression. Ethylene production targeted to the stamen primordia, but not carpel primordia, led to increased femaleness and changes in sex gene expression.

Materials and methods:

Plasmid construction and plant transformation

Construct development for the *AP3::ACS* and *CRC::ACS* plasmids was as described in

Little et al. (2007), for *AP3::etr1-1* and *CRC::etr1-1*, with the following changes. The *etr1-1* gene was removed from pCambia2300-*CRC::etr1-1*or pCambia2300-*AP3::etr1-1* by *BamH1* digestion and replaced with the petunia *ACS* from pCambia2300-*35S::ACS* (Papadopoulou et al., 2005), after polymerase chain reaction (PCR) amplification to introduce a *BamH1* site at the 5' and 3' ends using site-specific primers (Forward - 5'-CCCGGATCCCTCTCTCTCTCTCCTCGTGCC (RG280); Reverse – 5'GGGGGATCCGGGCGAATTGGGTACCGGGCC (RG281)).

The *AP3::ACS* and *CRC::ACS* constructs were transferred via *Agrobacterium tumefaciens* into the andromonoecious cv. Hale's Best Jumbo (Hollar Seed, Rocky Ford, Colorado) melon as described in Fang and Grumet (1990) with added revisions described in Little et al (2007). Putative transgenic regenerated shoots (T_0) were evaluated for *ACS* transgene presence using PCR and quantitative reverse transcriptase PCR (qRT-PCR) as described below. PCR-positive T_0 were self or cross-pollinated to produce T_1, BC_1 and BC_1S_1 progeny. T_1, BC_1 and BC_1S_1 plants were grown under greenhouse conditions as described in Little et al. (2007). T_1 *AP3::etr1-1*-3 plants were from Little et al. (2007).

PCR and qRT-PCR analysis

Genomic DNA was extracted from melon leaf tissue using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI), and PCR performed to amplify the inserted *ACS* transgene using construct specific primers. *CRC::ACS* was amplified with the *CRC* specific primer 5'-CTTGCAATCCCTAGCCAG (RG299) and 3' *ACS* primer 5'-

CCAGCGTTACTCTTCAATGC (RG310). *AP3::ACS* was amplified using the *AP3*-specific primer 5'-GCTTTGGTCCCCCTCTTTTACC (RG300) and the 3' *ACS* primer RG310.

For gene expression analyses by qRT-PCR, total RNA and cDNA was prepared from 200mg of melon apex tissue from T_1 *CRC::ACS* 168 and 169, BC_1S_1 *AP3::ACS* A4 and A5, and T_1 *AP3::etr1-1*-3 plants. Total RNA was isolated from liquid nitrogen frozen tissue using Trizol reagent based on manufacturer's recommendations (Invitrogen, Carlsbad, CA), followed by DNase treatment, and cleaned using RNeasy columns (Qiagen, Valencia CA). Quantification of RNA was performed using a spectrophotometer (ND-2000c; Nanodrop Technologies, Wilmington, DE); agarose gel electrophoresis was used to further assess quality. 2ug of RNA was used for first-strand cDNA synthesis using the SuperScript II reverse transcriptase kit (Promega, Madison, WI). Gene-specific primers were designed using Primer Express software (Applied Biosystems). The *ACS* transgene-specific primers were; Forward – 5'GCTTGCAACCATGTTATCCG (RG319) and Reverse 5'-CCAACCTGGTTCAGAACAATG (RG320). Reactions were carried out on the Stratagene Mx4000 machine using Power SYBR Green PCR Master Mix (Applied Biosystems). PCR quantification was performed via standard curve reference for each gene. *C. melo Polyubiquitin 7* (ICuGI: MU43954) was used as an endogenous control for normalization with the following primer sequences: 5'-GTTGGGGATGAGAAACGGC-3' (RG317), and 5'-GCCCACTACCACCCGAGC- 3' (RG318). Each experiment was conducted three times with 3-4 biological replicates per genotype (each biological replicate was composed of 3 lateral apices 3-4mm in size, removed from branches on nodes 5-6 on 2 month-old plants), and three technical replicates/sample.

Diurnal gene expression analysis and ethephon treatments

Hale's Best Jumbo seeds were pre-germinated on dampened Whatman #2 filter paper for 24 hours in the dark at 37°C and then sown into 4.8cm x 3.8cm plastic pots filled with Suremix Perlite soil medium (Michigan Grower Products Inc., Galesburg, MI). Seedlings were grown in a growth chamber with a 10 hour/14 hour light dark cycle at 24°C and 66.5% humidity. Apices were removed from 10 day-old plants (second true leaf beginning to emerge) with a razor blade one hour before the beginning of the light cycle (-1 hour) and at 3 hr, 6 hr, 9 hr and 12 hr time points for diurnal experiments. For the ethephon experiments, 20uL of 300ppm ethephon or water with 1% Tween (control) was dropped onto apices and collected at 0 hour (beginning of light cycle), 8 hours and 11 hours post treatment. RNA extraction and qRT-PCR analysis of native sex gene expression followed the above procedure. Each experiment was conducted twice with four apices/time point (one biological replicate) and three biological replicates using a completely random design.

Ethylene evolution measurements

Two lateral apices (~3-4mm in size) were excised from each 2 month-old melon plant using a razor blade from node positions 5 and 6 (1 replicate), weighed, and placed into a 10mL plastic syringe containing a 5mm diameter Whatman filter paper #2 disc moistened with distilled water (dH_2O). A rubber septum was fitted on the opposite end and wrapped with Parafilm to create a tight seal before the plunger was inserted to the 3mL mark after 1 hour rest period to allow for dissipation of wound ethylene. Headspace gas samples were taken after 2.5 hours with a 1mL syringe and analyzed on a gas chromatograph (HACH CARLE series 100 AGC, Linear

1200 recorder) with an activated alumina column and flame ionization detector with a 1ppm ethylene control. The experiment was conducted three times with six replicates/genotype. A subset of 3 replicates/genotype was selected for qRT-PCR analysis as described above to verify expression of the transgene after headspace samples were taken. Gene expression trends did not change during the period of time for sampling of ethylene evolution.

Promoter analysis

The *WIP1* promoter sequence has been published (Martin et al., 2009) and was used for regulatory element and putative transcription factor binding site analysis. The 3kb sequence upstream of the transcription start site of *ACS7* was kindly provided by Jordi Garcia-Mas at the Universitat de Barcelona in Barcelona, Spain and was used for identification of promoter sequences using the TSSP software (SoftBerry: http://linux1.softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=promoter). Regulatory element and transcription binding site analysis for the promoter regions utilized PlantPAN (Chang et al., 2008) and PLACE (Higo et al., 1999; Prestridge, 1991).

Sex expression and floral bud microscopy

The T_1/BC_1 or BC_1S_1 generation of *AP3::ACS* and *CRC::ACS* transgenic melon plants and their parental and azygous controls were grown under greenhouse conditions as described in Little et al. (2007). Each plant was observed for presence of staminate and/or carpel-bearing buds, flowers at anthesis, and first node of appearance, for 30 nodes on the main stem. Experiments were completed 2-3 times with 4-6 replicates per genotype per experiment in a randomized complete block design.

Examination of floral bud development via microscopy was completed on apices collected from greenhouse grown melon plants at the 1 true leaf (TL), 2 TL, 3 TL and 4 TL stages under greenhouse conditions as described in Little et al. (2007) with 5-6 replicates/genotype/plant age. Fixation, dehydration, and paraffin-embedding of collected apical tissue were performed based on procedures adapted from Takahashi et al. (2010), Ohtsu et al. (2007), Kidner and Timmermans (2006), and Jackson (1991), and briefly described here. Apices were collected with a razor blade and placed in an ice-cold mixture of 75% ethanol/25% acetate fixative solution in 15mL Falcon tubes and subjected to vacuum infiltration for 15 minute intervals, three to four times, followed by replacement of the fixative with fresh solution and placed on a rotator overnight at 4°C. The next morning samples were placed in fresh fixative for 30 minutes, followed by an ethanol dehydration series: 30% ethanol for 1 hour, 50% ethanol for 1 hour, and 70% ethanol overnight, all steps were completed on a rotator at 4°C. Tissues were then placed in 85% ethanol for 1 hour then 95% ethanol with 2% eosin added overnight; both were completed on a rotator at 4°C. The following morning, tissues were moved into glass bottles with 100% ethanol at room temperature on a rotator for 30 minutes then subjected to xylene series at room temperature and shaking: 25% xylene/75% ethanol for 30 minutes, 50% xylene/50% ethanol for 30 minutes, 75% xylene/25% ethanol for 30 minutes, and 100% xylene for 1 hour. The xylene was removed from the bottles and 100% xylene and ¼ volume paraplast chips (Sigma-Aldrich, St. Louis, MO) were added and let sit overnight. Samples were then placed at 42°C until chips melted and moved to 60°C with an addition of another ¼ volume of chips. Freshly melted paraplast wax was added to the bottles twice a day for three days before embedding into plastic molds for sectioning and stored at 4°C. Paraffin sections were cut at 10um using a Leica rotary microtome (2125RT; Leica Microsystems, Buffalo Grove, IL) and

imaged using a light microscope with Spot RT3 Digital Camera System (SPOT Imaging Solutions, Diagnostic Instruments Inc., MI). Developing floral buds were characterized using floral bud stages described in Bai et al. (2004) based on stamen and carpel primordia morphology, and the number of buds observed at specific stages was recorded for each apex.

Statistical analyses

Data were analyzed by analysis of variance (ANOVA) using Proc GLM or mixed protocol in SAS (SAS Institute, Cary, NC). Different letters represent significant differences at $P<0.05$ using LSD.

Results:

Gene integration and expression

The *CRC::ACS* and *AP3::ACS* transgenes were introduced into andromonoecious (cv. Hale's Best Jumbo) melon plants via *Agrobacterium*-mediated transformation, verified to contain the respective transgenes using PCR, and self-pollinated or back-crossed to wild type Hales to produce T_1, BC_1 and BC_1S_1 progeny (Appendix). *ACS* transgene expression was detected within apical tissue of PCR-positive progeny in all lines tested (Figure 2.2a-b). Apices from *AP3::ACS* plants had a 3-8 fold increase in ethylene evolution compared to controls (Figure 2.2c). Apical ethylene evolution from *CRC::ACS* line 169 T_1 plants showed two-fold higher ethylene, but not in the 168 line (Figure 2.2d).

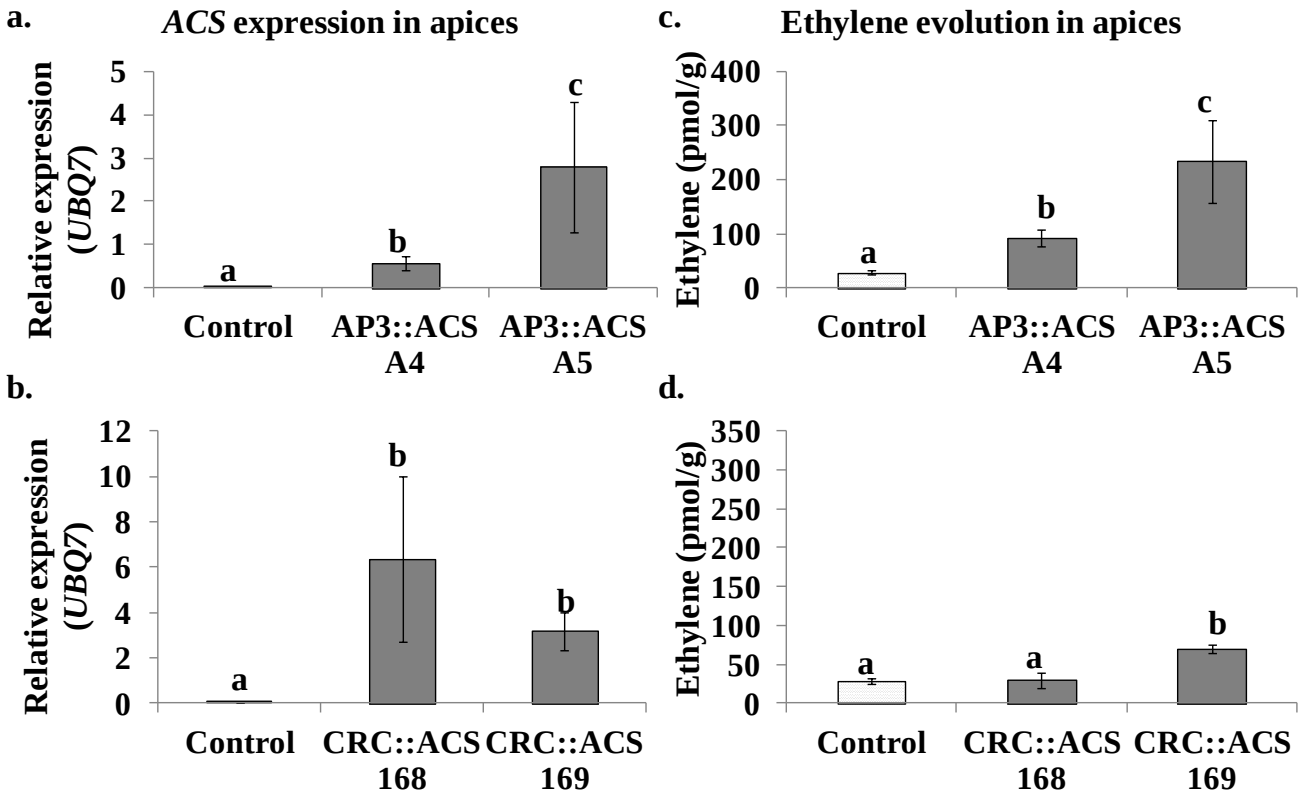


Figure 2.2. qRT-PCR analysis of *ACS* transgene expression (a,b) and ethylene evolution (c,d) in lateral apices of Control (WT and azygous), *AP3::ACS* A4 and A5 BC_1S_1 plants (a,c), and *CRC::ACS* 168 and 169 T_1 plants (b,d) generations. Values shown for qRT-PCR are the average of three biological replicates with three technical replicates/biological replicate $\pm$ SE. Values presented for ethylene evolution are the average of two experiments with 5-6 replicates with 1-2 plants/rep and 1-2 lateral apices/plant/genotype/experiment $\pm$ SE. Different letters represent significant differences by LSD at $P<0.05$.

Sex expression phenotypes

The typical sex expression phenotype of melon is a vegetative phase, followed by a phase with only male flowers, and then a mix of male and bisexual flowers. This pattern was observed in wild type Hale's Best Jumbo and azygous segregant progeny from the *CRC::ACS* and *AP3::ACS* lines (Figure 2.3a). No significant difference was observed in the sex expression pattern of transgenic *CRC::ACS* 168 and 169 plants (Figure 2.3a), or the number of male or bisexual buds and open flowers compared to wild type and azygous plants (Figure 2.3b).

In contrast, sex expression along the main stem of *AP3::ACS* plants showed an increased femaleness phenotype as manifested by a loss of the male-only phase, and gain of a bisexual-only phase, not observed in wild type and azygous siblings (Figure 2.4a). Onset of first carpel-bearing bud was not affected (Figure 2.4a). BC_1S_1 A4 and A5 *AP3::ACS* plants showed a marked decrease in the number of male buds and open flowers in PCR verified plants (Figure 2.4c-d). Variation in duration of the bisexual-only phase was observed in A5 plants, where approximately half of the plants with verified transgene presence completely lost their male buds, essentially becoming hermaphrodite plants (Figure 2.4b). The *AP3::ACS* A4 line also showed a significant increase in the number of carpel-bearing buds and open flowers (Figure 2.4e-f). Equivalent results were observed in both the BC_1 and BC_1S_1 generations.

Floral bud development within the apex

A possible explanation for the loss of male buds is that higher ethylene produced by stamen primordia in the *AP3::ACS* transgenic plants led to premature stamen primordia arrest and a subsequent failure of the bud to develop. Paraffin sectioned apices of wild type, azygous and both transgenic lines were examined to determine floral bud developmental stages (Figure

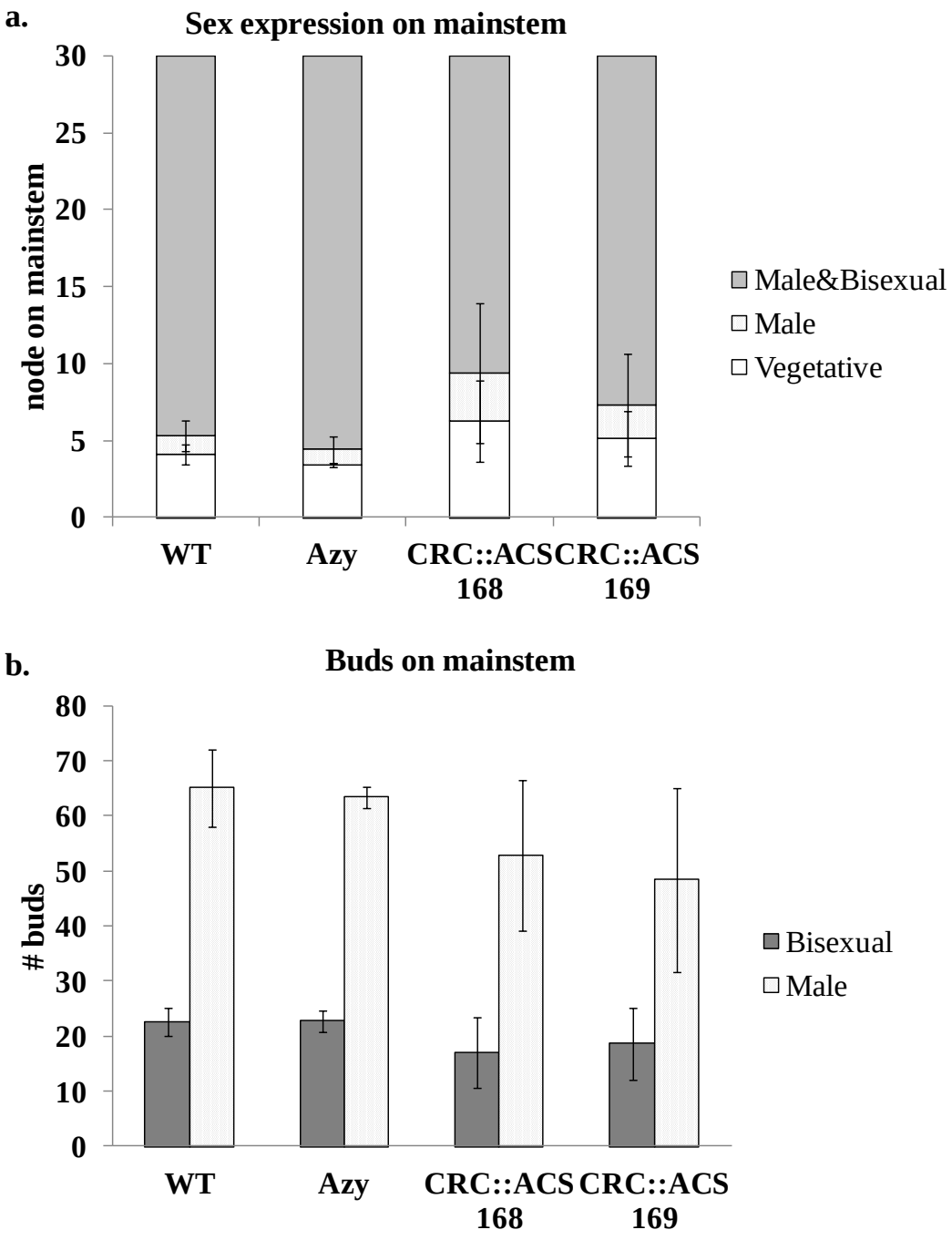


Figure 2.3. Greenhouse observations of sex expression for WT, azygous, and T_1 *CRC::ACS* 168 and 169 plants along the first 30 nodes of the main stem (a), and number of carpel-bearing (bisexual) and male buds (b). Values presented are the average of two experiments with 2-3 replicates/experiment ± SE.

