ROLE OF MEMBERS OF THE TOMATO ETHYLENE RECEPTOR FAMILY IN DETERMINING THE TIMING OF RIPENING

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2007

To my parents, who have supported every decision I have ever made and given everything to their children

ACKNOWLEDGMENTS

Thanks to my entire committee for their patience and constructive criticism throughout this process. I would like to especially thank my advisor, Dr. Harry Klee, for guiding me through my Ph.D. and not only teaching me how to be a scientist but how to present myself and my science. I thank the entire Klee lab for all their help throughout the years. Thanks to my bench-mate Michelle Zeigler whose attention to detail has helped me to become a better scientist. I thank Denise Tieman for sharing her knowledge in the lab and Mark Taylor for generating all the transgenic plants used in my experiments. Thanks to Peter Bliss for taking care of my plants in the greenhouse and doing just about everything around the Klee lab. I thank Valeriano Dal Cin for all of this help on the mapping project.

Thanks to everyone in the lab of Dr. Andrew Hanson for all their help and great friendship. I would especially like to thank Dr. Gilles Basset and Dr. Sebastian Klaus for teaching me everything they know about protein expression. Additionally I thank Dr. Gale Bozzo, Dr. Rocio Diaz de la Garza, Dr. Giuseppe Orsomando, Dr. Aymeric Goyer, and Tariq Ahktar for being there when I needed a break and to have some fun. Thanks to the lab of Dr. David Clark for allowing me to come over and do my RNA extractions in their hood and also to bother them when I needed a break. I thank Carol Dabney-Smith for teaching me all she knows about custom antibodies, without this help I would not have been able to finish all my work.

Most importantly, thank you to my family for always being there for me when I needed them. Also for understanding that moving from Ohio to Florida was what was best for my career even though it was so far. I also thank all of my friends back in Ohio and Michigan for staying in touch and giving me plenty of fun times outside of Gainesville. Lastly, thanks to Stephanie Violi from the bottom of my heart for being the person I have leaned on for the past three years. She

has made me laugh when I needed it and always put things in perspective. Even though we haven't been together she has remained the driving force in my life and is the love of my life.

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December 2007

Chair: Harry John Klee
Major: Plant Molecular and Cellular Biology

Tomatoes are an economically important crop and a significant dietary source of important phytochemicals, such as carotenoids and flavonoids. While it has been known for many years that the plant hormone ethylene is essential for ripening of climacteric fruits, its role in fruit growth and maturation is much less understood. In an attempt to better understand tomato fruit ripening we utilized both biotechnology and traditional breeding strategies. The multigene ethylene receptor family has been shown to negatively regulate ethylene signal transduction and suppress ethylene responses. Here, we demonstrate that a reduction in the levels of either of two family members, *LeETR4* or *LeETR6*, causes an early ripening phenotype. We provide evidence that the receptors are rapidly degraded in the presence of ethylene and that degradation likely occurs through the 26S proteasome-dependent pathway. Ethylene exposure of immature fruits causes a reduction in the amount of receptor protein and earlier ripening. Fruit-specific suppression of the ethylene receptor *LeETR4* causes early ripening while fruit size, yield and flavor-related chemical composition are largely unchanged. These results demonstrate that ethylene receptors likely act as biological clocks regulating the onset of tomato fruit ripening.

In order to better understand the mechanism controlling the timing of ripening we screened a *Lycopersicon hirsutum* introgression population for QTLs responsible for reduced time from

anthesis to breaker and/or increased ripening-associated ethylene biosynthesis. The *L. hirsutum* population was chosen because of unusual ripening characteristics and significantly higher levels of ethylene biosynthesis at maturity of *L. hirsutum*. A number of lines were identified that showed statistically significant differences from the control for both phenotypes. These lines are currently being refined for possible map-based cloning of loci controlling these phenotypes. These results demonstrate the power of using both molecular biology and traditional breeding for gene isolation/characterization and crop improvement.

CHAPTER 1
LITERATURE REVIEW

Ethylene in Plant Biology

The phytohormone ethylene is an important signaling molecule that is involved in many plant processes including but not limited to abscission, leaf and flower senescence, germination, sex determination and fruit ripening (Abeles et al., 1992). Ethylene also functions in both biotic and abiotic stress responses. Exposure to environmental stresses like flooding, wounding, herbivory, chilling or pathogen attack can enhance ethylene production (Boller, T. 1991; Abeles et al., 1992). This ethylene then slows growth until the stress is removed. Interest in ethylene's importance as a plant hormone has resulted in thousands of peer-reviewed publications in the last 100 years and has laid the foundation for a real understanding of ethylene's involvement in plant growth and development.

Ethylene is a small, gaseous, two-carbon molecule that has the ability to diffuse through hydrophilic and hydrophobic environments. This property allows it to pass into any compartment in the plant cell. The ability of ethylene to alter plant development has been known for centuries, with farmers from many cultures using smoke and wounding to induce flowering and ripening (Abeles et al., 1992). Damage to city and greenhouse plants in the late 19th and early 20th centuries was found to be caused by leaking illuminating gas that was used at the time for lighting. Work done by Dimitry Neljubov in 1901 proved that ethylene was in fact the active component in illuminating gas that resulted in the plant damage (Abeles et al., 1992). Subsequent work showed that ethylene was clearly important for fruit ripening but many scientists at the time believed that the other phenotypes of endogenously produced ethylene were a by-product of the ripening process (Abeles et al., 1992). Work done in the 1960s by the Burgs provided definitive proof that ethylene is important for plant development beyond its involvement in fruit

ripening (Burg and Burg, 1962; Burg 1962). Their work was instrumental in classifying ethylene as a plant hormone.