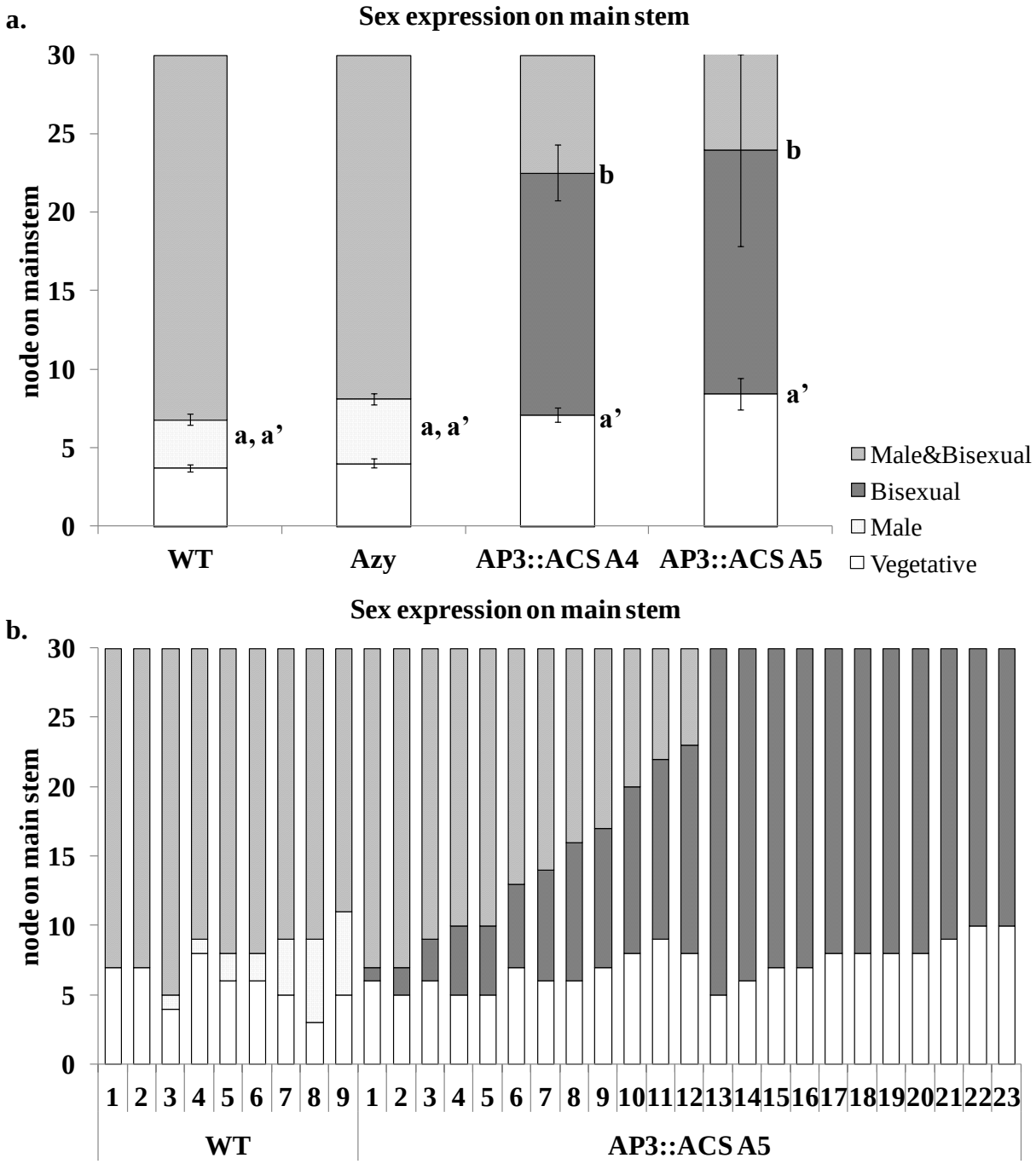
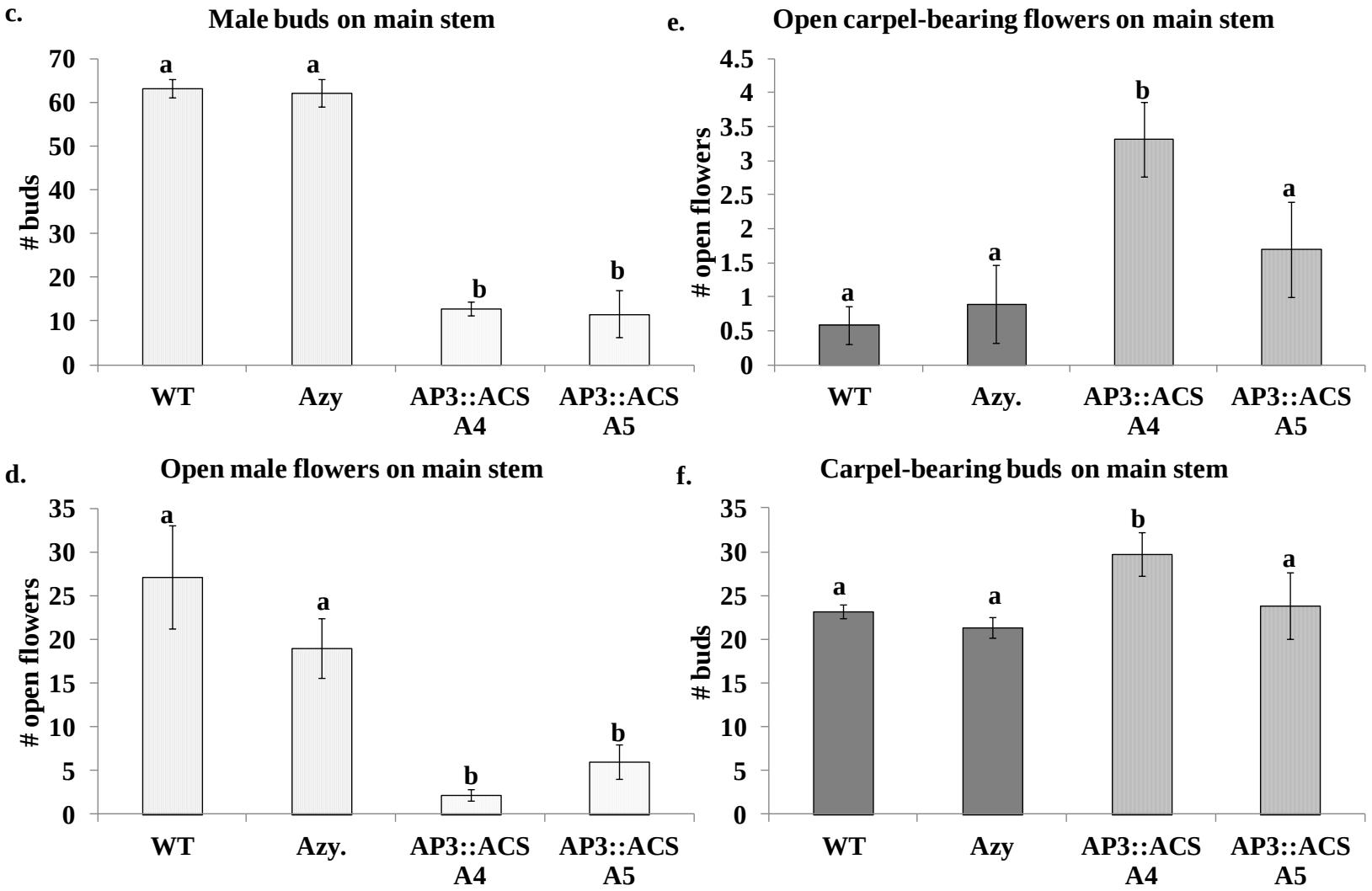


Figure 2.4. Greenhouse observations of WT, azygous, and BC_1S_1 *AP3::ACS* A4 and A5 plants for sex expression along the first 30 nodes of the main stem (a), individual WT and *AP3::ACS* A5 plants (b), number of male buds (c) and open flowers (d), and number of carpel-bearing (bisexual) buds(e) and open flowers (f). Values presented for a,c,d,e and f are the average of two experiments with 2-3 plants/genotype/replicate and 6 rep/experiment $\pm$ SE. Equivalent results were observed in both the BC_1 and BC_1S_1 generations. Different letters represent significant differences by LSD at $P<0.05$. In panel (a), a,b notation refers to onset of male and bisexual phase; a,a' notation refers to first node of carpel-bearing buds.

Figure 2.4 (cont'd)



2.5a-h). A lower percentage of buds were seen in the post-sex determination stages (5-9) in the *AP3::ACS* A4and A5 lines with an average of 37% in the post-sex determination for wild type and azygous versus 15% for *AP3::ACS* lines (*P*= 0.055) (Figure 2.5i).

Native sex gene expression

qRT-PCR analysis of the two major native sex loci in melon, *G* (*WIP1*) and *A* (*ACS7*), was performed on apical tissue of *CRC::ACS* T_1, and *AP3::ACS* BC_1S_1transgenic plants to investigate the effect of the introduced gene. No significant difference in gene expression for the two loci was detected in *CRC::ACS* plants; however *AP3::ACS* A4 showed a significant down-regulation of *WIP1*, and both *AP3::ACS* lines showed up-regulation of *ACS7* (Figure 2.6). Conversely, transgenic *AP3::etr1-1* melons, which showed an almost complete loss of carpel-bearing buds (Little et al., 2007), showed increased *WIP1* expression and decreased expression of *ACS7* (Figure 2.6).

To obtain baseline information about *WIP1* and *ACS7* expression in wild type plants, Hales was grown under controlled conditions over a time course of 12 hours. Control apices showed a diurnal pattern of *WIP1* expression, peaking late in the light cycle (Figure 2.7a). *ACS7* expression was generally constant throughout the cycle (Figure 2.7a).

The effect of increased ethylene on the *G* and *A* sex genes also was tested by application of ethylene application to apices at the beginning of the light cycle (0 hour). *G* and *A* expression was subsequently examined at 8 hours (diurnal peak for *WIP1*) and 11 hours (dark) post-treatment. Expression of the known ethylene responsive gene, *Ethylene Response Factor1* (Solano et al., 1998) from melon (*CmEBF1*), increased with ethephon treatment (Figure 2.7b).

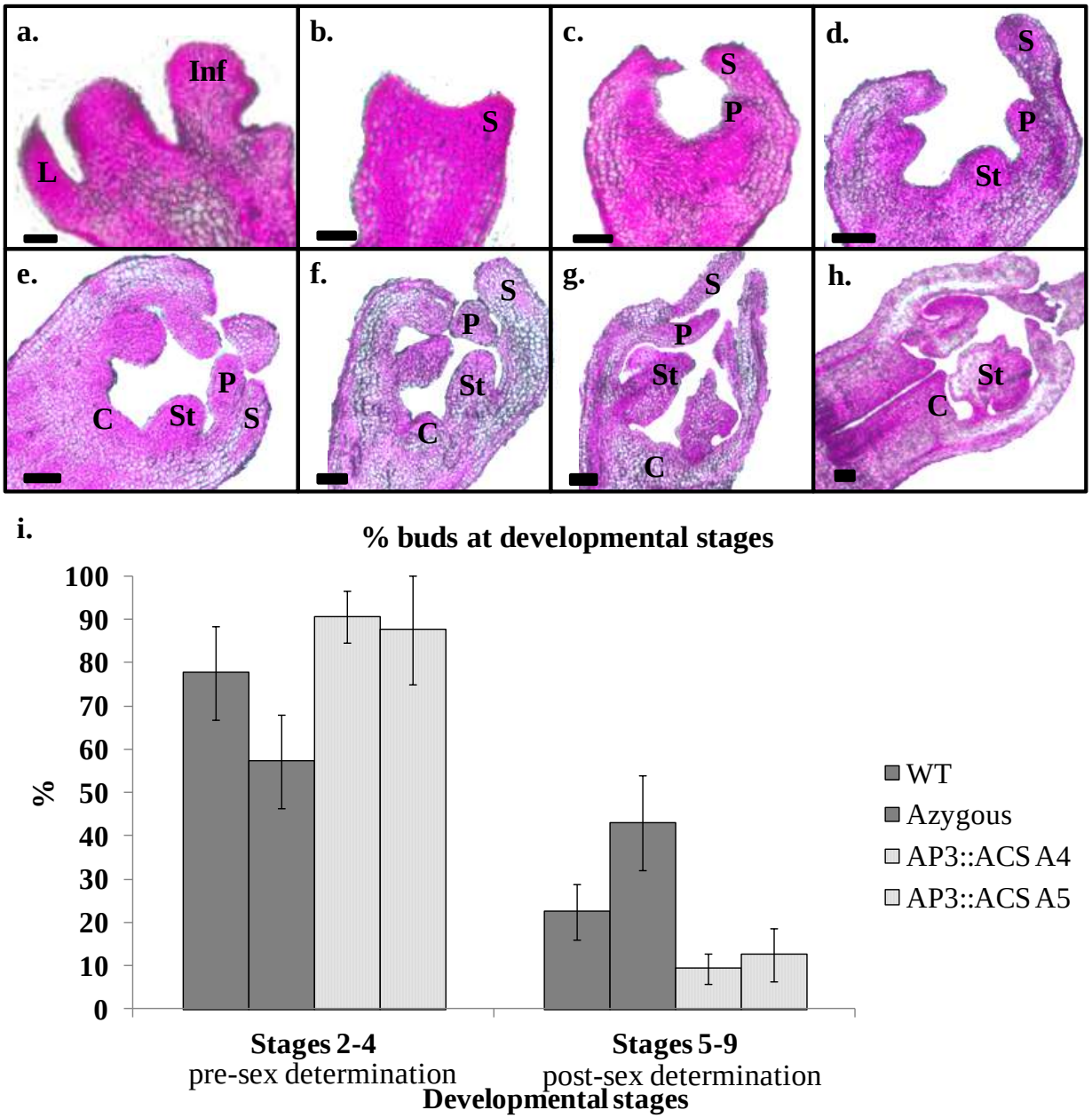


Figure 2.5. Microscopy images of melon floral buds at inflorescence stage 1(a), stage 2 (b), stage 3 (c), stage 4 (d), stage 5 (e), stage 6 (f), stage 7 (g), and bisexual bud at stage 8 (h) based on Bai et al. (2004) cucumber stages. L= leaf primordia, Inf= inflorescence meristem, S= sepal primordia, P= petal primordia, St= stamen primordia, and C= carpel primordia. Black bars represent 50 µm at 100x magnification (a-g), and 40x magnification (h). Percentage of floral buds at different stages of development observed in microtome-sectioned apices of WT, azygous, and BC_1S_1 *AP3::ACS* A4 and A5 under light microscopy (i).Values presented are an average of 5-6 replicates/time point $\pm$ SE.

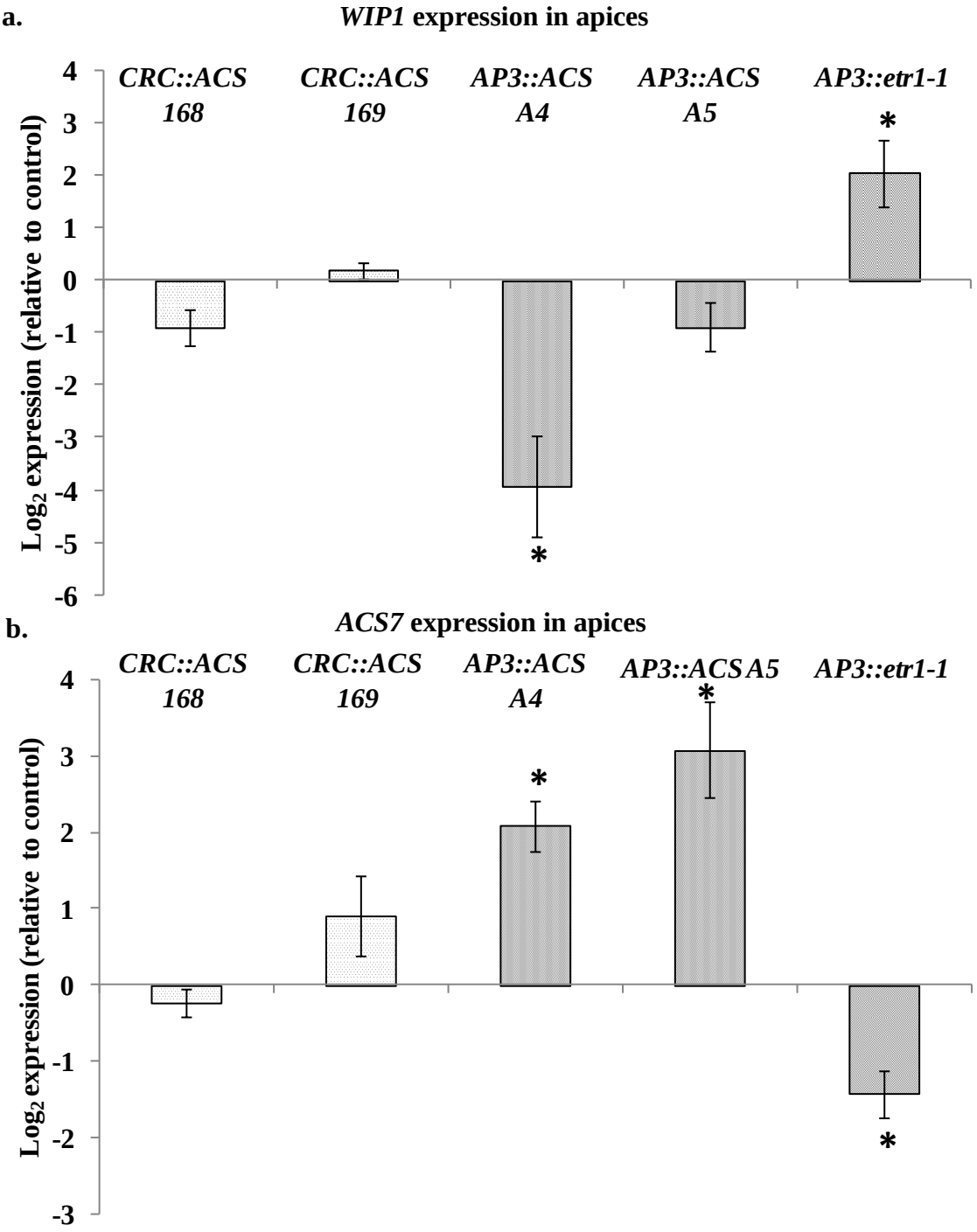


Figure 2.6. qRT-PCR analysis of the melon sex genes *WIP1* (a) and *ACS7* (b) in apices of T_1 *CRC::ACS* 168 and 169, BC_1S_1 *AP3::ACS* A4 and A5, and T_1 *AP3::etr1-1* plants relative to expression of wild type controls. Values shown are the average of three biological replicates with three technical replicates/biological replicate $\pm$ SE. * represent significant differences by t test at $P<0.05$.

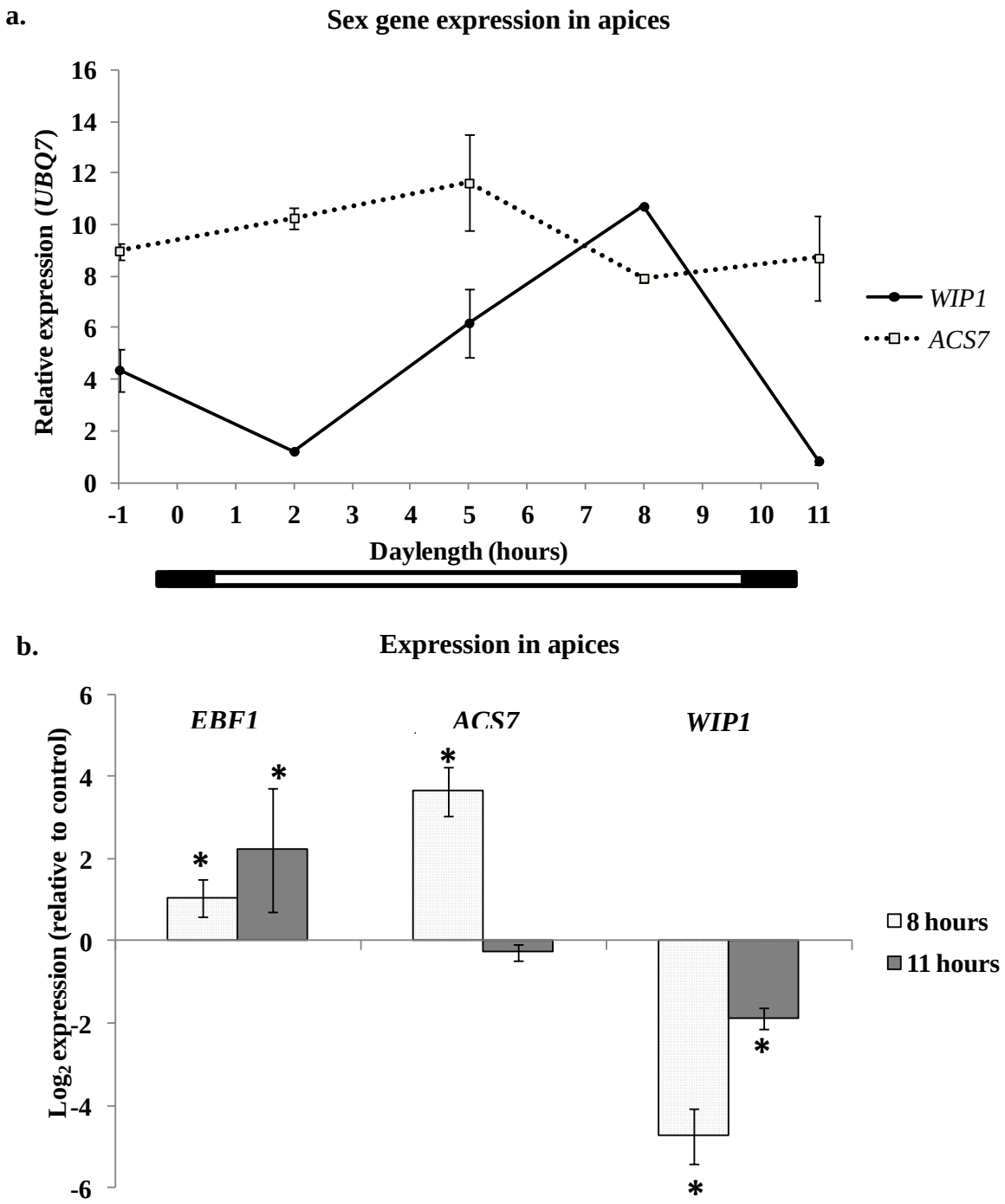


Figure 2.7. qRT-PCR analysis of *ACS7* and *WIP1* expression (a) in wild type melon apices taken at -1 hr, 2 hr, 5 hr, 8 hr and 11 hr relative to a 10 hour light cycle, and *EBF1*, *ACS7*, and *WIP1* expression (b) in ethephon treated apices at 8 hr (light) and 11 hr (dark) post-treatment relative to untreated controls (log_2). Values shown are the average of two experiments with three biological replicates with three technical replicates/biological replicate $\pm$ SE. Trends were equivalent for both experiments. * represent significantly different from untreated control by t test at $P<0.05$.

As was observed in the apices of *AP3::ACS* transgenic melons (Figure 2.6), *WIP1* expression decreased in response to ethylene; *ACS7* expression showed a transient increase in expression (Figure 2.7).

WIP1 and ACS7 promoter elements

Analysis of the promoter areas of *WIP1* for known regulatory elements and transcription factor binding sites showed a densely packed set of ethylene and light responsive elements in the first 500bp of sequence. There was an ethylene response element (ERE) at -340bp (Itzhaki et al., 1994); a putative ERFBP binding site at -442bp (Riechmann and Meyerowitz, 1998); and multiple light-regulatory elements: GATA box, GT1 motif, and circadian motif, at positions -83bp, -90bp, -359bp, and -485bp (Figure 2.9a) (Terzaghi and Cashmore, 1995; Arguello-Astorga and Herrera-Estrella, 1998). Three promoter regions were predicted for *ACS7*at positions -263bp, -2386bp, and -2859bp. The longest predicted promoter was used for analysis based on average predicted promoter lengths of *CsACS1G* and *AtACS7* (Wang et al., 2004; Wu et al., 2012). The *ACS7* promoter had ERE elements at -2733bp and -1409, and a putative binding site for a tobacco EIN3-like protein (NbEIL) at -2279 (Figure 2.9b)(Kosugi and Ohashi, 2000). No light-regulatory elements or putative light-regulated protein binding sites were predicted for the *ACS7* promoter.

Discussion:

Ethylene evolution and native sex genes

Ethylene has long been identified as the predominant hormone involved in sex

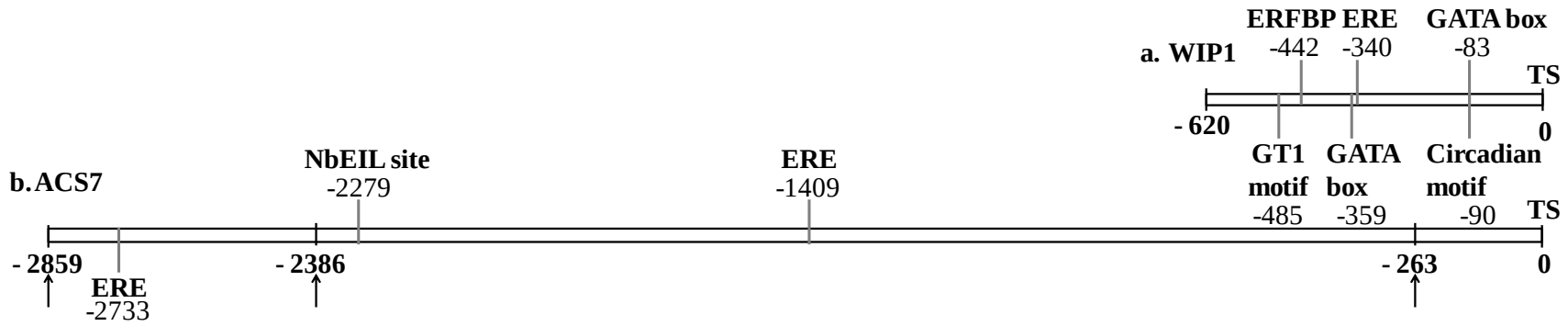


Figure 2.8. Diagram of the promoter of *WIP1* (a) and *ACS7* (b) with positions of putative regulatory elements and transcription factor binding sites for ethylene responsive element (ERE), tobacco EIN3-like binding site (NbEIL), ERF binding site (ERFBP), GATA box, and GT1 motif (Itzhaki et al., 1994; Terzaghi and Cashmore, 1995; Riechmann and Meyerowitz, 1998; Arguello-Astorga and Herrera-Estrella, 1998). Arrows indicate predicted promoter start sites.

determination in cucurbit species as evidenced by effects of exogenous application, sex genotype-related differences, and endogenous ethylene levels (Rudich, 1990; Perl- Treves, 1999; Grumet and Taft, 2011). Cloning of the key sex expression genes, *F* and *M* in cucumber, and *A* in melon (Trebitsh 1997; Knopf and Trebitsh, 2006; Boualem et al., 2008, 2009) which all encode *ACS* genes, and the effect of increased internal ethylene level via *35S::ACS* expression in transgenic melons (Papadopoulou et al., 2005) further show the importance of ethylene production in promoting femaleness, either by stimulating carpel production or suppressing stamens. Here we produced transgenic melons expressing *ACS* under floral primordia-targeted promoters to study the effect of location and timing of ethylene production during the sex determination process, and to investigate the role of cross-talk between the sex organ primordia in regulation of development.

The *CRC::ACS* and *AP3::ACS* melon plants showed a 2-8 fold increase in apical ethylene relative to wild type plants. Numerous studies measuring ethylene evolution from gynoecious cucumber apices have observed 2-3 fold differences between gynoecious and monoecious ethylene levels, providing evidence for the biological significance of two-fold differences (Byers et al., 1972; Rudich et al., 1972, 1976; Yamasaki et al., 2001, 2003).

Stamen primordia-targeted expression of ACS leads to feminization

Transgenic melons expressing *ACS* in the stamen and petal primordia showed increased femaleness manifested by gain of a bisexual-only phase not typically seen in wild type, a decrease in male buds and open flowers, and loss of the male-only phase along the main stem. In the extreme case, plants became phenotypically hermaphrodite rather than andromonoecious.

This increased femaleness phenotype is consistent with our previous data showing that ethylene perception in the stamens is required to promote femaleness (Little et al., 2007). However, in those experiments, increased femaleness was manifested by increased carpel development (Little et al., 2007); here the primary effect was loss of male buds. Studies with exogenous ethylene have indicated that timing and concentration are key factors determining whether carpel or stamen development is affected. Loss of male buds was more frequently observed in andromonoecious and monoecious melons treated with ethrel at a very young age (second leaf stage) (Rudich et al., 1969; Karchi, 1970). Increase in carpel-bearing buds occurred more frequently when plants were treated at later stages (i.e. visual flower bud and first flower at anthesis). Inhibition of stamens within carpel-bearing buds causing female rather than bisexual flowers also occurred more frequently when plants were treated at later stages, consistent with a time when carpel-bearing buds would have been already developing (Karchi, 1970). Thus, it has been suggested that there are critical windows in floral bud development when sex organs are sensitive to the effects of ethylene (Galun et al., 1963; Yamasaki et al., 2003).

Furthermore, higher concentrations (300ppm-600ppm) of ethrel were required to inhibit stamens in carpel-bearing buds than to promote production of carpel-bearing buds (Karchi, 1970). Andromonoecious and monoecious melons require concentrations of ethrel at 500ppm or higher to inhibit staminate buds (Karchi, 1970; Rudich et al., 1969). When low (150ppm) concentrations of ethrel were used, carpel-bearing buds were promoted (Rudich et al., 1969; Karchi, 1970).

The loss of male buds on the *AP3::ACS* plants suggested the possibility that bud abortion may be occurring, rather than continued development of asexual flowers that would result from premature inhibition of stamen primordia. If increased endogenous ethylene levels in the stamen

primordia were sufficiently high early in development to cause inhibition of stamen primordia prior to successful establishment of carpel development, this would result in asexual flowers. Consistent with this possibility that abortion occurred at an early stage of bud development, fewer *AP3::ACS* buds transitioned from the pre-sexual stages into the sex differentiation stages. We also did not observe the production of any asexual buds or flowers. Similarly, exogenous application of ACC to monoecious cucumber led to abortion of staminate buds before female flowering (Trebitsh et al., 1997; George, 1971). Loss of male flowers was also observed in the *Cucurbita pepo* 'Vegetable Spaghetti' genotype treated with ethephon (Manzano et al., 2011), and different melon genotypes treated with ethrel (Karchi, 1970).

Despite increased ethylene production, the *AP3::ACS* plants did not cause a conversion of bisexual flowers to female. This may result from the higher threshold required for stamen suppression than carpel promotion. Only those buds whose ethylene levels were not sufficiently high to suppress stamens would be able to proceed further in development, thus resulting in bisexual flowers.

In contrast to the effects of *AP3::ACS*, targeting of ethylene production to the carpel and nectary primordia using the *CRC* promoter did not cause a significant difference in number of male or carpel-bearing buds and open flowers, or sex expression pattern along the main stem. These results are consistent with the previous report by Little et al. (2007) showing that lack of ethylene perception by the carpel primordia did not prevent subsequent carpel development, and the role of stamen primordia in promoting carpel development.

Native sex genes

The *ACS* transgene also influenced native sex gene expression. Apices of *AP3::ACS* melons demonstrated a decrease in expression of the native sex gene, *WIP1*, the suppressor of carpel development, consistent with a phenotype of increased femaleness. Conversely, inhibition of ethylene perception in the *AP3::etr1-1* plants, which show a near loss of carpel-bearing buds, was associated with increased *WIP1* expression. *WIP1* has not previously been reported to be affected by ethylene, and yeast one-hybrid studies showed no direct interaction between the WIP1 transcription factor and *ACS7* promoter (Martin et al., 2009). These results provide evidence that ethylene acts to regulate *WIP1* gene expression, consistent with increased exogenous and endogenous ethylene levels causing increased carpel-bearing buds and flowers (McMurray and Miller, 1968; Robinson et al., 1969; Rudich et al., 1969; Karchi, 1970; Shimotsuma and Jones, 1972; Augustine et al., 1973; Owens et al., 1980; Papadopoulou et al., 2005). *WIP1* gene expression was also reduced by exogenous ethylene. The *WIP1* promoter was found to include the known ethylene response element (ERE) and ERFBP found within the promoters of the ethylene-responsive genes carnation *glutathione-S-transferase* (*GST1*) and tomato *E4* (Montgomery et al., 1993; Itzhaki et al., 1994; Xu et al., 1996).

Interestingly, the *WIP1* gene showed a clear diurnal pattern of expression with a peak occurring mid-day. Promoter analysis for *cis*-regulatory sites predicted multiple light regulatory motifs found within known light regulated genes, i.e. *RuBisCo* (*rbcS*) (Gidoni et al., 1989) and chlorophyll a/b-binding proteins of photosystem II (*Lhcb*) (Giuliano et al., 1988). Studies in cucumber have observed that sex expression can be influenced by day length; short days increase femaleness and long days increase maleness (Takahashi et al., 1983; Yamasaki et al., 2003b). These promoter elements may contribute to day length related effects on sex expression.

Ethylene levels were also shown to follow a diurnal pattern with a peak at mid-day, which was enhanced under short day conditions and correlated with increased femaleness (Yamasaki et al., 2003b).

Boualem et al. (2008) reported that increased ethylene produced by the fully functional ACS7 enzyme leads to stamen inhibition in carpel-bearing flowers. *AP3::ACS* plants showed an increase in *ACS7* sex gene expression in the apex. However, the mutant *ACS7* present in andromonoecious melon is unable to suppress stamens due to reduced enzymatic activity (Boualem et al., 2008).

Ethylene threshold model for sex expression in melon

Our objective for this study was to examine the effects of location and timing of ethylene production during floral bud development. Yin and Quinn et al. (1995) proposed an ethylene threshold model in which differing levels of sensitivity in stamen or carpel primordia could allow the primordia to react to different ranges of ethylene concentrations, and thus inhibit or promote each sex independently. The ethylene results presented here provide further evidence for ethylene thresholds acting within the apex during floral bud development, and indicate critical developmental stages and levels for sex differentiation for promotion or inhibition of sex organs (Figure 2.9).

At floral bud stage 4, the stamen, but not carpel, whorls have initiated. At stage 5, at low levels of ethylene, carpels are suppressed, as would occur in monoecious (*GA*) or andromonoecious (*Ga*) plants. At intermediate levels, G expression is suppressed in andromonoecious or monoecious plants, or not expressed in gynoecious (*gA*) or bisexual (*ga*)

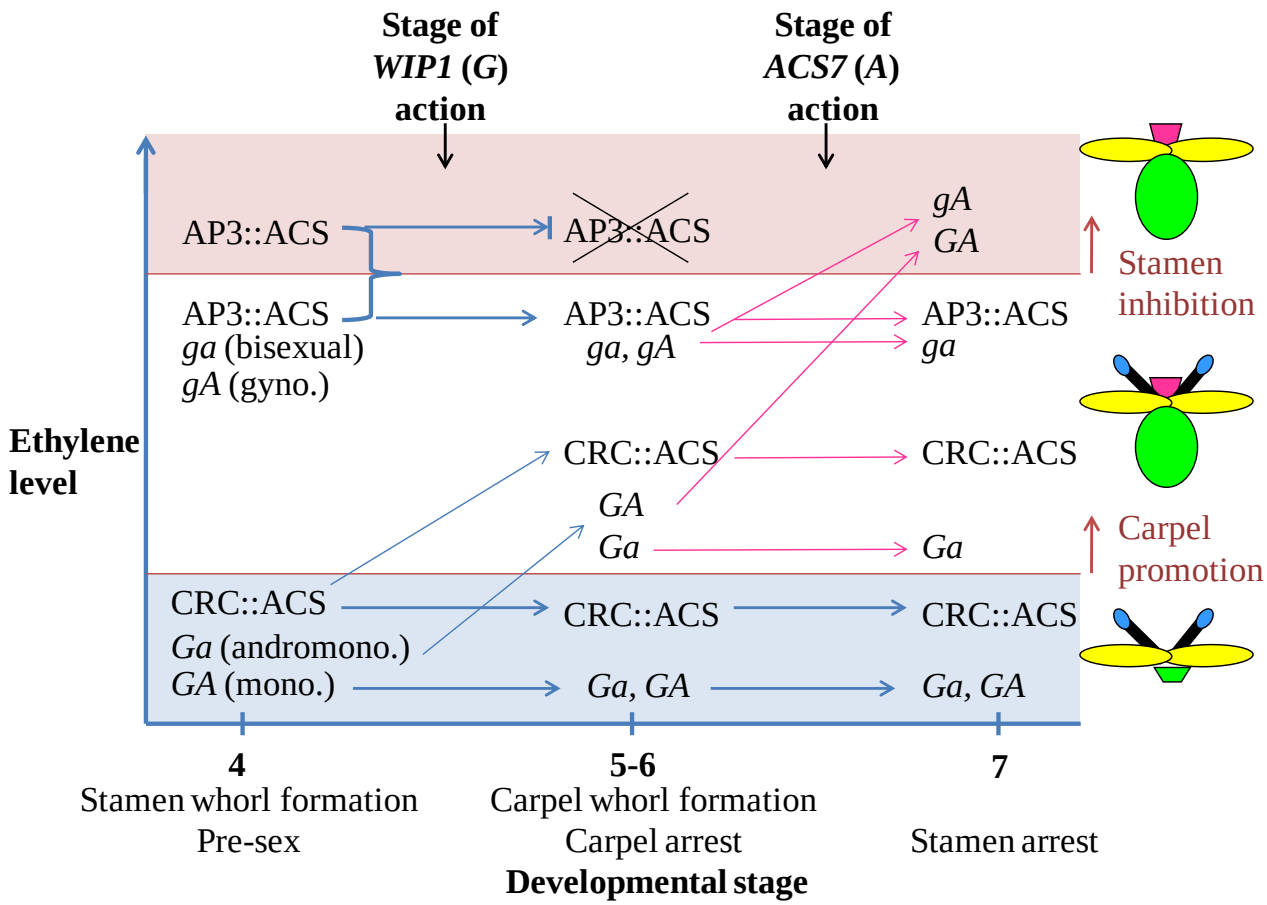


Figure 2.9. Ethylene threshold model of melon floral bud development including known developmental stages (stamen whorl formation/pre-sex, 4; carpel whorl formation/carpel arrest, 5-6; stamen arrest, 7), stages of sex gene action (*WIP1*/*G*; *ACS7*/*A*), genotypes (*GA*, *Ga*, *gA*, *ga*), and sex phenotypes (monoecious (mono.), andromonoecious (andromono.), gynoecious (gyno.), and bisexual).

plants, and carpels are promoted. If ethylene levels at this stage are too high, the stamen primordia are inhibited, leading to bud abortion. As the floral bud moves into stages 5-6, if sufficient ethylene is perceived by the stamen primordia, the carpel primordia continue to develop; levels that are too low to promote carpels lead to a staminate bud. Carpel-bearing buds on hermaphrodite and gynoecious plants continue to develop. At stage 7, if ethylene levels in carpel-bearing buds are sufficiently high, as occurs in gynoecious or monoecious plants, the stamen primordia will fail to develop producing female flowers. If ethylene levels do not exceed this threshold, the stamens continue to develop to become a bisexual flower.

This information makes evolutionary sense when building a unisexual flower. After initiation of the whorls, the stamen or carpel primordia must be inhibited to become staminate or carpel-bearing, respectively. If development of the stamen primordia was arrested before the initiation of the carpels, energy to sustain fruit set would be wasted to produce an asexual flower. Regulation of stamens by the carpels prevents this occurrence. Similarly, regulation of carpels by the stamens, which develop first, minimizes resources allocated to carpels in flowers destined to be male. Finally, a higher ethylene threshold for suppression of stamens than carpels, also ensures that stamens are not inhibited prior to establishment of carpels.

APPENDIX

PCR segregation of the two lines of *CRC::ACS* (168 and 169) matched a single-gene insertion. One line of *AP3::ACS* (A4) showed a PCR segregation ratio matching a two gene insertion model, the other line (A5) was consistent with a three-gene insertion.

Table A1. PCR segregation ratios of transgenic *CRC::ACS* T_1 and *AP3::ACS* BC_1/BC_1S_1 lines.

Line	**Generation**	**Segregation (+ : -)**	**Expected ratios**	X^2
CRC::ACS 168	T_1	17:9	3:1	0.82 ns
CRC::ACS 169	T_1	23:6	3:1	0.10 ns
AP3::ACS A4	BC_1	26:8	3:1	0.29 ns
AP3::ACS A5	BC_1	23:0	15:1	0.22 ns
AP3::ACS A4	BC_1S_1	15:2	15:1	0.05 ns
AP3::ACS A5	BC_1S_1	16:3	225:1	0.44 ns

LITERATURE CITED

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Arguello-Astorga G and Herrera-Estrella, L (1998). Evolution of light regulated plant promoters. Annu Rev Plant Phys 49: 525-555.

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

Bai SL, Peng YB, Cui JX, Gu HT, Xn LY, Li YQ, Xu ZH, Bai SN (2004). Developmental analyses reveal early arrest of the spore-bearing parts of reproductive organs in unisexual flowers of cucumber (*Cucumis sativus* L.). Planta 220: 230-240.

Boualem A, Fergany M, Fernandez R, Troadec C, Martin A, Morin H, Sari M, Collin F, Flowers J, et al. (2008). A conserved mutation in an ethylene biosynthesis enzyme leads to andromonoecy in melons. Science 321: 836-838.

Bowman JL and Smyth DR (1999). *CRABS CLAW*, a gene that regulates carpel and nectary development in *Arabidopsis*, encodes a novel protein with zinc finger and helix-loop-helix domains. Development 126: 2387-2396.

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

Chang WC, Lee TY, Huang HD, Huang HY, Pan RL (2008). PlantPAN: Plant Promoter Analysis Navigator, for identifying combinatorial cis-regulatory elements with distance constraint in plant gene group. BMC Genomics 9: 561.

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

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

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

Gidoni D, Brosio P, Bond-Nutter D, Bedbrook J, Dunsmuir P (1989). Novel *cis*-acting elements in petunia *Cab* gene promoters. Mol Genet Genomics 215: 337-344.

Giuliano G, Pichersky E, Malik VS, Timko MP, Scolnik PA (1988). An evolutionarily conserved protein-binding sequence upstream of a plant light-regulated gene. P Natl Acad Sci USA 85: 7089-7093.

Goffinet M (1990). Comparative ontology of male and female flowers of *Cucumis sativus*. In: Biology and Utilization of the Cucurbitaceae. Bates DM, Robinson RW, Jefferey C (eds). Cornell Univ Press, NY: 288-304.

Grumet R and Taft JA (2011). Sex expression in cucurbits In: Genetics, Genomics and Breeding in Cucurbits. Kole C, Wang YH, Behera TK, Kole C (eds). Science Publishers Inc. Enfield NH and CRC Press (Taylor & Francis) group, Boca Raton, FL. P. 353-375.

Hao YJ, Wang DH, Peng YB, Bai SL, Xu LY, Li YQ, Zu ZH, Bai SN (2003). DNA damage in the early primordial anther is closely correlated with stamen arrest in the female flower of cucumber (*Cucumis sativus* L.). Planta 217: 888-895.

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999). Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res 27: 297-300.

Ishiki Y, Oda A, Yaegashi Y, Orihara Y, Arai T, Hirabayashi T, Nakagawa H, Sato T (2000). Cloning of an auxin-responsive 1-aminocyclopropane-1-carboxylate synthase gene (CMe-ACS2) from melon and the expression of ACS genes in etiolated melon seedlings and melon fruits. Plant Sci 159: 173-181.

Itzhaki H, Maxson JM, Woodson WR (1994). An ethylene-responsive enhancer element is involved in the senescence-related expression of the carnation *glutathione-S-transferase* (*GST1*) gene. P Natl Acad Sci 91: 8925-8929.

Jackson D (1991). In-situ hybridization in plants. In: Molecular Plant Pathology: A Practical Approach. Bowles DJ, Gurr SJ and McPherson M (eds). Oxford University Press.

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

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

Kidner C and Timmermans M (2006). *In situ* hybridization as a tool to study the role of miRNAs in plant development. Method Mol Cell Biol 342: 159-179.

Kosugi S and Ohashi Y (2000). Cloning and DNA-binding properties of a tobacco Ethylene-Insensitive3 (EIN3) homolog. Nucleic Acids Res 28**:** 960–967.

Lasserre E, Bouquin T, Hernandez JA, Bull J, Pech JC, Balague C (1996). Structure and expression of three genes encoding ACC oxidase homologs from melon (*Cucumis melo* L.). Mol Genet Genomics 251: 81-90.

Little HA, Papadopoulou E, Hammar SA, Grumet R (2007). The influence of ethylene perception on sex expression in transgenic melon as assessed by expression of the mutant ethylene receptor gene, *At-etr1-1*, under control of constitutive and floral-targeted promoters. Sex Plant Reprod 20: 123-136.

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

Manzano S, Martinez C, Megias Z, Gomez P, Garrido D, Jamilena M (2011). The role of ethylene and brassinosteriods in the control of sex expression and flower development in *Cucurbita pepo*. Plant Growth Regul 65: 213-221.

Martin A, Troadec C, Boualem A, Mazen R, Fernandez R, Morin H, Pitrat M, Dogimont C, Bendhamane A (2009). A transposon-induced epigenetic change leads to sex determination in melon. Nature 461: 135-1139.

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

Miki T, Yamamoto M, Nakagawa H, Ogura N, Mori H, Imaseki H, Sato T (1995). Nucleotide-sequence of a cDNA for 1-aminocyclopropane-1-carboxylate synthase from melon fruits. Plant Physiol 107:297-298.

Montgomery J, Goldman S, Deikman J, Margossian L, Fischer RL (1993). Identification of an ethylene-responsive region in the promoter of a fruit ripening gene. P Natl Acad Sci 90: 5339-5343.

Ohtsu K, Smith MB, Emrich SJ, Borsuk LA, Zhou B, Chen T, Zhang X, Timmermans MCP, Beck J, Buckner B et al. (2007). Global gene expression analysis of the shoot apical meristem of maize (*Zea mays* L.). Plant J 52: 391-404.

Owens KW, Peterson CE, Tolla GE (1980). Production of hermaphrodite flowers on gynoecious muskmelon by silver nitrate and aminoethoxyvinylglycine. HortScience 15:654-655.

Papadopoulou E, Little HA, Hammar SA, Grumet R (2005). Effect of modified endogenous ethylene production on sex expression, bisexual flower development, and fruit production in melon (*Cucumis melo* L.). Sex Plant Reprod 18: 131-142.

Prestridge DS (1991). SIGNAL SCAN: A computer program that scans DNA sequences for eukaryotic transcriptional elements. CABIOS 7: 203-206.

Riechmann JL and Meyerowitz EM (1998). The AP2/EREBP family of plant transcription factors. Biol Chem 379: 633-646.

Robinson RW, Shannon S, La Guardia MD (1969). Regulation of sex expression in the cucumber. BioScience 19: 141-142.

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

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

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

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

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

Solano R, Stepanova A, Chao Q, Ecker JR (1998). Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes and Dev 12: 3703-3714.

Takahashi H, Saito T and Suge H (1983).Separation of the effects of photoperiod and hormones on sex expression in cucumber. Plant Cell Physiol 24: 147-154.

Takahashi H, Kamakura H, Sato Y, Shiono K, Abiko T, Tsutsumi N, Nagamura Y, Nishizawa NK, Nakazomo M (2010). A method for obtaining high quality RNA from paraffin sections of plant tissues by laser microdissection. J Plant Res 126: 807-813.

Terzaghi WB and Cashmore AR (1995). Light regulated transcription. Annu Rev Plant Phys 46**:** 445–474.

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

Wang NN, Shih MC, Li N (2004). The GUS reporter-aided analysis of the promoter activities of *Arabidopsis* ACC synthase genes *AtACS4*, *AtACS5*, and *AtACS7* induced by hormones and stresses. J Exp Bot 56: 909-920.

Wu T, Qin Z, Feng Z, Zhou X, Xin M, Du Y (2012). Functional analysis of the promoter of a female-specific cucumber CsACS1G gene. Plant Mol Biol Rep 30: 235-241.

Xu RL, Goldman S, Coupe S, Deikman J (1996). Ethylene control during tomato fruit ripening involves two cooperative *cis*-elements. Plant Mol Biol 31: 1117-1127.

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

Yamasaki S, Fujii N, Takahashi H (2003a). Characterization of ethylene effects on sex determination in cucumber plants. Sex Plant Reprod 16:103-111.

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

CHAPTER 3

Effect of carpel primordia-targeted inhibition of ethylene perception on sex expression and fruit set, development, and ripening in melon (*Cucumis melo L.*)

Introduction:

Angiosperms typically produce bisexual flowers expressing both male and female organs (stamens and carpels, respectively). The Cucurbitaceae family is unique in that it contains species exhibiting a variety of heritable sex phenotypes. The Cucumis species cucumber (*C. sativus*) and melon (*C. melo*) are model organisms for studies of sex determination (Roy and Saran, 1990; Grumet and Taft, 2011). The common sex expression pattern in melon is andromonoecious. Progressing along the main stem, vegetative nodes are followed by nodes bearing male flowers, and then by nodes with a mixture of male and bisexual flowers.

Ethylene is a key factor in the regulation of sex determination in cucurbits (Perl-Treves, 1999; Grumet and Taft, 2011). Exogenous application of ethylene-releasing chemicals leads to increased femaleness in both melon and cucumber. Transgenic melons over-expressing the ethylene biosynthetic enzyme *ACS*, under the 35S promoter showed increased femaleness (Papadopoulou et al., 2005). Inhibition of ethylene biosynthesis with aminoethoxyvinylglycine (AVG), or ethylene perception with silver ions leads to increased maleness (Den Nijs and Visser, 1980; Owens et al., 1980). Similarly, constitutive expression of the *Arabidopsis thaliana* ethylene receptor mutant *etr1-1*, conferring loss of ethylene perception in transgenic melons, resulted in almost complete loss of carpel-bearing nodes (Little et al., 2007). These results demonstrated that ethylene perception is required for carpel primordia development during melon sex determination (Little et al., 2007). Furthermore, our previous studies targeting expression of *etr1-1* to either the stamen and petal primordia with the *APETALA3* (*AP3*) promoter, or carpel and nectary primordia with the *CRABS CLAW* (*CRC*) promoter, indicated that ethylene perception by stamen primordia, but not carpel primordia, is essential for the production of carpel bearing buds (Little et al., 2007). Stamen targeted expression of *etr1-1*

caused a near-complete loss of carpel-bearing buds. In contrast, transgenic andromonoecious melon plants expressing *etr1-1* under the control of the carpel and nectary primordia-directed *CRABS CLAW* (*CRC*) promoter showed increased and earlier carpel bearing buds and flowers in the greenhouse (Little et al., 2007).

Ethylene is also a key factor regulating fruit ripening in fleshy fruits such as tomato, apple and melon (Barry and Giovannoni, 2007). Fruit can be classified based on whether they undergo a rise in ethylene levels and respiration at early ripening stages (climacteric), or not (non-climacteric). In climacteric fruit, ethylene is a major factor in controlling fruit firmness, color, taste, and aroma (Barry and Giovannoni, 2007; Bapat et al., 2010; Paul et al., 2012). Studies in melon, which has climacteric and non-climacteric members, have elucidated aspects of ethylene's role in the ripening process (Paul et al., 2012). Research by Guis et al. (1997) and Pech et al. (2008) using ACO-antisense melons under the constitutive 35S promoter have characterized ethylene-dependent and independent pathways of ripening. Loss of chlorophyll, carotenoid increase in the rind, peduncular abscission, aroma production, and a majority of the flesh softening process were found to be ethylene-dependent processes, while acidity level changes, and flesh carotenoid and sucrose accumulation were ethylene-independent (Pech et al., 2008).

In this work, we studied transgenic melons targeting expressing of *etr1-1* to the carpel and nectary primordia under control of the *CRABS CLAW* (*CRC*) promoter (*CRC::etr1-1*) to determine if the increased femaleness phenotype previously observed in the greenhouse allows for earlier or increased fruit set. Fruit development was also assessed to determine if ethylene perception inhibition in the carpels has an effect on fruit growth and ripening. We observed increased femaleness and a conversion of bisexual-to-female flowers, not seen in wild type

plants. The *CRC::etr1-1* plants also exhibited increased and earlier fruit set, and modified fruit ripening. These results demonstrate the potential to modulate fruit set and ripening by targeted ethylene inhibition to the carpel and nectary primordia.

Materials and methods:

Plant material and growth conditions

Melon genotypes used for these experiments were wild type, andromonoecious Hale's Best Jumbo (Hales; Hollar Seed, Rocky Ford, CO) and two *CRC::etr1-1* transgenic lines, M5 and M15, that were originally produced from Hales as described by Little et al. (2007). T_1 M5 and M15 plants were self-pollinated in the greenhouse to obtain T_2 seed. Segregating progeny were verified for transgene presence by PCR analysis using a *CRC* specific primer (5' - CTTGCAATCCCTAGCCAG -3', RG299) and *etr1-1* specific primer (5'-GCTCATGGGACACACAACTCGG-3', RG306), and for transgene expression as described below. PCR positive T_2 M5 and M15 plants were grown under greenhouse conditions as described by Little et al. (2007). Ovary diameter and length was recorded on M5 and M15 plants in the greenhouse.

Field experiments

Field trials were performed in East Lansing, MI during the summer of 2009 with Hales and T_2 *CRC::etr1-1* M5 and M15 plants in a randomized complete block design with 6 replications with 6 plants/plot, and in the summer of 2011using 4 replications and 10 plants/plot. Three week-old greenhouse-grown plants were transplanted into the field at 1m intervals into

1.5m wide black plastic rows with 3m between rows. The plot was surrounded on all sides by a border of Hales plants. Standard practices were used for fertilization and pesticide application. In 2009, the main stems of 4 plants/plot were scored for number of male and carpel-bearing buds and open flowers, and first node appearance of carpel-bearing buds and open flowers. In 2011, the plants were observed for first node on the main stem of set fruit and total fruit set on the whole plant throughout the growing season. Fruit development and ripening was monitored on selected fruit by tagging fruit of equivalent diameter from each of the genotypes at 26, 40, and 46 days after planting. At final harvest (10 weeks after transplanting), ripening stage for all fruit was characterized by rind color as: green, turning (mix of green and orange), or orange. Fruit length, diameter, and weight were recorded for all fruit.

qRT-PCR analysis

Total RNA was prepared from 200mg of liquid nitrogen frozen melon apex tissue (~3-4mm in size) removed from plants at the 2 leaf stage, or from lateral branches (5-6 nodes in length) using Trizol (Invitrogen, Carlsbad, CA). cDNA preparation was performed using Superscript II reverse transcriptase (Promega, Madison, WI) following manufacturer's recommendations. RNA was also prepared from 200-300mg of fruit mesocarp or exocarp (minus rind) from 10mm and 60mm diameter field grown fruit (~3 days post pollination (dpp), and ~14 dpp), and greenhouse grown 35 dpp fruit (~107mm diameter, turning stage). Gene-specific primers for the *etr1-1*transgene were designed using Primer Express 3.0 software (Applied Biosystems); the primer sequences are Forward -GTTGGGGATGAGAAACGGC-3' (RG317), and Reverse -GCCCACTACCACCCGAGC- 3' (RG318). qRT-PCR analysis was carried out using a Stratagene Mx4000 system with Power SYBR Green PCR Master Mix

(Applied Biosystems) for PCR quantification via standard curve reference. *C. melo Polyubiquitin 7* (ICuGI: MU43954) was used as an endogenous control for normalization; the primer sequences are Forward -GACCGGGAAGGAAATTGAGAT-3' (RG541) and Reverse -CCGCTCCTTAATACGATCAATTGG-3' (RG543).

Ethylene measurements

Ethylene measurements were performed on apices of greenhouse grown Hales, *CRC::etr1-1* M5 and M15 plants. Lateral apices (~3-4mm in size) were excised from branches 8-10 nodes long using a razor blade, and weighed to record fresh weight. Apices were placed in a 10mL plastic syringe with a 5mm diameter Whatman filter paper #2 disc moistened with distilled water, and placed at room temperature for 1 hour to allow for diffusion of wound ethylene. Ethylene measurements were performed by gas chromatography as described in Papadopoulou et al. (2005). The experiment was repeated twice with six plants/genotype in a randomized complete block design. Three replicates/plant were taken for ethylene analysis with two lateral apices/replicate.

To perform fruit ethylene measurements, fruit harvested from the field were classified by rind color as green (green rind, unripe), turning (mixture of green and orange), or orange (fully orange rind, ripe) and weighed. Three fruit/rind color category (one replicate) were placed in separate 10 gallon buckets and sealed for 1 hour with air tight covers with a rubber septum prior to sampling for ethylene concentration. Internal fruit ethylene was measured from each fruit after whole fruit head space was analyzed. An 18 gauge needle attached to a 1mL syringe was inserted in each fruit to draw air from the cavity. The experiment was repeated on three harvest dates, with three replicates/fruit rind category/genotype/harvest date.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using Proc GLM or Mixed protocol in SAS (SAS Institute, Cary, NC).

Results:

Ethylene and sex expression

Introduction of the *CRC::etr1-1* transgene into Hales via *Agrobacterium*-mediated transformation was described in Little et al. (2007). qRT-PCR analysis of T_2 transgenic plants detected *etr1-1* transgene expression in apical tissue of M5 and M15 plants (Figure 3.1a). Inhibition of ethylene perception is frequently associated with increased ethylene production (Wilkinson et al., 1997; Little et al., 2007). A 2-6 fold increase in apical ethylene evolution was observed in transgenic PCR positive plants (Figure 3.1b). Studies of ethylene evolution in gynoecious cucumber apices have shown two-fold greater ethylene compared to their monoecious counterparts, providing evidence for the biological significance of two-fold differences in modulating sex determination (Rudich et al., 1972; Yamasaki et al., 2003).

Our previous greenhouse studies of T_1 plants (Little et al., 2007) observed an earlier onset and increased production of carpel-bearing flowers. These phenotypes were also observed in greenhouse trials with T_2 progeny. The *CRC::etr1-1* plants showed a significant increase in number of open carpel-bearing flowers on the main stem, and earlier onset by an average of 6 nodes (Table 3.1). A second phenotype observed in the T_1 progeny and again in the T_2 generation, was conversion of the *CRC::etr1-1* carpel-bearing flowers from bisexual to female

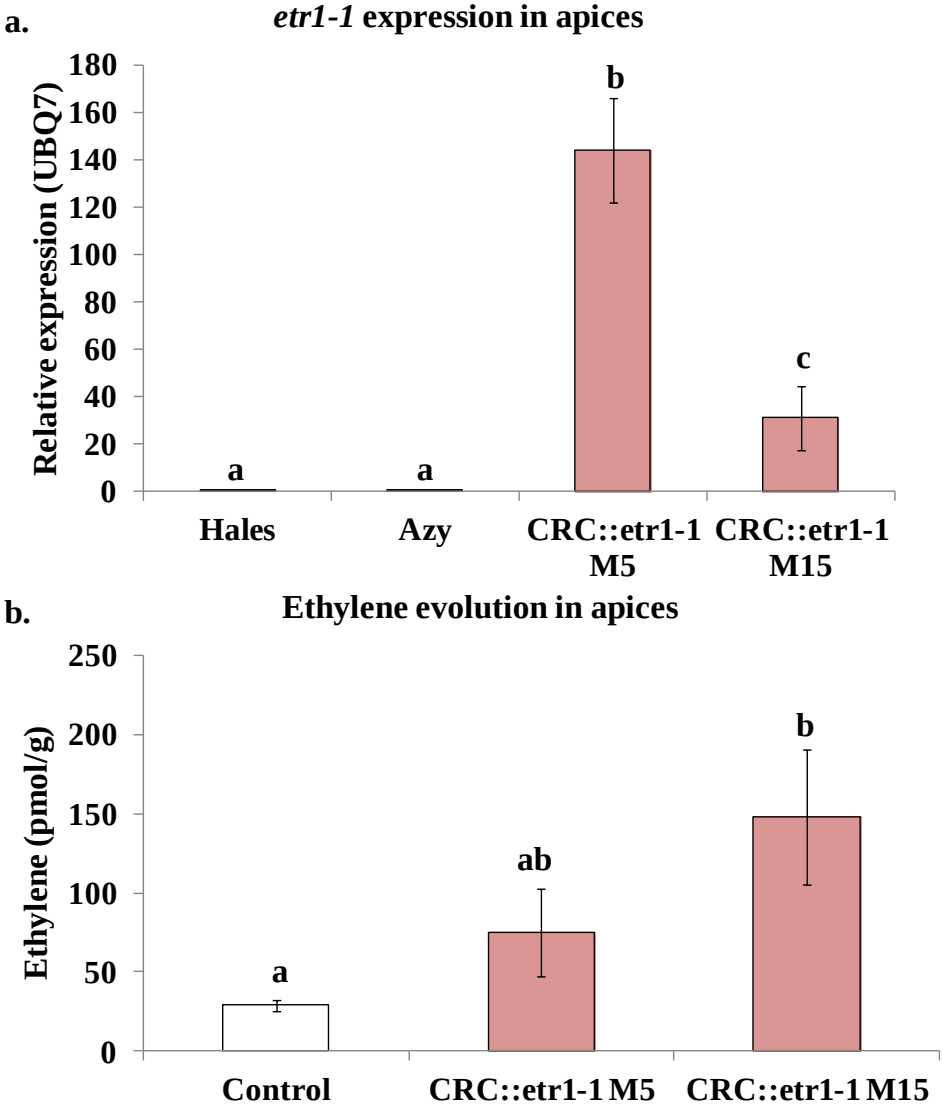


Figure 3.1. qRT-PCR analysis of expression of the *etr1-1* transgene (a), and ethylene evolution (b) in apices of Hales (WT), Azygous (Azy), and *CRC::etr1-1* M5 and M15 plants (Control in (b) is a combination of Hales and Azygous samples). Values in qRT-PCR are the average of three biological replicates with three technical replicates/biological rep ± SE. Values presented in ethylene evolution are the average of two experiments with 5-6 replicates with 1-2 plants/rep and 1-2 samples/plant/genotype/experiment ± SE. Different letters represent significant differences by LSD at $P<0.05$.

Table 3.1. Greenhouse observations of T_2 generation of *CRC::etr1-1* M5 and M15.

Genotype	# carpel-bearing buds	First node of carpel-bearing bud	# open carpel-bearing flowers	First node of open carpel-bearing flower	% open bisexual flowers	% open female flower
Hales	22.7 ± 0.7a	9.6 ± 0.6a	3.0 ± 1.2a	19.5 ± 4.2a	100 ± 0.0a	0 ± 0.0a
Azygous	23.3 ± 0.7a	9.0 ± 0.8a	1.7 ± 0.32a	17.8 ± 4.6a	100 ± 0.0a	0 ± 0.0a
CRC::etr1-1 M5	24.5 ± 0.7a	10.0 ± 0.9a	5.8 ± 1.2**b**	11.8 ± 0.9**b**	59.6 + 7.4**b**	40.4 + 7.4**b**
CRC::etr1-1 M15	26.0 ± 1.6a	11.2 ± 1.2a	7.0 ± 0.3**b**	13.5 ± 1.1**b**	57.3 + 4.9**b**	42.7 + 4.9**b**

Values presented are the average of two experiments with 6 replicates/genotype/experiment ± SE. Different letters represent significant differences by LSD at $P<0.05$.

(i.e. suppression of stamens). Approximately 40% of the *CRC::etr1-1* carpel-bearing flowers were female in both lines, a phenotype that is not observed in the andromonoecious melons (Table 3.1).

Earlier and increased carpel-bearing flowers could be of possible value for melon production if it is manifested in the field and results in increased and earlier fruit set. The first field experiment was conducted to examine sex expression phenotypes. Both the M5 and M15 *CRC::etr1-1* lines showed an increase in the number of carpel-bearing buds and open flowers, and earlier onset of the first open carpel-bearing flower on the main stem by approximately 7-10 nodes, or 8-10 days (Figure 3.2a-c). A ~50% conversion of the typically bisexual flowers to female flowers also was observed in the field, coinciding with our greenhouse studies (Figure 3.2d). The plants did not exhibit obvious differences in vegetative characteristics.

Fruit set and yield

The second field experiment was conducted to assess the influence of the *CRC::etr1-1* transgene on fruit set, development, and yield. M5 and M15 transgenic fruit showed increased average fruit set, especially early in the season, consistent with the earlier appearance of carpel-bearing flowers (Figure 3.3). Total number of fruit at harvest was increased in the *CRC::etr1-1* lines compared to wild type (Table 3.2). Total yield, as measured by kg/plot, did not significantly differ from wild type plants (Table 3.2). The transgenic fruit was generally smaller, i.e. had lower fruit weight (Table 3.2).

Fruit development and ripening

M5 and M15 transgenic fruit were more elongated, as evidenced by a higher

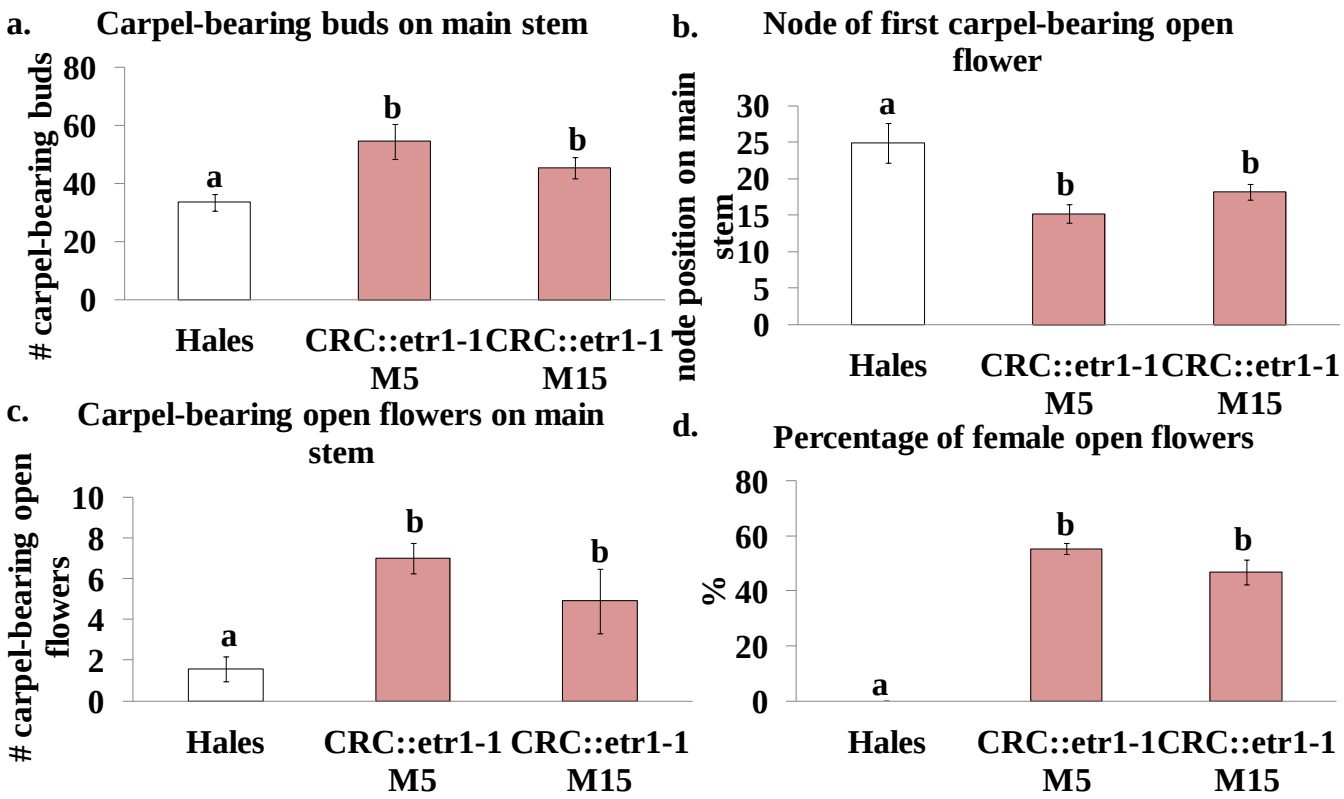


Figure 3.2. Field observations of number of carpel-bearing buds (a), open flowers (b), node of first carpel-bearing open flower on the main stem (c), and percentage of female vs. bisexual open flowers (d) of Hales (WT), and *CRC::etr1-1* M5 and M15. Values for sex expression data are presented as the average of 6 replicates with 2-3 plants/rep ± SE. Different letters represent significant differences by LSD at $P<0.05$.

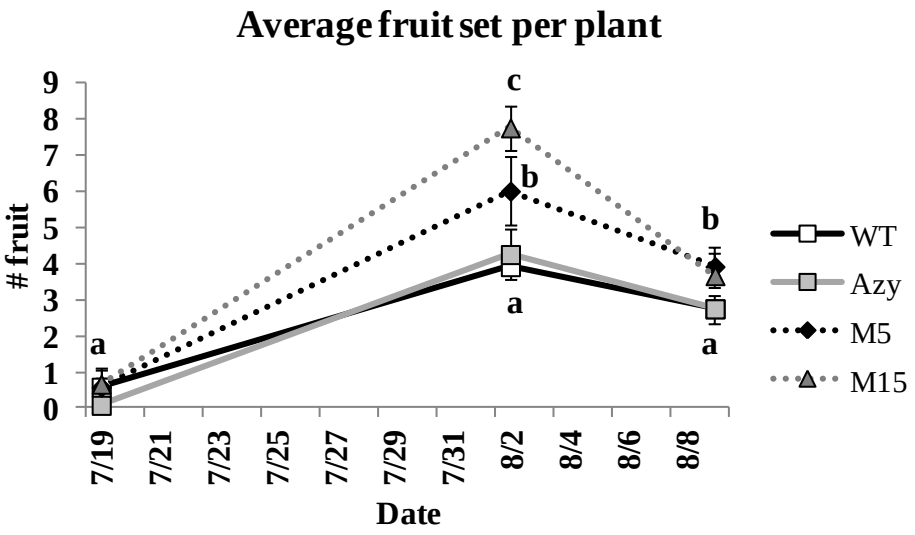


Figure 3.3. Average fruit set per plant for Hales (WT), azygous, and *CRC::etr1-1* M5 and M15 plants in the summer of 2011. Values are the average of 4 replicates with 3 plants/rep ± SE. Different letters represent significant differences by LSD at $P<0.05$.

Table 3.2. Field observations of fruit yield parameters.

Parameter	Hales (WT)	Azygous	*CRC::etr1-1*		Non-transgenic	*CRC::etr1-1*
			M5	M15		
Total fruit/plot	42 ± 4.2[z]	40 ± 3.8	52.5 ± 4.6	51.5 ± 4.1	41 ± 2.6**a**[y]	52 ± 2.9**b**
Total fruit yield (kg/plot)	60.3 ± 4.6	56.0 ± 4.7	63.2 ± 6.8	60.5 ± 6.1	58.2 ± 3.1a	61.9 ± 4.3a
Average fruit weight (kg)	1.5 ± 0.0	1.40 ± 0.1	1.20 ± 0.0	1.17 ± 0.0	1.45 ± 0.1**a**	1.19 ± 0.0**b**

[z]Values are presented are the average ± SE of 4 replicates with 10 plants/genotype/rep.

[y]Comparison of non-transgenic (Hales and azygous) vs. *CRC::etr1-1* transgenic plants by orthogonal contrast. Different letters represent significant differences at $P<0.05$.

length/diameter ratio (LD) (Figure 3.4a). Examination of carpel-bearing flowers showed that this difference was already apparent in ovaries of female and bisexual flowers (Figure 3.4b). No difference however was seen between the LD of female versus bisexual flowers (Figure 3.4b).

To determine effects on fruit ripening, fruit exocarp color at final harvest was categorized as: green, turning (mix of green and orange), or orange. Fruit at full slip were orange, but could be easily removed from the vine. At the final harvest date, 100% of the of the *CRC::etr1-1* M5 fruit showed external ripening (classified as turning, orange, or full slip), compared to ~50% of wild type, and ~30% of the M15 line (Figure 3.5a). Despite equivalent timing of fruit set, a majority of the M15 fruit did not show external ripening signs. Examination of abscission showed that a significantly reduced portion of M15 fruits had reached full slip compared to both M5 and wild type fruit (Figure 3.5b). Although there was a lack of external ripening phenotypes, when full size M15 green fruits were cut open, the mesocarp was orange and frequently accompanied by over-ripe characteristics (i.e., mesocarp degradation, septum separation from seeds, and/or septum degradation)(Figure 3.6a). Measurements of ethylene evolution from whole fruit showed that orange fruit of all lines had higher ethylene evolution than green fruit (Figure 3.6b). Similarly, samples of internal ethylene levels from taken from the seed cavity of green fruit showed that Hales, azygous, and M5 lines all had low ethylene levels. However, M15 green fruit showed high ethylene levels comparable to those of orange fruit (Figure 3.6c).

A possible explanation for the green exterior, but overly ripe interior, is that expression of the *etr1-1* transgene was inhibiting perception of ethylene in the exocarp but not the mesocarp. There was a general trend of increased *etr1-1* expression as the transgenic fruit developed

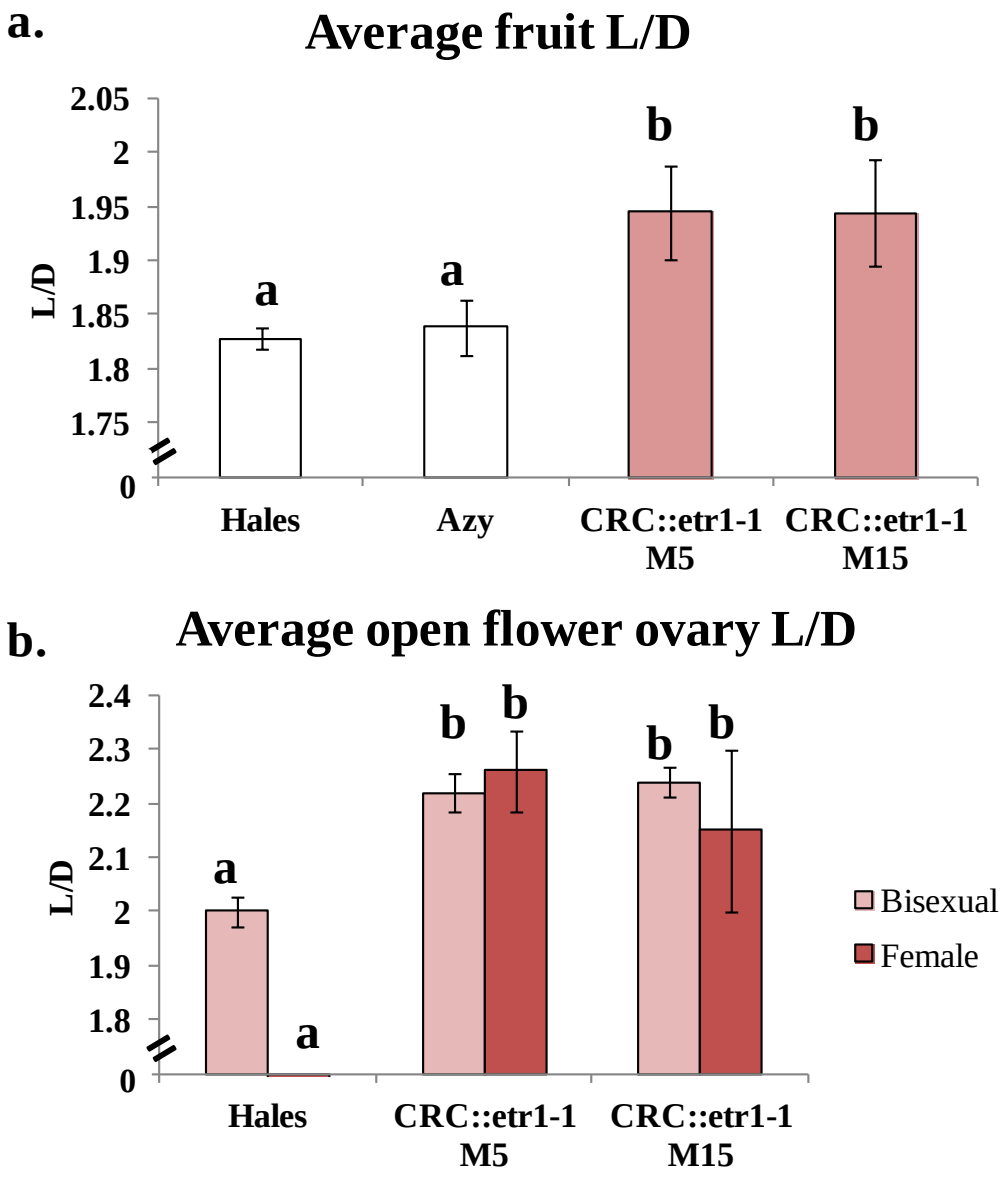


Figure 3.4. Average fruit (a) and open bisexual and female flower ovary (b) length/diameter ratio on wild type (WT) and *CRC::etr1-1* M5 and M15. Values for fruit L/D are presented as the average ± SE of 35-40 fruit/genotype. Values for ovary L/D are the average of data combined from two experiments with 6 plants/genotype/experiment ± SE. Different letters represent significant differences by LSD at $P<0.05$.

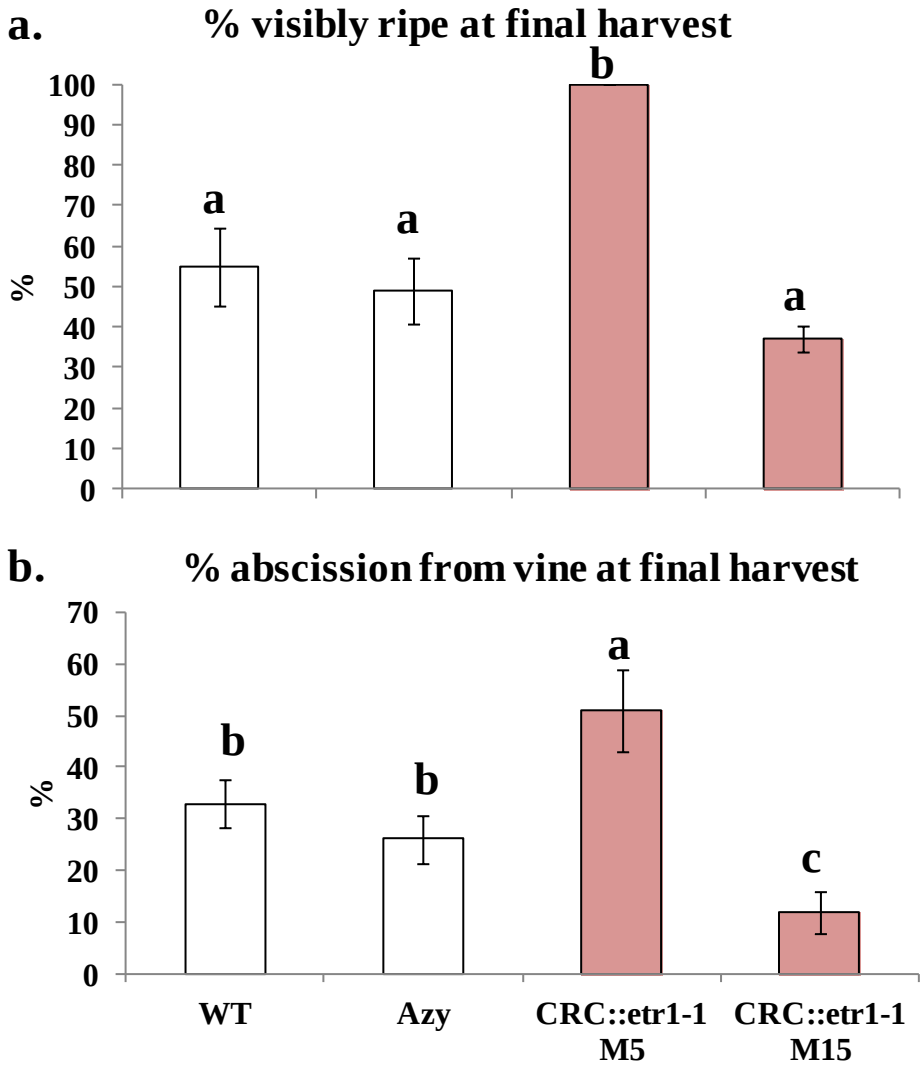


Figure 3.5. Percentage of harvested fruit that are visibly ripe (visibly ripe fruit were classified as turning, orange and full slip) (a), and abscission from the vine (b) at final harvest. Values are average ± SE of four replicate plots (35-40 fruit/genotype). Different letters represent significant differences by LSD at $P<0.05$.

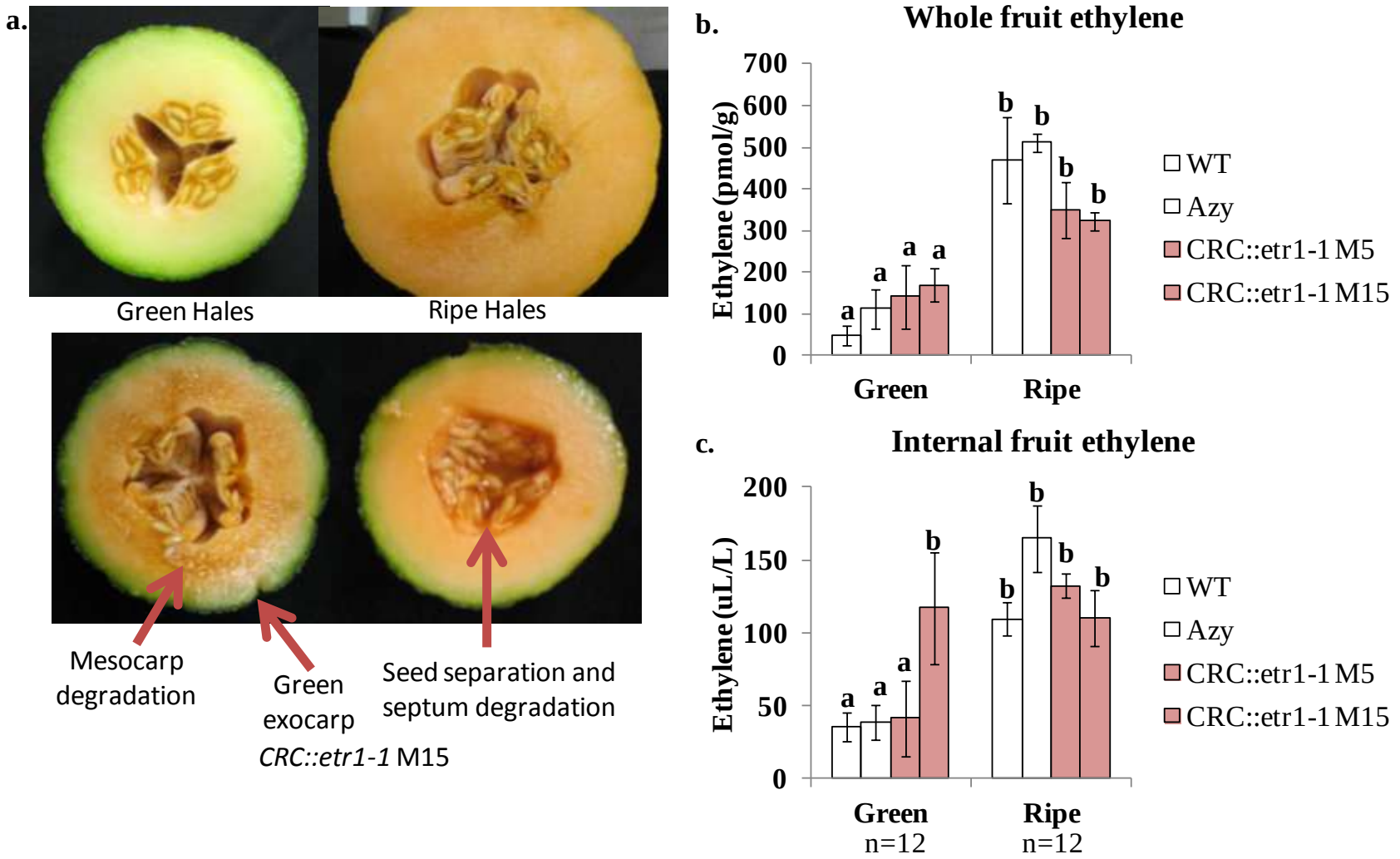


Figure 3.6. Mesocarp of Hales (green and ripe), and *CRC::etr1-1 M15* fruit (a), whole fruit ethylene evolution (b) and internal fruit ethylene (c) of wild type, azygous, and *CRC::etr1-1* M5 and M15. Values for whole and internal fruit ethylene are the average of data combined from three sample dates with three replicates of 3 fruit/rep ± SE. Different letters represent significant differences by LSD at $P<0.05$.

(Figure 3.7). Fruit approaching the turning stage (35dpp) showed an increase in transgene expression in the exocarp, especially in M15 fruit (Figure 3.7).

Discussion:

Greenhouse and field observations of the T_2 generation of *CRC::etr1-1* M5 and M15 plants showed increased and earlier onset of carpel-bearing flowers, increased early fruit set and modified fruit ripening characteristics. The phenotypes observed for the *CRC::etr1-1* lines were specific to floral and fruit tissue, unlike *35S::etr1-1* melons, which showed a variety of vegetative and developmental phenotypes (Little et al., 2007). Typically increased femaleness has been associated with exogenous application or increased endogenous ethylene (Rudich et al., 1969; Karchi 1970; Papadopoulou et al., 2005). Transgenic melons constitutively expressing the ethylene biosynthetic enzyme, *ACS*, or *ACS* targeted to stamen primordia, showed higher ethylene evolution, and increased and earlier onset of bisexual flowers (Papadopoulou et al., 2005; Taft et al., 2010). Here we observed increased femaleness in the *CRC::etr1-1* plants, despite inhibition of ethylene perception by carpel primordia. A possible explanation is that failure of the carpel primordia to perceive ethylene results in increased ethylene production due to lack of feedback inhibition, as has been observed frequently in non-ethylene perceiving mutants and transgenics (Barry and Giovannoni, 2007), and was observed in the *CRC::etr1-1* apices. The increased ethylene produced by the *CRC::etr1-1* apices, could still be perceived by the stamen primordia, which are the organs that regulate carpel development in andromonoecious melons (Little et al., 2007). Ethylene perception by stamen primordia is required to promote carpel development, and would result in increased production of carpel-bearing buds.

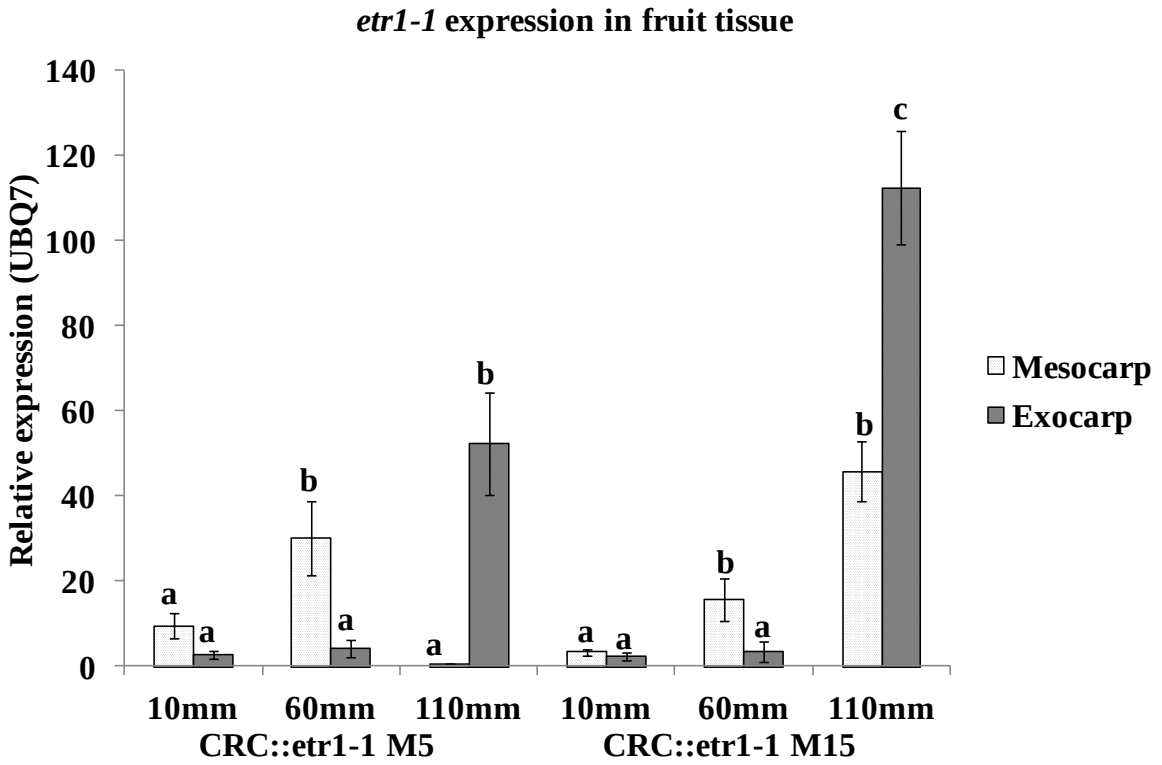


Figure 3.7. qRT-PCR analysis of expression of the *etr1-1* transgene in 10mm diameter (~3dpp), 60mm diameter (~14dpp) and 35dpp fruit exocarp and mesocarp of *CRC::etr1-1* M5 and M15 fruits. Values are the average of three biological replicates with three technical replicates/biological replicate ± SE. Different letters represent significant differences by LSD at $P<0.05$.

The conversion of approximately 50% of the bisexual flowers to female which was observed in *CRC::etr1-1* plants, may be a further manifestation of this phenotype. One of the major sex genes in melon, the *A* locus, which is responsible for stamen inhibition in carpel-bearing flowers is an ACS enzyme (Boualem et al., 2008), and inhibition of stamens within bisexual flowers has been observed as a response to application of ethylene in melon (Rudich et al., 1969; Karchi, 1970). Increased apical ethylene evolution that can be perceived by the stamens during carpel bud development could subsequently lead to their own inhibition.

Increased or earlier carpel-bearing flower production was associated with increased fruit set on the *CRC::etr1-1* plants, especially early in the season. Total yield, however, was not different from wild type. It appears that the increased initial fruit set on the *CRC::etr1-1* lines was compensated for by a combination of abscission of young fruit, and smaller final fruit size. This suggests that higher competition for photosynthetic resources of the plant might have occurred in the *CRC::etr1-1* plants, either due to increased fruit number or earlier fruit set on younger, smaller plants. Competition for resources has been observed to regulate the balance between number of carpel-bearing flowers and fruit set in *Cucurbita* species (Stephenson et al. 1988*;* Krupnick et al. 1999; Avila-Sakar et al. 2001). Cucumber thinning has been demonstrated to increase fruit size and decreased assimilates within the plant, leading to fruit abortion of young fruits (Hikosaka and Sugiyama, 2005).

At final harvest, all of the M5 transgenic fruit showed a ripe phenotype. The earlier occurrence of ripe fruit in this line is likely due to earlier fruit set. In contrast, the M15 line did not show this phenotype; instead a higher proportion did not appear ripe based on external rind color and failure to slip from the vine. However, when M15 fruit classified as green were cut open, a majority showed that over-ripening had occurred within the mesocarp. Externally

evolved ethylene levels were consistent with those of green fruit for wild type and M5 fruit but, internal ethylene measurements of these fruit were on the level of ripe (orange) fruit of wild type and M5. The elevated ethylene would be expected during climacteric ripening (Barry and Giovannoni, 2007; Paul et al., 2012), and is consistent with the accelerated mesocarp ripening that was not externally visible.

The non-ripening rind (green) and elongated phenotype is similar to the spontaneous tomato mutant *Never-ripe 2* which was linked to ethylene perception inhibition (Barry et al., 2005). The external and internal ripening phenotypes observed could be explained by expression of the *etr1-1* transgene under the *CRC* promoter, targeting ethylene insensitivity to the carpel and ultimately to the exocarp/rind. A study by Fernandez et al. (2009) looking at tomato plants with the *CRC* promoter fused to GUS observed that expression was specifically localized to the fruit epidermis, but not mesocarp, throughout the ripening process. The expression of the *etr1-1* transgene was elevated in the exocarp of M15 fruit approaching the age that would be turning stage in wild type fruit, possibly explaining the observed external non-ripening. Earlier work with transgenic *35S::ACO*-antisense melons inhibiting the ethylene ripening processes showed that rind yellowing, abscission, and a majority of flesh softening processes are ethylene-dependent (Ayub et al., 1996; Pech et al., 2008). Although elevated expression was also observed in the exocarp of the M5 line at 35dpp, it was comparable to levels within mesocarp of M15 fruit. This suggests that *etr1-1* expression within the M5 exocarp and the M15 mesocarp was not high enough to inhibit ripening, while the greater expression within the M15 exocarp was sufficient to inhibit ethylene-dependent external ripening.

In summary, these studies show that inhibition of ethylene perception targeted to the carpel and nectary primordia modifies sex expression in the field as manifested by earlier onset

and increased carpel-bearing flowers, and a ~50% loss of stamens in carpel-bearing flowers (conversion from bisexual-to-female). Fruit set and total fruit number was increased, with earlier ripening apparent in the M5 line, however, M15 fruit showed inhibited external ripening. These results provide insight into the role of ethylene production and perception in carpel development, translating into earlier and increased fruit set. If regulated properly, manipulation of the ethylene signaling pathway could provide a shorter field growing season with increased and earlier fruit set, however a balance needs to be struck to maintain desired fruit size. These studies also provide further evidence for ethylene's involvement in external fruit ripening (i.e. rind degreening and abscission from the vine) in melon, and coordinated regulation of exocarp and mesocarp during normal ripening processes. Collectively, these results demonstrate potential for moderating melon fruit set and ripening by targeted regulation of ethylene perception.

LITERATURE CITED

LITERATURE CITED

Avila-Sakar G, Krupnick GA, Stephenson AG (2001). Growth and resource allocation in *Cucurbita pepo* spp. *texana*: effects of fruit removal. Int J Plant Sci 162: 1089-1095.

Ayub R, Guis M, BenAmor M, Gillot L, Roustan JP, Latche A, Bouzayen M, Pech JC (1996). Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits**.** Nat Biotechnol 14: 862-866.

Bapat VA, Trivedi PK, Ghosh A, Sane VA, Ganapathi TR, Nath P (2010). Ripening of fleshy fruit: Molecular insight and the role of ethylene. Biotechnol Adv 28: 94-107.

Barry CS, McQuinn RP, Thompson AJ, Seymour GB, Grierson D, Giovannoni JJ (2005). Ethylene insensitivity conferred by the *Green-ripe* and *Never-ripe 2* ripening mutants of tomato. Plant Physiol 138: 267-275.

Barry CS and Giovannoni JJ (2007). Ethylene and fruit ripening. Plant Growth Regul 26: 143-159.

Boualem A, Fergany M, Fernandez R, Troadec C, Martin A, Morin H, Sari M, Collin F, Flowers J, et al. (2008). A conserved mutation in an ethylene biosynthesis enzyme leads to andromonoecy in melons. Science 321: 836-838.

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

Fernandez AI, Viron N, Alhagdow M, Karimi M, Jones M, Amsellem Z, Sicard A, Czerednik A, Angenent G, Grierson D, May S et al. (2009). Flexible tools for gene expression and silencing in tomato. Plant Physiol 151:1729-1740.

Grumet R and Taft JA (2011). Sex expression in cucurbits In: Genetics, Genomics and Breeding in Cucurbits. Kole C, Wang YH, Behera TK, Kole C (eds). Science Publishers Inc. Enfield NH and CRC Press (Taylor & Francis) group, Boca Raton, FL. p. 353-375.

Guis M, Botondi R, BenAmor M, Ayub R, Bouzayen M, Pech JC, Latche A (1997). Ripening-associated biochemical traits of cantaloupe Charentais melons expressing an antisense ACC oxidase transgene. J Amer Soc Hort Sci 122: 748-751.

Hikosaka S and Sugiyama N (2005). Effect of fruit-load on growth patterns of fruit at the middle nodes of gynoecious-type cucumbers. J Hortic Sci Biotech 80:130-134.

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

Krupnick GA, Brown KM, Stephenson AG (1999). The influence of fruit on the regulation of internal ethylene concentration and sex expression in *Cucurbita texana*. Int J Plant Sci 160: 321-330.

Little HA, Papadopoulou E, Hammar SA, Grumet R (2007). The influence of ethylene perception on sex expression in melon (*Cucumis melo* L.) as assessed by expression of the mutant ethylene receptor, *At-etr1-1*, under the control of constitutive and floral targeted promoters. Sex Plant Reprod 20: 123-136.

Owens KW, Peterson CE, Tolla GE (1980). Production of hermaphrodite flowers on gynoecious muskmelon by silver nitrate and aminoethoxyvinylglycine. HortScience 15:654-655.

Papadopoulou E, Little HA, Hammar SA, Grumet R (2005). Effect of modified endogenous ethylene production on sex expression, bisexual flower development, and fruit production in melon (*Cucumis melo* L.). Sex Plant Reprod 18: 131-142.

Paul V, Pandey R, Srivastava, GC (2012). The fading distinction between classical patterns of ripening in climacteric and non-climacteric fruit and the ubiquity of ethylene – An overview. J Food Sci Tech Mys 49: 1-21.

Pech JC, Bouzayen M, Latche A (2008). Climacteric fruit ripening: Ethylene-dependent and independent regulation of ripening pathways in melon fruit. Plant Sci 175: 114-120.

Perl-Treves R (1999). Male to female conversion along the cucumber shoot: approaches to study sex genes and floral development in *Cucumis sativus*. In: Sex Determination in Plants (eds. CC Ainsworth). BIOS Scientific, Oxford: 189-215.

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

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

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

Stephenson AG, Devlin B, Horton JB (1988). The effects of seed number and prior fruit dominance on the pattern of fruit production in *Cucurbita pepo* (zucchini squash). Annu Bot 62: 653-661.

Taft J, Little H, Hammar S, Grumet R (2010). Effect of floral primordia-targeted ethylene production on sex determination in melon (*Cucumis melo* L). In: Cucurbitaceae 2010 Proceedings. Thies JA, Kousik S, Levi A (eds). American Society of Horticultural Science, Alexandria VA: 159-162.

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

Yamasaki S, Fujii N, Takahashi H (2003). Characterization of ethylene effects on sex determination in cucumber plants. Sex Plant Reprod 16:103-111.

CHAPTER 4

Expression characterization of sex genes and ethylene biosynthetic genes in different sex genotypes of melon (*Cucumis melo* L.)

Introduction:

Melon (*Cucumis melo* L.) plants have the capacity for a variety of sex expression phenotypes including andromonoecious (male and bisexual flowers), monoecious (male and female flowers), gynoecious (female-only flowers), and hermaphrodite (bisexual-only flowers). Differentiation to produce unisexual flowers occurs by suppression of either carpel primordia or stamen primordia at key development stages (Goffinet, 1990; Bai et al., 2004; see chapter 2). Control of melon (*Cucumis melo* L.) sex expression involves two major loci, *Gynoecious* (*G*) and *Andromonoecious* (*A*), as well as possible modifier loci (Kubicki, 1969; Kenigsbuch and Cohen, 1990; Roy and Saran, 1990). The *G* locus is involved in inhibition of early carpel development within male floral buds, allowing for separate male flowers to occur in dominant *G* genotypes, i.e. andromonoecious (*GGaa*) and monoecious (*GGAA*) (Kenigsbuch and Cohen, 1990; Roy and Saran, 1990). Stamen inhibition in carpel-bearing flowers is controlled by the *A* locus, wherein dominant *A* leads to suppression of stamens to produce a female flower instead of bisexual (Kenigsbuch and Cohen, 1990; Roy and Saran, 1990). Table 4.1 outlines the different sex genotypes of melon and their corresponding phenotypes.

Ethylene is a key factor regulating melon sex determination (Rudich, 1990; Grumet and Taft, 2011). Increased exogenous and endogenous ethylene levels are known to increase femaleness as manifested by increased carpel-bearing bud production and/or suppression of stamens (Rudich et al., 1969; Karchi, 1970; Papadopoulou et al., 2005). In contrast, inhibition of ethylene production or perception causes increased maleness as shown by increased male bud number or the conversion of female flowers to bisexual flowers (Den Nijs and Visser, 1980; Owens, 1980; Little et al., 2007). The ethylene biosynthetic pathway begins

Table 4.1. Melon sex expression patterns with corresponding genotypes and phenotypes.

Sex expression pattern	Genotype	Phenotype
Andromonoecious	*Ga*	♂⚥ - Male and bisexual flowers
Monoecious	*GA*	♂ ♀ - Male and female flowers
Gynoecious	*gA*	♀ - Female flowers only
Hermaphrodite	*ga*	⚥ - Bisexual flowers only

with the *S*-AdoMet precursor acted upon by ACC synthase (ACS) to produce ACC that is then oxidized by ACC oxidase (ACO) to form ethylene (Argueso et al., 2007).

The molecular identity of the *A* and G loci has recently been determined. The *A* locus encodes an ethylene biosynthetic gene, *ACC synthase 7* (*CmACS7*) that is expressed in carpel primordia (Boualem et al., 2008). A fully functional ACS7 can inhibit stamen primordia to consequently form a female flower, however mutant ACS7 (reduced enzymatic activity) cannot suppress stamens, thereby forming bisexual flowers (Boualem et al., 2008).

The *G* locus encodes a transcription factor named Wound Inducible Protein1 (CmWIP1). An insertion of a transposon into the *WIP1* promoter leads to methylation and thus silencing of the *G* locus, resulting in plants that make only female (gynoecious) or only bisexual (hermaphrodite) flowers (Martin et al., 2009). Our studies have shown that expression of *WIP1* is regulated by ethylene. Increased ethylene causes a decrease in *WIP1* transcript levels (see chapter 2). The source of ethylene regulating *WIP1* has not been determined, and may potentially act as a modifier of sex expression. The melon *G* locus may be contrasted with the corresponding *F* locus in cucumber (Trebitsh et al., 1997; Knopf and Trebitsh, 2006), Unlike *G*, *F* encodes an ACC synthase, further indicating importance of ethylene in preventing suppression of carpel primordia and potential involvement of ethylene biosynthetic enzymes in both sex determination steps (Trebitsh et al., 1997; Knopf and Trebitsh, 2006).

Melon has several members in each of the *ACS* and *ACO* gene families that have variable expression across plant tissues. Several of the *ACS* members (*ACS1, ACS2*, and *ACS3*) and *ACO1* were first identified in melon fruit as potential factors involved in the ripening process, or in response to hormone application on seedlings (Miki et al., 1995; Lasserre et al., 1996; Ishiki et al., 2000). Further characterization looked at expression of *ACS* and *ACO* in other plant tissues

such as seedlings, leaf and flowers, or response to stress (Lasserre et al., 1997; Ishiki et al., 2000). A majority of these members have not been tested for expression in apex, floral tissue, or in different sex genotypes of melon. With the recent sequencing of the melon genome (Garcia-Mas, et al., 2012) and transcriptome data available for the closely related cucumber, we can also ask if there are previously unreported members of the *ACS* and *ACO* family with potential roles in sex determination.

In this study, we sought to characterize the *ACS* and *ACO* gene family members in different melon sex genotypes to provide gene expression profiles within apices. Monoecious and hermaphrodite lines were developed through a gynoecious x andromonoecious cross, and segregation ratios matched a four gene model implicating two major loci and two modifying loci. Three new *ACS* gene family members were identified from the available melon genome: *CmACS5*, *CmACS10*, and *CmACS12*. Gene expression analysis of apices showed that transcript levels of a majority of the *ACS* members and all of the *ACO* members were higher in the gynoecious and hermaphrodite lines.

Materials and methods:

Plant genotypes and growing conditions

The gynoecious breeding line, Wisconsin 998 (WI998; *ggAA*) (kindly provided by Dr. J. Staub, Univ Wisconsin) was crossed to the commercial andromonoecious line Hale's Best Jumbo (Hales; *GGaa*) to develop monoecious and hermaphrodite lines. The F_1 hybrid progeny (*GgAa*) were self pollinated to produce the F_2 generation. Two plantings of the F_2 were performed in the greenhouse, each with 200- 400 seeds, to determine segregation ratios of sex phenotypes. Plants were selected for those that only produced female flowers (gynoecious;

ggAA), or bisexual flowers (hermaphrodite; *ggaa*) along the first 10-15 nodes of the main stem. To allow room for seed production, F_2 plants that produced male flowers were removed from the greenhouse at the appearance of the first male bud ; thus numbers for andromonoecious and monoecious were pooled when testing F_2 segregation ratios. The hermaphrodite plants were self pollinated to produce F_3 families, which were screened for homozygosity. Andromonoecious (*GGaa*), monoecious (*G-A-*), gynoecious (*ggAA*), and hermaphrodite (*ggaa*) genotypes were grown under greenhouse conditions described in Little et al. (2007), and used for analysis of apical gene expression as described below.

Identification of ACS gene family members

Previously published sequences for *ACS1*, *ACS2*, *ACS3*, *ACS7*, *ACO1*, *ACO2*, and *ACO3* were available at NCBI or ICuGI and used for primer design described below. To identify new members of the *ACS* family, available cucumber 454 pyrosequencing data from Ando and Grumet (2010) was searched for ACC synthases that had homology to other known *ACS* genes. A corresponding melon EST was identified in ICuGI (MU48956; referred to here as *CmACS10*) using the cucumber sequence to BLAST the melon EST database. Two additional members were identified with homology to Arabidopsis *ACS4*, *5*, *8*, and *ACS12*, respectively, referred to here as *CmACS5* and *CmACS12*, using the newly sequenced melon genome (Garcia-Mas et al., 2012), and provided to us by Jordi Garcia-Mas and Walter Sanseverino (ILTA, Spain).

qRT-PCR analysis

Total RNA was prepared from 200mg of liquid nitrogen frozen melon apex tissue (~3-

4mm in size) removed from plants at the 2 leaf stage (developing floral buds just entering stage 5; beginning of sex differentiation) using the Trizol method (Invitrogen, Carlsbad, CA). cDNA preparation was performed using Superscript II reverse transcriptase (Promega, Madison, WI) following manufacturer's recommendations. Gene-specific primers for the *ACS* and *ACO* gene family members were designed using Primer Express 3.0 software (Applied Biosystems); the primer sequences are listed in Table 4.2. qRT-PCR analysis was carried out using a Stratagene Mx4000 system with Power SYBR Green PCR Master Mix (Applied Biosystems). *C. melo Polyubiquitin 7* (ICuGI: MU43954) was used as an endogenous control for normalization. qRT-PCR experiments were repeated twice with three biological replicates and three technical replicates/biological replicate.

Statistical analysis

WIP1 and *ACS* member gene expression data were $\log_2$ transformed. Data were analyzed by analysis of variance (ANOVA) using Proc Mixed protocol in SAS (SAS Institute, Cary, NC).

Results and discussion:

Segregation analysis of a gynoecious x andromonoecious cross

To study natural variation of gene expression in melon, four sex genotypes were produced by a cross between andromonoecious Hales (*GGaa*) and gynoecious WI998 (*ggAA*). The resulting F_1 progeny (*GgAa*) showed the expected monoecious phenotype. Segregation analysis of the sex phenotypes of the F_2 progeny were tested for a 2 gene, 3 gene, or 4 gene model. The ratios were not consistent with a two gene model as the expected number of the

Table 4.2. Primers of melon *ACS* and *ACO* gene members for qRT-PCR analysis.

Primer	Forward sequence	Reverse sequence
qRT ACS1 3F(RG742) qRT ACS1 3R (RG743)	5'-AAGACATGCAAGGTTCACAGAAGC-3'	5'-TCCTGCATTGTATTCAAGCA-3'
Cm-ACS2-3F qRT (RG634) Cm-ACS2-3R qRT (RG635)	5'-GCAGAGCCCGGTTTCATTAG-3'	5'-CGCATTCAACGTCGTTCATG-3'
Cm-ACS3-2F qRT (RG636) Cm-ACS3-2R qRT (RG637)	5'-TTGGTTACGAAGTGGGTTTGAATAT-3'	5'-ACCCTGGCTCAGTGCAATG-3'
qRT ACS5 1F (RG748) qRT ACS5 1R (RG749)	5'-GGTTGGTTTAGAATGTGCTTTGC-3'	5'-ACGATGCATGGCCACCTT-3'
qRT-ACS7F (RG465) qRT-ACS7R (RG464)	5'-TTTAGCGTCCATGTTGTCGAACCG-3'	5'-TCATACCGTTTCTTGAGCCTGTCC-3'
newACS-2F (RG644) (ACS10) newACS-2R (RG645)	5'-AGAGTGCAAACGGTTTCCGA-3'	5'-AGGTTAAGCTTTTGAGCTTGCTCGT-3'
qRT ACS12 F (RG744) qRT ACS12 R (RG745)	5'-CAAAGGGAATGGGCATGGTG-3'	5'-CCACGAATTCAAAAGAAAGCTGGT-3'
qRT WIP1F (RG680) qRT WIP1R (RG681)	5'-TAGGGCTTCCAACTCCTTCCTCT-3'	5'-CTTGCAATTGATGGGTGTGATCTTCTTG-3'
qRT ACO1F (RG477) qRT ACO1R (RG476)	5'-GCTGCACAAGCTGAAGTTAATGA-3'	5'-TCAGGAAGATGGCGTAAGAAAAA-3'
qRT ACO2F (RG475) qRT ACO2R (RG474)	5'-GACGGCAGATGGTTCAAAATC-3'	5'-TCGCCGATGTTGACGAAAA-3'
qRT ACO3F (RG473) qRT ACO3R (RG472)	5'-AAGGAATTTGCAGATGAATTGGA-3'	5'-AGCCCAAGATTCTCACACAACAA-3'
CmUBQ7-3F (RG541) CmUBQ7-3R (RG543)	5'-GACCGGGAAGGAAATTGAGAT-3'	5'-CCGCTCCTTAATACGATCAATTGG-3'

double recessive hermaphrodite phenotype in a total of 600 plants would be 37.5, however only 5 plants were hermaphrodite (Table 4.3). F_3 progeny testing of the F_2 progeny verified that the hermaphrodite F_2 plants were true-breeding for sex type, as expected for homozygous recessive individuals. X^2 values support a four gene model implicating two modifiers acting along with the two major sex loci to produce a hermaphrodite plant. Prior analyses have led to sex expression models for melon including only two genes, or as many as four genes (2 major and 2 modifying sex loci) (Kubicki, 1969; Kenigsbuch and Cohen, 1990). Differences among the results likely reflect different modifier alleles present in the starting parental genotypes.

Analysis of sex genes in different sex genotypes

Expression of the sex genes, *G* (*WIP1*) and *A* (*ACS7*), was examined in apices of the different sex genotypes. Apices were collected from plants at the two leaf stage where the most developed buds are just entering developmental stage 5 (Taft chapter 2). At stage 5 all four floral whorls have been initiated and sex differentiation begins; carpel primordia can be either inhibited or promoted based on ethylene levels and *WIP1* gene expression (Martin et al., 2009; Taft chapter 2). *WIP1* expression was highest in monoecious (*G-A-*) apices compared to apices of andromonoecious (*G-aa*), gynoecious (*ggAA*) and hermaphrodite (*ggaa*) plants (Figure 4.1a). This result matched the gene expression analysis in Martin et al. (2009) where monoecious buds showed higher expression compared to gynoecious and hermaphrodite buds; andromonoecious plants were not used for comparison in their study. This is expected as the *G* locus, which encodes the transcription factor *WIP1*, is silenced in recessive genotypes due to a transposon inserted upstream of the gene, however dominant genotypes do not have this insertion allowing

Table 4.3. Segregation for F_2 population of gynoecious (WI998) x andromonoecious (Hales) cross.

Genotype	**Flower sex phenotype**	**Models: Expected**			**Exp.1**	**Exp.2**	
		Two gene	**Three gene**	**Four gene**	**Observed**	**Observed**	**Total**
GA	Male, female	9	36	189	**196**	**393**	**589**
Ga (P2)	Male, bisexual	3 **450**	12 **450**	63 **590**			
gA(P1)	Female[1]	3 **112.5**	9 3 **112.5**	3 **8**	**3**	**3**	**6**
ga	Bisexual	1 **37.5**	4 **37.5**	1 **2**	**1**	**4**	**5**
	X^2:	35.53**	29.93**		0.50	2.00	3.22
	P	0.01	0.01		0.80	0.40	0.20

[1]May also include gynomonoecious (female and bisexual) sex phenotype. ** denotes statistical significance at $P<0.01$.

for expression of *WIP1* (Martin et al., 2009). *WIP1*expression level was lower in andromonoecious than monoecious, however the reason for this difference is not known.

Expression of the *A* gene, *ACS7*, which suppresses stamens in carpel-bearing buds (Boualem et al., 2008) was nearly exclusively expressed in gynoecious apices compared to andromonoecious, monoecious, and hermaphrodite apices, respectively (Figure 4.1b). This nearly exclusive expression is expected since expression of the *A* locus would be higher in a female genotype compared to bisexual, and buds developing within the apex at the two leaf stage in monoecious plants are mostly male (Taft chapter 2). This profile matches the results for gynoecious and hermaphrodite plants observed by Boualem et al. (2008) and Martin et al. (2009), where *ACS7* showed higher expression in female buds compared to bisexual buds. The equivalent locus in cucumber, *M* locus, which is involved in stamen inhibition in carpel-bearing buds, also showed the same expression profile for gynoecious and hermaphrodite lines (Boualem et al., 2009).

Identification of ACS and ACO gene family members

There are 4 melon *ACS* and 3 *ACO* gene family members that have been previously identified; *ACS1*, *ACS2*, *ACS3*, *ACS7*, *ACO1*, *ACO2*, and *ACO3* (Miki et al., 1995; Lasserre et al., 1996, 1997; Ishiki et al., 2000; Boualem et al., 2008; Martin et al., 2009). Examination of our available cucumber 454 pyrosequencing data set from Ando and Grumet (2010) identified a new member of the ACS family with homology to Arabidopsis *ACS10* and *12*, and a corresponding

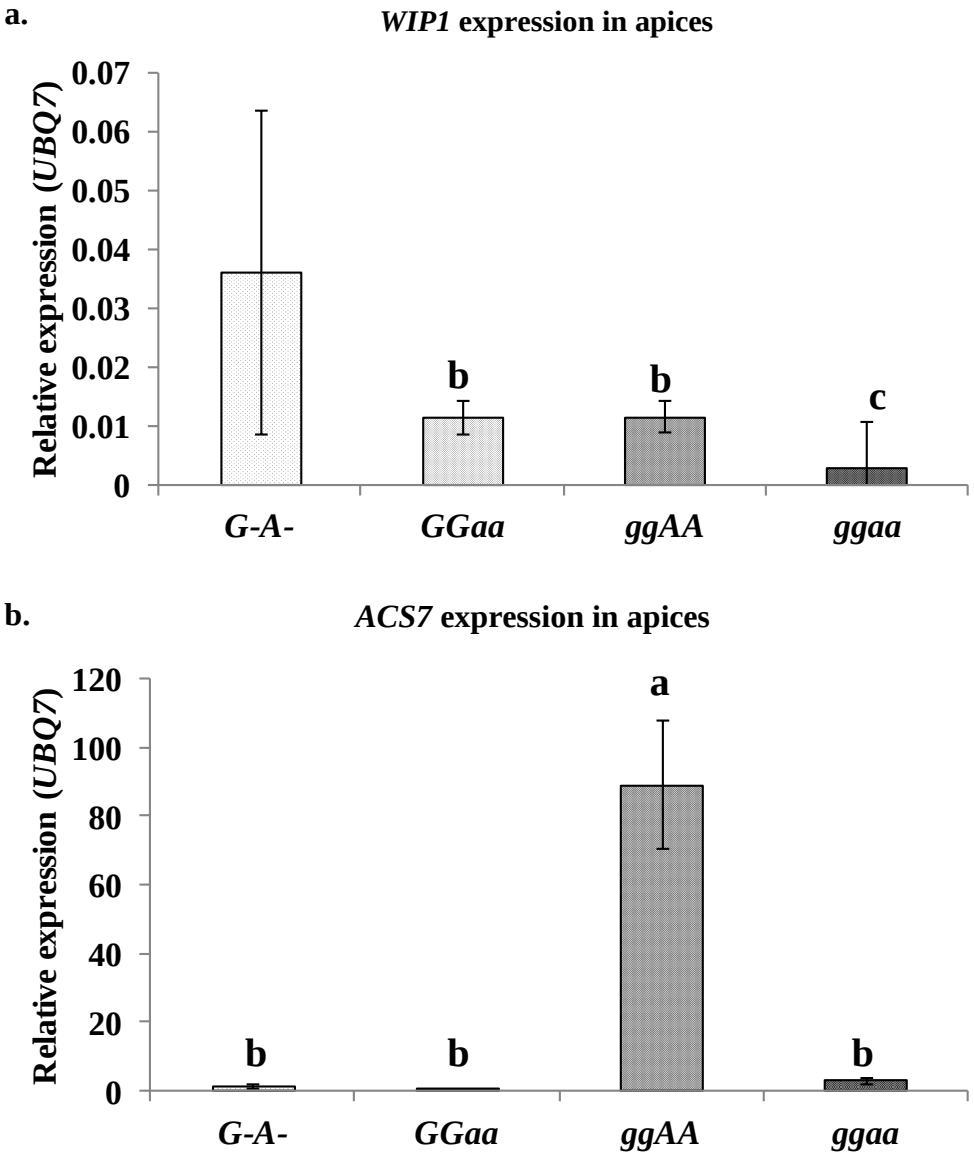


Figure 4.1. Expression of the melon sex genes *WIP1* (a) and *ACS7* (b) in monoecious (*G-A-*), andromonoecious (*GGaa*), gynoecious (g*gAA*), and hermaphrodite (*ggaa*). Values are the average of three biological replicates with three technical replicates/biological rep $\pm$ SE. Different letters represent significant differences by LSD at $P<0.05$.

melon EST was identified in ICuGI (MU48956; referred to here as *CmACS10*). A phylogenetic tree of the *ACS* gene family in melon was constructed by Sanserevino et al. (personal communication) using sequences from the newly available melon genome (Figure 4.2) (Garcia-Mas et al., 2012). Two additional members were identified with homology to Arabidopsis *ACS4/ 5/ 8* clade, and *ACS12*, referred to here as *CmACS5* and *CmACS12*. Examination of the *ACS* phylogenetic tree shows that the melon *ACS* members are most closely related to cucumber *ACS* members, as would be expected; the same set of family members was identified in both species. No new *ACO* gene family members were identified from the melon genome beyond the three already known.

Analysis of ACS and ACO gene family members in different sex types

Expression of the different *ACS* and *ACO* gene family members was examined in young apices of the four sex genotypes (Figure 4.3). *ACS2*, *ACS5,* and *ACS10* were detectable within the apex (Figure 4.3a). Apices from gynoecious (*ggAA*) plants showed the highest expression of *ACS2*, *ACS5,* and *ACS10*, while hermaphrodite showed higher expression of *ACS5* and *ACS10* compared to monoecious and andromonoecious (Figure 4.3a).Similarly, expression of all three *ACO* members also was highest in the gynoecious apices with second highest values in hermaphrodite buds compared to andromonoecious and monoecious genotypes (Figure 4.3b).

Expression of *ACS5*, *ACS10*, and all the *ACO* members (*ACO1*, *ACO2* and *ACO3*) were higher in recessive *g* genotypes (gynoecious and hermaphrodite) compared to dominant *G* (Figure 4.3b). A possible explanation is that the increased expression of *ACS* and *ACO* members increases ethylene levels, reducing *WIP1* (*G*) expression in order to inhibit male bud production

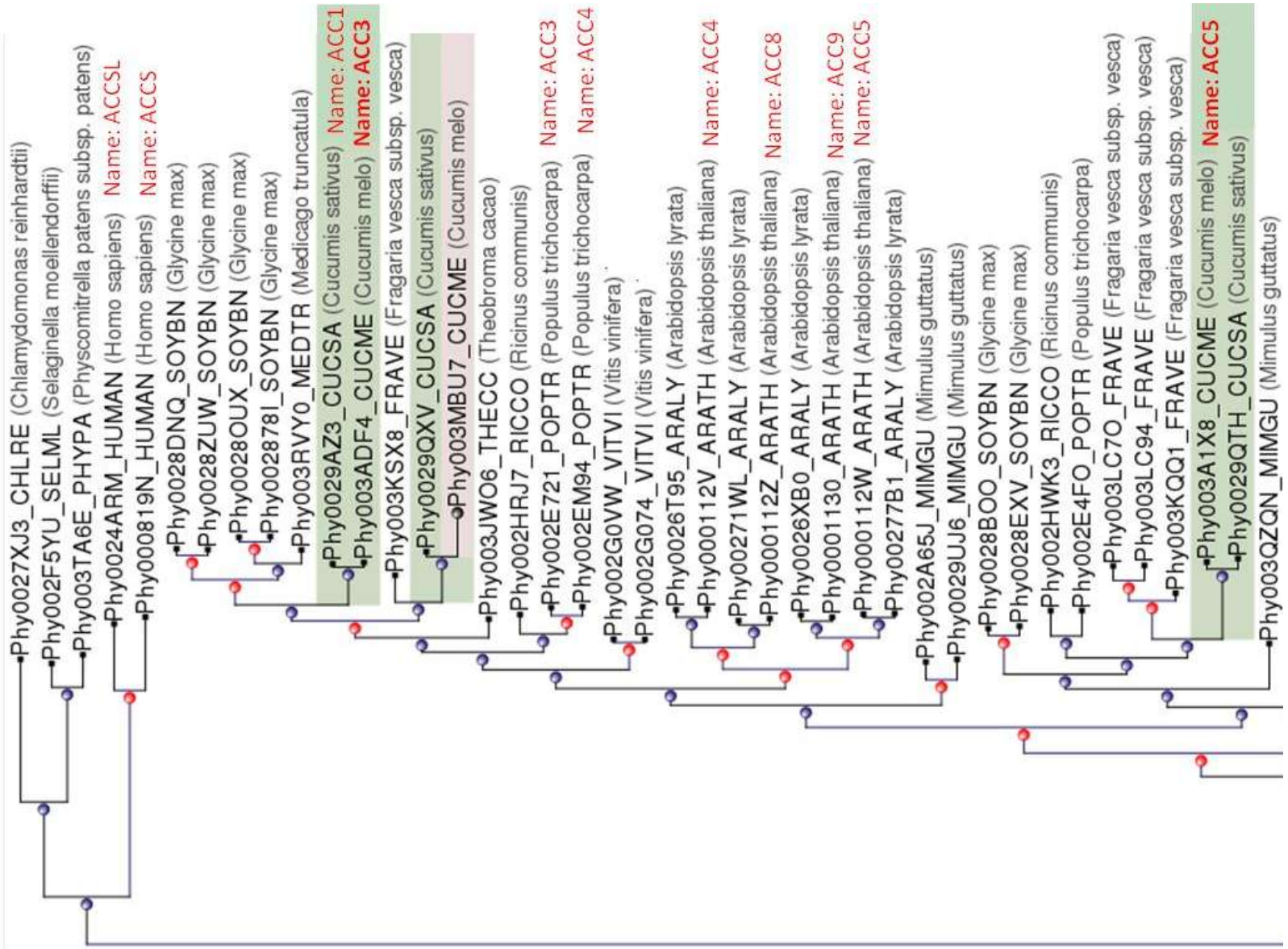


Figure 4.2. Rooted phylogenetic tree of *ACS* gene family members across different species provided by Jordi Garcia-Mas and Walter Sanseverino (personal communication).

Figure 4.2 (cont'd)

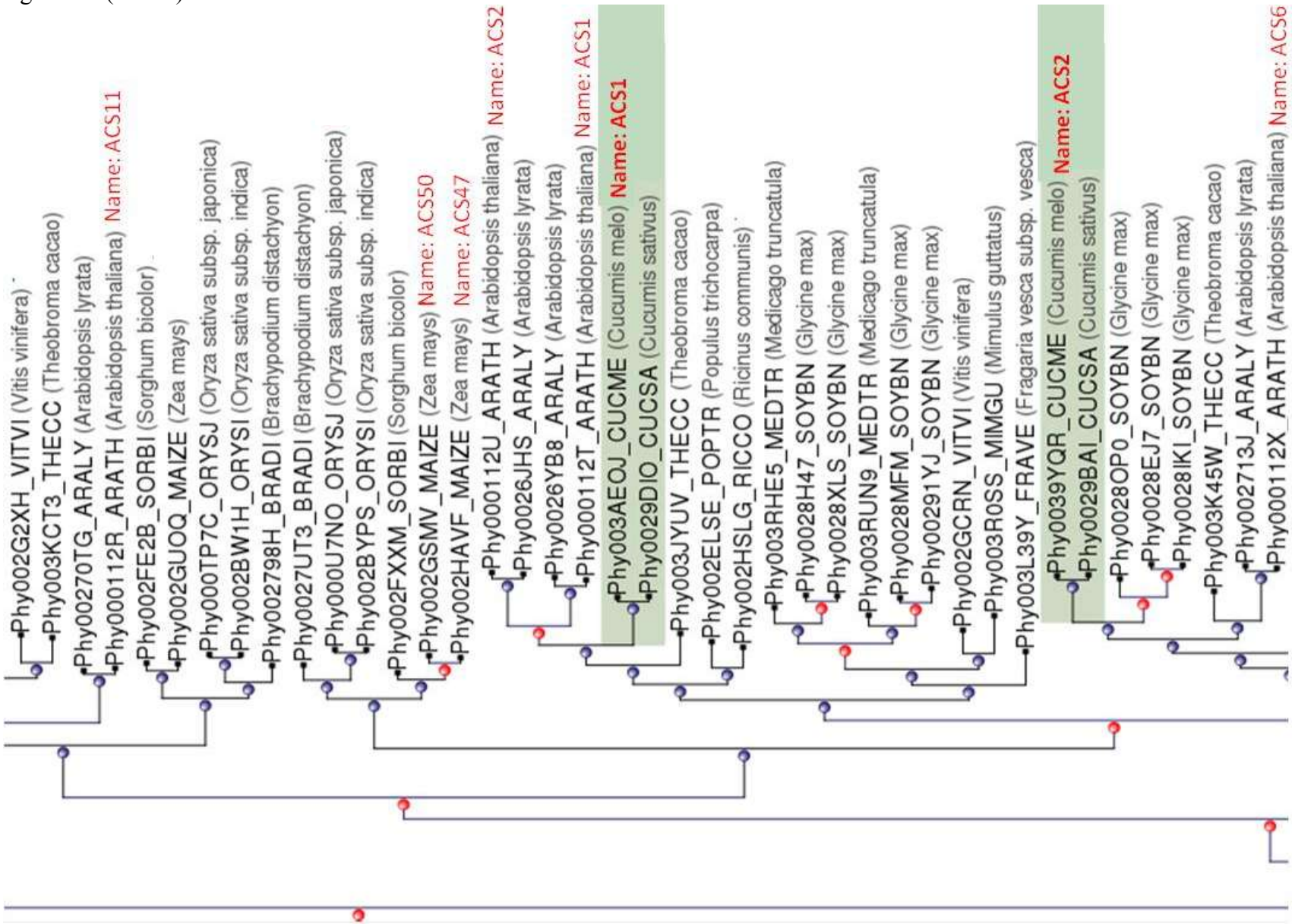


Figure 4.2 (cont'd)

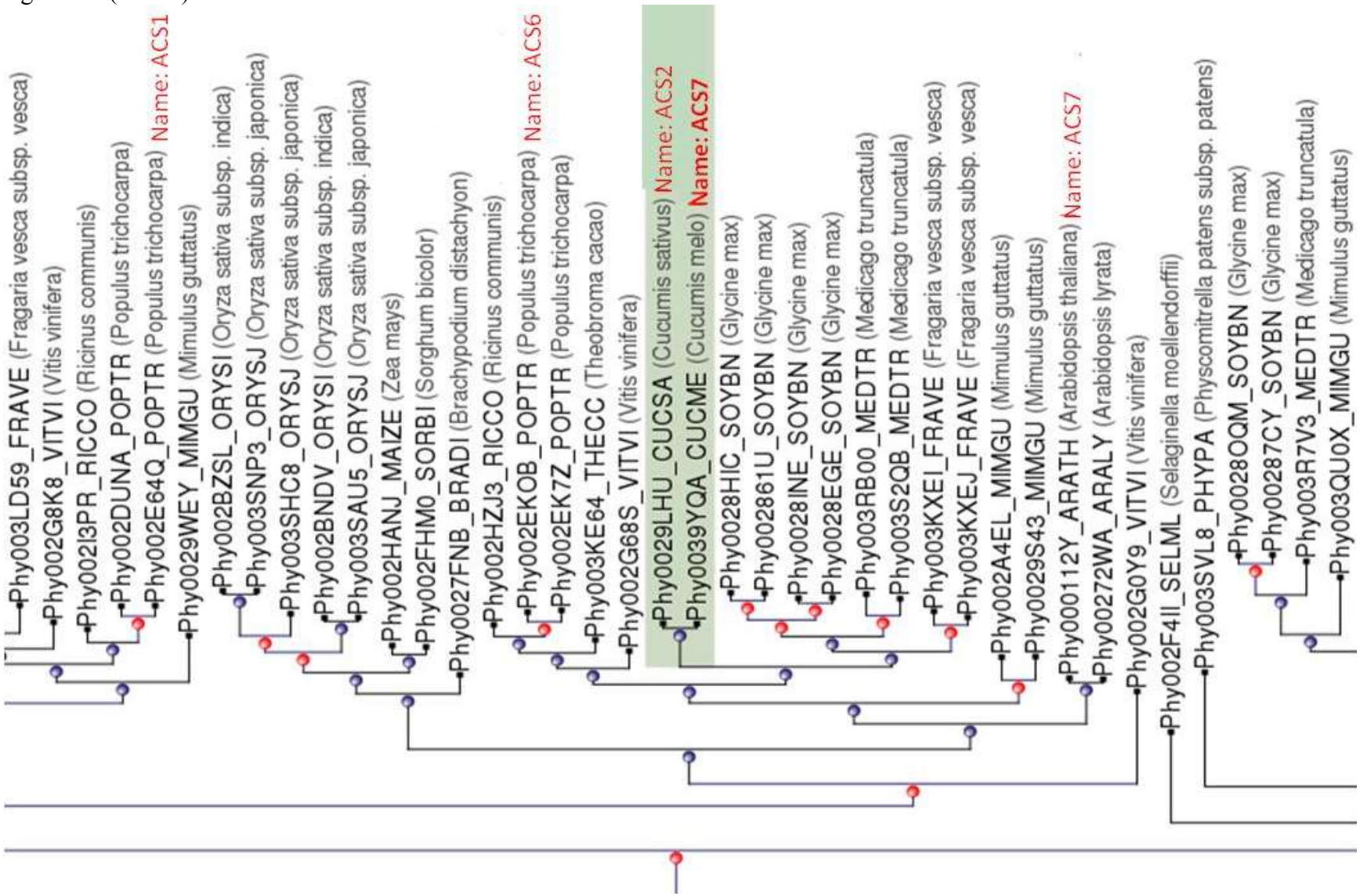
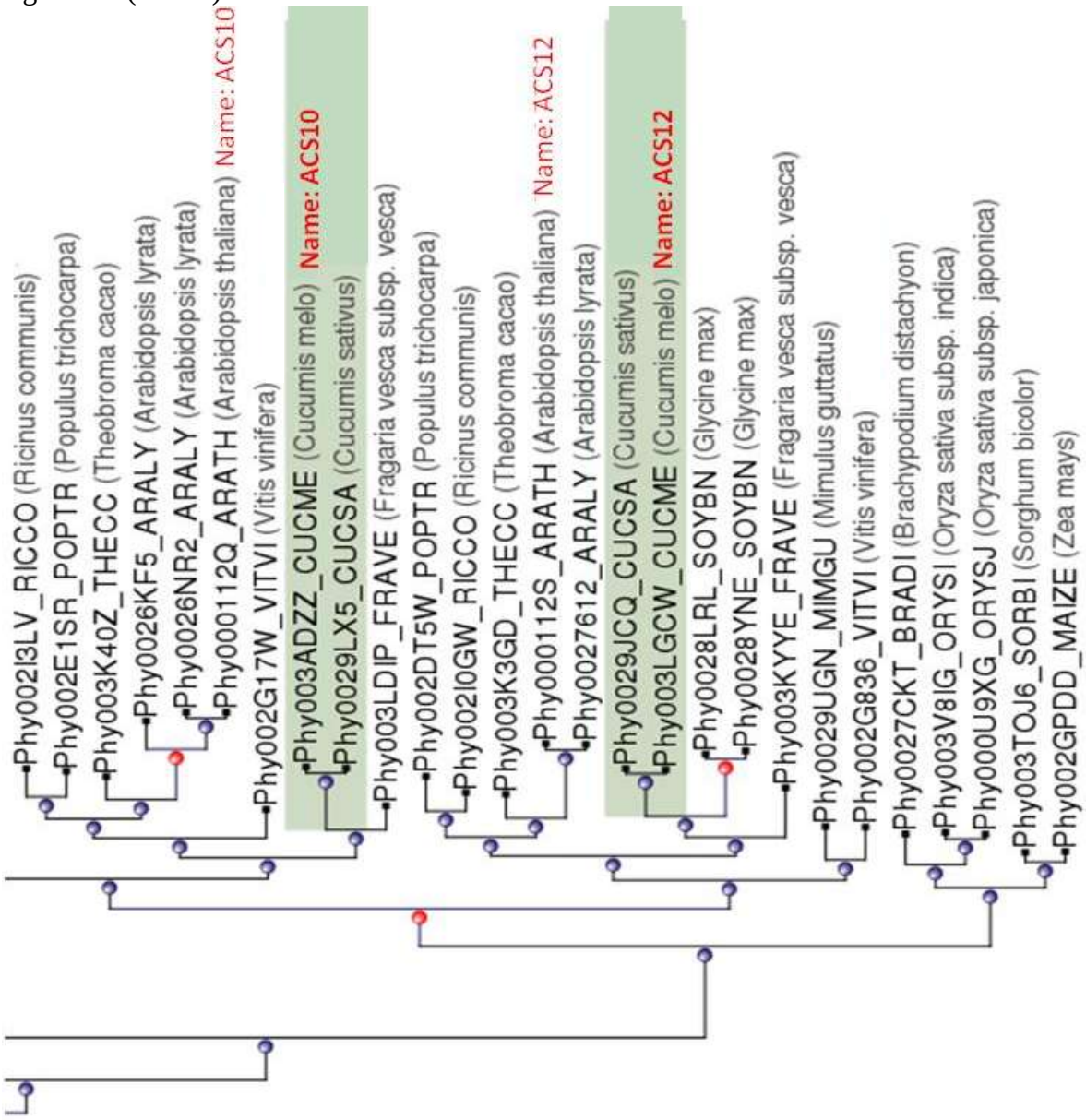


Figure 4.2 (cont'd)



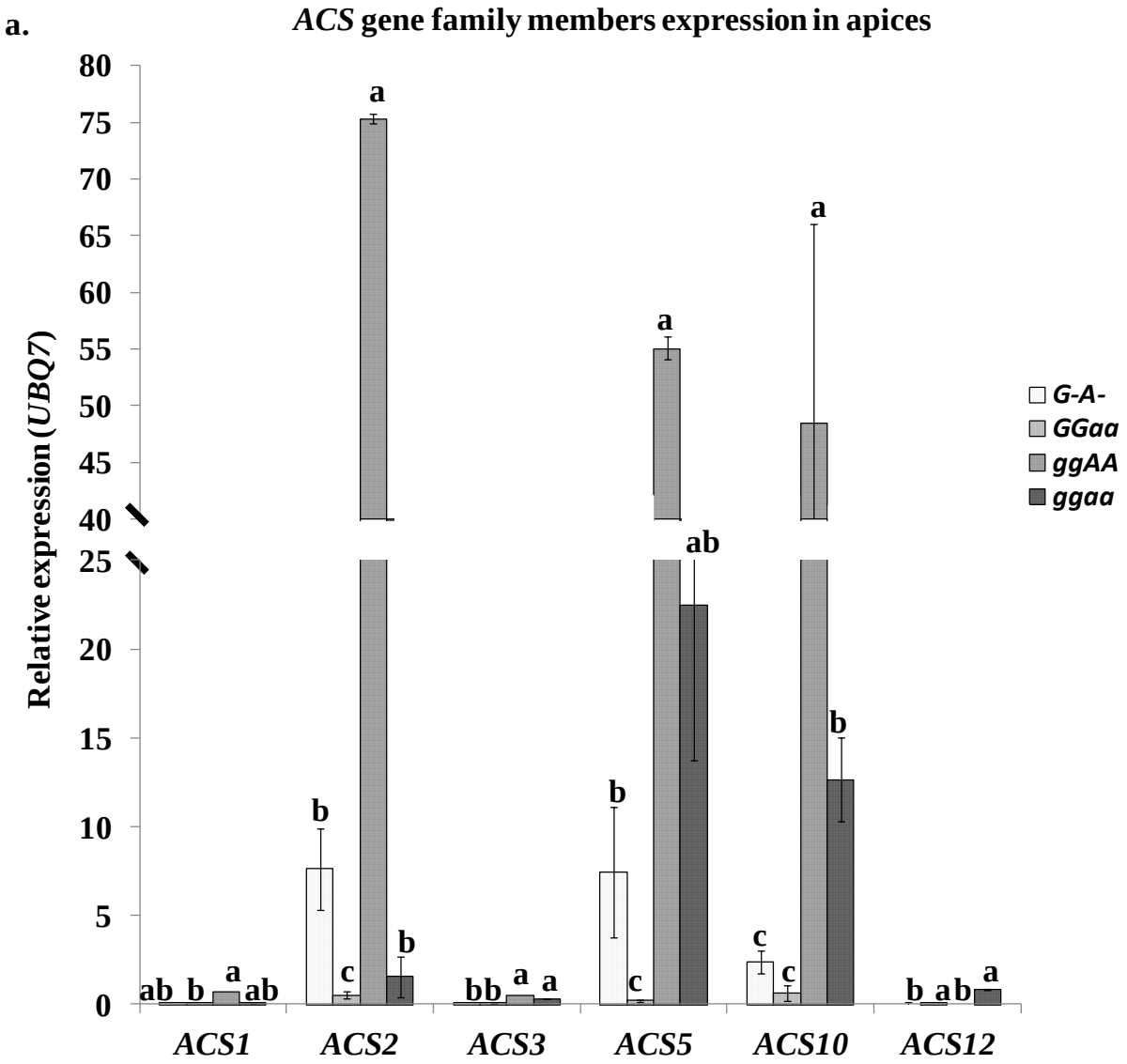
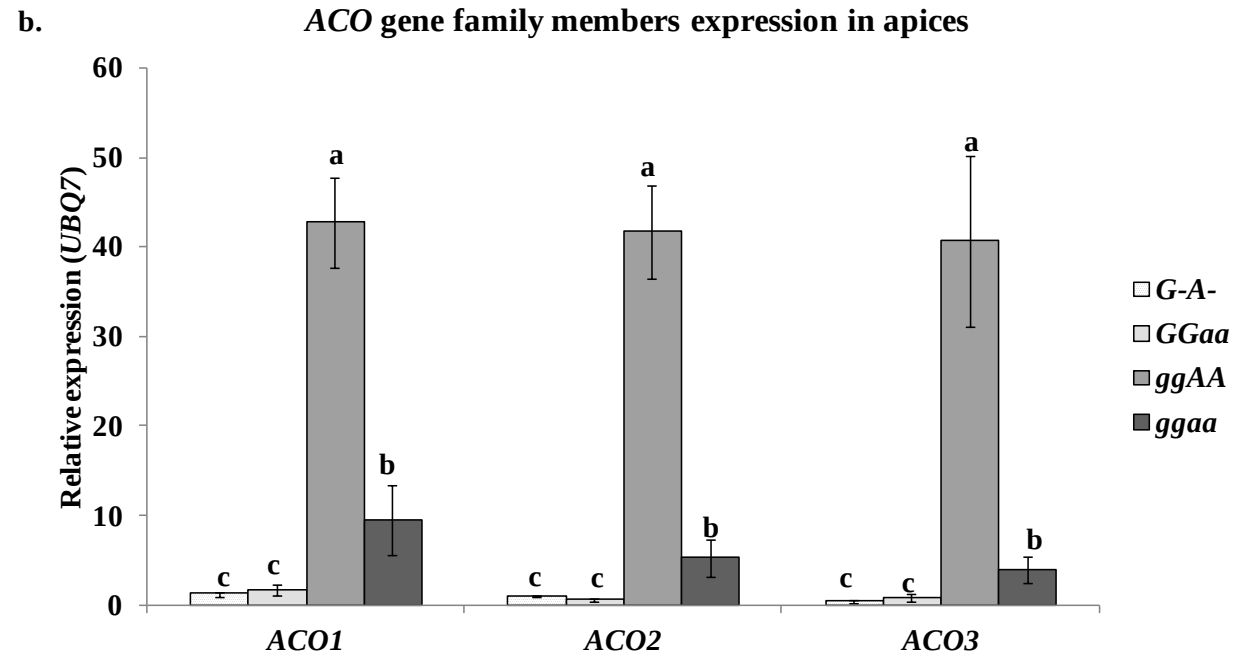


Figure 4.3. Expression of *ACS* (a) and *ACO* (b) gene family members in monoecious (*GA*), andromonoecious (*Ga*), gynoecious (*gA*), and hermaphrodite (*ga*). Values are the average of three biological replicates with three technical replicates/biological rep ± SE. Different letters represent significant differences by LSD at $P<0.05$.

Figure 4.3 (cont'd)



to facilitate all carpel-bearing phenotypes (*g* genotypes). However, the *g* locus in all carpel-bearing genotypes is recessive due to silencing of the *WIP1* transcription factor and loss of the inhibition of carpel development, thus ethylene would have to interfere with methylation of the *WIP1* transcription factor in order to cause male flowers and change to a *G* genotype. Alternatively, it is possible that the reduced expression of the *ACS* and *ACO* genes in andromonoecious and monoecious genotypes is an effect of the dominant *G* locus, leading to suppressed expression of ethylene related.

Unlike the recessive *g allele* in melon, the cucumber allele responsible for preventing suppression of carpel development, is the dominant *F*, *F* encodes an extra *ACS1*, namely *ACS1G* and was a product of gene duplication (Trebitsh et al., 1997; Knopf and Trebitsh, 2006). The homolog to *ACS1G* in melon is *CmACS3;* unlike *CsACS1* and *1G* which are located in tandem in cucumber, *CmACS1* and *CmACS3* in melon are located on linkage groups 11 and 8, respectively. Expression of *CmACS3* had not been examined in apices to determine if it is related to sex determination, however it is a possible modifier candidate. *ACS3* expression was higher in gynoecious and hermaphrodite, however compared to other *ACS* members, the gene expression was very low.

In summary, gene expression profiles of the *ACS* and *ACO* gene family members in different melon sex genotypes were examined within apices to determine if these ethylene production genes were implicated in sex determination. A cross between gynoecious x andromonoecious plants was used to develop monoecious and hermaphrodite lines. Segregation of the F_2 generation matched a four gene model with two modifying loci in order to form the hermaphrodite genotype. Expression profiles of the ethylene production gene family members of *ACS* and *ACO* showed that genotypes that produce all carpel-bearing buds (gynoecious and

hermaphrodite) tended to show higher expression of all family members within the apex. It is possible that higher ethylene produced by higher gene expression of the biosynthetic enzymes suppresses a functional *G* (*WIP1*), leading to promotion of carpels and a greater number of carpel-bearing buds. An alternative hypothesis is that a functional *G* effects gene expression of the *ACS* and *ACO* genes within the apex to reduce their expression and increase the number of male buds. This suggests that one of more *ACS* or *ACO* members may act as modifiers in sex determination.

LITERATURE CITED

LITERATURE CITED

Ando K and Grumet R (2010). Transcriptional profiling of rapidly growing cucumber fruit by 454-pyrosequencing analysis. J Amer Soc Hort Sci 135: 291-302.

Argueso CT, Hansen M, Kieber JJ (2007). Regulation of ethylene biosynthesis. J Plant Growth Regul 26: 92-105.

Bai SL, Peng YB, Cui JX, Gu HT, Xn LY, Li YQ, Xu ZH, Bai SN (2004). Developmental analyses reveal early arrest of the spore-bearing parts of reproductive organs in unisexual flowers of cucumber (*Cucumis sativus* L.). Planta 220: 230-240.

Boualem A, Fergany M, Fernandez R, Troadec C, Martin A, Morin H, Sari M, Collin F, Flowers J, et al. (2008). A conserved mutation in an ethylene biosynthesis enzyme leads to andromonoecy in melons. Science 321: 836-838.

Boualem A, Troadec C, Kovalski I, Sari M, Perl-Treves R, Bendhamane A (2009). A conserved ethylene biosynthesis enzyme leads to andromonoecy in two *Cucumis* species. PLoS ONE 4: e6144.

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

Duan QH, Wang DH, Xu ZH, Bai SN (2008). Stamen development in Arabidopsis is arrested by organ-specific overexpression of a cucumber ethylene synthesis gene *CsACO2*. Planta 228: 537-543.

Garcia-Mas J, Benjak A, Sanseverino W, Bourgeois M, Mir G, Gonzalez VM, Henaff E, Camara F, Cozzuto L et al. (2012). The genome of melon (*Cucumis melo* L.). P Natl Acad Sci 109: 11872-11877.

Goffinet MC (1990). Comparative ontogeny of male and female flowers in *Cucumis sativus*. In: Biology and Utilization of the *Cucurbitaceae*. DM Bates, RW Robinson, C Jeffery (eds). Cornell University Press, New York: 288-304.

Grumet R and Taft J (2011). Sex expression in cucurbits. In: Genetics, Genomics and Breeding in Cucurbits. C Kole, YH Wang, TK Behera (eds). Science Publishers Inc., Enfield and CRC Press (Taylor and Francis) group, Boca Raton: 353-375.

Ishiki Y, Oda A, Yaegashi Y, Orihara Y, Arai T, Hirabayashi T, Nakagawa H, Sato T (2000). Cloning of an auxin-responsive 1-aminocyclopropane-1-carboxylate synthase gene (CMe-ACS2) from melon and the expression of ACS genes in etiolated melon seedlings and melon fruits. Plant Sci 159: 173-181.

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

Kenigsbuch D and Cohen Y (1990). The inheritance of gynoecy in muskmelon. Genome 33: 317-320.

Knopf R and Trebitsh R (2006). The female-specific *CsACS1G* gene of cucumber. A case of gene duplication and recombination between the non-sex specific 1-aminocyclopropane-1-carboxylate synthase gene and a branched chain amino acid transaminase gene. Plant Cell Physiol 47: 1217-1228.

Kubicki B (1969). Sex determination in muskmelon (*Cucumis melo* L.) Genet Pol 10: 145-163.

Lasserre E, Bouquin T, Hernandez JA, Bull J, Pech JC, Balague C (1996). Structure and expression of three genes encoding ACC oxidase homologs from melon (*Cucumis melo* L.). Mol Genet Genomics 251: 81-90.

Lasserre E, Godard F, Bouquin T, Hernandez JA, Pech JC, Rody D, Balague C (1997). Differential activation of two ACC oxidase gene promoters from melon during plant development and in response to pathogen attack. Mol Genet Genomics 256: 211-222.

Little HA, Papadopoulou E, Hammar SA, Grumet R (2007). The influence of ethylene perception on sex expression in transgenic melon as assessed by expression of the mutant ethylene receptor gene, *At-etr1-1*, under control of constitutive and floral-targeted promoters. Sex Plant Reprod 20: 123-136.

Martin A, Troadec C, Boualem A, Mazen R, Fernandez R, Morin H, Pitrat M, Dogimont C, Bendhamane A (2009). A transposon-induced epigenetic change leads to sex determination in melon. Nature 461: 135-1139.

Miki T, Yamamoto M, Nakagawa H, Ogura N, Mori H, Imaseki H, Sato T (1995). Nucleotide-sequence of a cDNA for 1-aminocyclopropane-1-carboxylate synthase from melon fruits. Plant Physiol 107:297-298.

Owens KW, Peterson CE, Tolla GE (1980). Production of hermaphrodite flowers on gynoecious muskmelon by silver nitrate and aminoethoxyvinylglycine. HortScience 15:654-655.

Papadopoulou E, Little HA, Hammar SA, Grumet R (2005). Effect of modified endogenous ethylene production on hermaphrodite flower development and fruit production in melon (*Cucumis melo* L.). Sex Plant Reprod 18: 131-142.

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

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

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

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

CONCLUDING REMARKS

Melon and cucumber are model species of unisexual flower development that can be governed by developmental stage, hormones, and genetics in order to cause a variety of sex expression phenotypes (Roy and Saran, 1990; Perl-Treves, 1999; Grumet and Taft, 2011). The development of a comprehensive model for melon sex determination that includes known phenotypes, developmental stages, ethylene influences, and gene expression will provide a greater understanding of the molecular aspects involved in this process.

To examine the influence of ethylene production that is perceived by the stamen primordia during early flower development (Little et al., 2007), transgenic plants targeting production to the stamen and anther (*AP3::ACS*), or carpel and nectary primordia (*CRC::ACS*) were used. Melon plants with ethylene production targeted to the stamen primordia showed high levels of ethylene evolution in the apex, and increased femaleness, as manifested by gain of a bisexual-only phase not seen in wild type, and loss of male buds and open flowers along the main stem. The most dramatic phenotype observed was a conversion to hermaphrodite instead of andromonoecious. Microscopic analysis of apical tissue found that a smaller proportion of pre-sex determination buds transitioned into the sex determination stages within the transgenic *AP3::ACS* melons. In contrast, carpel primordia-targeted plants did not show a difference in sex expression or gene expression for the native sex genes, providing further evidence in support of stamen's involvement in carpel development.

Native sex gene expression was also influenced in *AP3::ACS* plants as transcript levels of the *A* locus (involved in stamen inhibition in carpel-bearing flowers; *ACS7*; Boualem et al., 2008) were increased, and expression of the *G* locus (involved in carpel inhibition in male buds;

WIP1; Martin et al., 2009) was decreased. Exogenous application of ethylene also showed a reduction in *WIP1* gene expression, and promoter analysis identified ethylene-related motifs. This provides evidence for the regulation of *WIP1* expression by ethylene, and a link to genetic control of sex expression by the *G* locus and ethylene. Taken together, these results provide further evidence for ethylene thresholds acting within the apex during floral bud development, and indicate critical developmental stages and levels for sex differentiation for promotion or inhibition of sex organs.

Previous studies conducted in our lab using carpel and nectary primordia-targeted ethylene perception inhibition (*CRC::etr1-1*) showed increased femaleness under greenhouse conditions (Little et al., 2007). *CRC::etr1-1* melons were then tested in a field setting to determine if the floral sex phenotype would also be observed in the field, and if so, if there was an effect on fruit set, development, and ripening. Increased femaleness was observed in the field as manifested by earlier onset and increased carpel-bearing flowers, and a conversion of bisexual-to-female flowers, consistent with greenhouse observations.

Transgenic plants set earlier and higher numbers of fruit compared to wild type, however the fruit was smaller resulting in similar total kg/plot yields, most likely caused by resource competition in the plants leading to young fruit abscission. Two different ripening phenotypes were observed in *CRC::etr1-1* fruit; one line (M5) showed 100% ripe fruit at final harvest, likely due to earlier fruit set, and the other (M15) showed a comparable percentage to wild type, however less M15 fruit abscised from the vine compared to wild type and M5. Externally, these fruit had a green rind, similar to unripe melon, however internally the mesocarp showed signs of over-ripening (mesocarp degradation, seed separation from septum, and/or septum degradation). Internal ethylene levels of externally green M15 were comparable to those seen in orange (ripe)

fruit of M5 and wild type. Expression of the *etr1-1* transgene using the *CRC* promoter could explain this phenotype as expression was higher in the exocarp, targeting ethylene perception inhibition to the exocarp, not mesocarp, and thus leading to the observable external non-ripening. These results show potential for regulating melon fruit set and ripening by targeting ethylene perception to the gynoecium.

Gene models of melon sex determination have been proposed with two major sex loci and one or two modifier loci (Kubicki, 1969; Kenigsbuch and Cohen, 1980). One of the major sex genes in melon was identified as a member of the ACS ethylene biosynthetic enzyme family, *ACS7* (Boualem et al., 2008). Melon has multiple *ACS* and *ACO* members (Miki et al., 1995; Lasserre et al., 1996; Ishiki et al., 2000; Boualem et al., 2008) which have the potential to be modifiers of sex determination. In order to examine this possibility, monoecious and hermaphrodite sex genotypes from a gynoecious and andromonoecious cross were developed to examine gene expression in the apices of these four genotypes. Segregation ratios of the F_2 matched a four gene model, and three new *ACS* members were identified using available cucumber sequences and the melon genome. Gene expression analysis revealed that transcript levels of a majority of the *ACS* members and all of the *ACO* members were higher in recessive *g* genotypes (gynoecious and hermaphrodite). However, gynoecious showed higher expression compared to hermaphrodite. These results implicate that dominant *G* genotypes, namely a functional WIP1 transcription factor (Martin et al., 2009), can suppress gene expression of ethylene production genes.

Cumulatively, the results presented here provide insight into the molecular aspects of unisexual floral development in melon and its link to ethylene. The molecular mechanism of ethylene regulation of the *G* locus (*WIP1*) still needs to be investigated. Promoter analysis

identified putative sites for regulation by an ethylene-regulated protein, and examination of this activity will be essential to solidify this connection. Another question is how does the stamen's perception of ethylene pass this signal to the carpels to allow for continued carpel development? If it isn't ethylene itself, what could this signal be? How does it affect *WIP1* gene expression to inhibit carpel development, or inhibit *WIP1* to promote carpel development? What is the actual identity of the two modifying genes involved in sex differentiation? Are the ethylene perception genes also a player in sex expression? These questions should be addressed in the future to better understand the molecular intricacies of carpel development.

LITERATURE CITED

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Boualem A, Fergany M, Fernandez R, Troadec C, Martin A, Morin H, Sari M, Collin F, Flowers J, et al. (2008). A conserved mutation in an ethylene biosynthesis enzyme leads to andromonoecy in melons. Science 321: 836-838.

Grumet R and Taft JA (2011). Sex expression in cucurbits In: Genetics, Genomics and Breeding in Cucurbits. Kole C, Wang YH, Behera TK, Kole C (eds). Science Publishers Inc. Enfield NH and CRC Press (Taylor & Francis) group, Boca Raton, FL. p. 353-375.

Ishiki Y, Oda A, Yaegashi Y, Orihara Y, Arai T, Hirabayashi T, Nakagawa H, Sato T (2000). Cloning of an auxin-responsive 1-aminocyclopropane-1-carboxylate synthase gene (CMe-ACS2) from melon and the expression of ACS genes in etiolated melon seedlings and melon fruits. Plant Sci 159: 173-181.

Kenigsbuch D and Cohen Y (1990). The inheritance of gynoecy in muskmelon. Genome 33: 317-320.

Kubicki B (1969). Sex determination in muskmelon (*Cucumis melo* L.) Genet Pol 10: 145-163.

Lasserre E, Bouquin T, Hernandez JA, Bull J, Pech JC, Balague C (1996). Structure and expression of three genes encoding ACC oxidase homologs from melon (*Cucumis melo* L.). Mol Genet Genomics 251: 81-90.

Little HA, Papadopoulou E, Hammar SA, Grumet R (2007). The influence of ethylene perception on sex expression in transgenic melon as assessed by expression of the mutant ethylene receptor gene, *At-etr1-1*, under control of constitutive and floral-targeted promoters. Sex Plant Reprod 20: 123-136.

Martin A, Troadec C, Boualem A, Mazen R, Fernandez R, Morin H, Pitrat M, Dogimont C, Bendahamane A (2009). A transposon-induced epigenetic change leads to sex determination in melon. Nature 461: 1135-1139.

Miki T, Yamamoto M, Nakagawa H, Ogura N, Mori H, Imaseki H, Sato T (1995). Nucleotide-sequence of a cDNA for 1-aminocyclopropane-1-carboxylate synthase from melon fruits. Plant Physiol 107: 297-298.

Perl-Treves R (1999). Male to female conversion along the cucumber shoot: approaches to study sex genes and floral development in *Cucumis sativus*. In: Sex Determination in Plants (eds. CC Ainsworth). BIOS Scientific, Oxford: 189-215.

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