Early feeding experiments suggested that the amino acid methionine is a precursor of ethylene (Lieberman et al., 1966; Burg and Clagett, 1967). Later work provided evidence that oxygen is necessary for the production of ethylene. It was then hypothesized that if fruit tissue was held in an anaerobic environment the precursor should build up and provide enough compound to allow identification. This work led to the subsequent isolation of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of endogenous ethylene (Adams and Yang, 1979). ACC is synthesized from S-adenosyl methionine (SAM) by a pyridoxal phosphate-requiring enzyme termed ACC synthase (ACS). The conversion of ACC to ethylene is subsequently performed by the oxygen-requiring enzyme ACC oxidase (ACO). The conversion of ACC by ACO results in the production of CO_2 and HCN in addition to ethylene.

Most tissues synthesize low levels of ethylene. Synthesis can be stimulated by a number of means, including wounding, submergence, chilling and pathogen attack. Synthesis of ACC is considered to be the rate limiting step in ethylene production. Thus, increased ethylene production requires modulation of ACS expression and/or activity.

While ethylene is often characterized as the ripening hormone, not all fruit require ethylene to complete the ripening process. Species are often characterized by the presence or absence of a large increase in ethylene production concomitant with increased respiration at the onset of ripening. Species whose fruit exhibit these increases are termed “climacteric” while those that do not are referred to as “non-climacteric.” Climacteric species include apple, avocado, banana, peach and tomato while non-climacteric species include strawberry, grape, cherry and citrus. The increase in ethylene production associated with climacteric ripening is essential for ripening.

Blockage of either ethylene biosynthesis or perception results in an inability of the fruit to complete its ripening program.

Tomato is an excellent model for the study of ethylene's involvement in fleshy fruit development because of a relatively short life cycle, ease of genetic manipulation and a wealth of genetic resources. In addition, the tomato genome is being sequenced, which will be a tremendous resource to those working on this species.

Ethylene's involvement in ripening, fruit softening, volatile production and lycopene accumulation has been well documented. Ethylene biosynthesis during tomato fruit development generally goes through three distinct stages. There is a slight burst of synthesis after successful pollination that then falls to low levels until the onset of climacteric ethylene production at the onset of ripening. Ethylene production during immature fruit development has been termed system I and is characterized as low level production which cannot be stimulated by treatment with exogenous ethylene (Yang, 1987). Ethylene biosynthesis in mature fruit, referred to as system II, is autocatalytic, meaning it can induce its own synthesis (Yang, 1987). The induction of ethylene synthesis at the onset of ripening is believed to be due to developmental induction of an ethylene-inducible ACS (Barry et al., 2000; Nakatsuka et al., 1998).

Although immature tomato fruit do not produce significant levels of ethylene they do respond to ethylene, but in a different manner to that of ripening fruit. This response manifests as a change in gene expression but to a smaller set of genes to that of ripening fruits (Alba et al., 2005). This difference in response suggests that there is developmental control of gene expression in addition to that of ethylene. The developmental control of ethylene regulated genes has been best characterized by research done on the *E4* and *E8* genes found in tomato. Expression of *E4* is ethylene inducible throughout fruit development while *E8* is only ethylene

inducible in ripening fruit (Lincoln et al. 1987, Wilkinson et al., 1995). Treatment of immature fruit with ethylene induces a set of genes, proving a response to the hormone, but it does not induce immediate ripening. However, that ethylene exposure does hasten the onset of ripening as compared to untreated fruit of similar age, suggesting that the fruit can measure cumulative ethylene exposure (Burg and Burg, 1962; Lyons and Pratt, 1964; McGlasson et al., 1975; Yang, 1987). Treatment of immature green tomato fruit with ethylene, or its analog propylene, could reduce the time from anthesis to breaker by half that seen in non-treated controls (Lyons and Pratt, 1964; McGlasson et al., 1975). The way fruits measure this ethylene exposure is unknown. Along with temporal control of fruit ripening there is also a spatial aspect of control. Fruit do not ripen evenly across the entire fruit, they begin to ripen at the basal end of the fruit and proceed towards the calyx. Since ethylene is diffusible throughout the fruit, and accumulates to high levels within the fruit, there appears to be a developmental control within individual fruit that controls the spatial ripening of the fruit.

In addition to ethylene's role in fruit development it also plays an important part in seedling emergence (Clark et al., 1999). During germination seedlings must be able to force their way through any soil between them and a light source. When a seedling encounters a barrier in the soil it often becomes slightly wounded which can induce ethylene production. Dark grown seedlings, like those found underground, are often tall and spindly in the presence of air alone. Upon exposure to ethylene its growth habit changes and exhibits growth that is referred to as the "triple response." This response manifests as a shortening of both the hypocotyl and root, radial thickening of the hypocotyl and an exaggeration of the apical hook. These changes allow the seedling to push through any barriers without damaging the meristem. While this mechanism has evolutionary importance, the ability to exploit this response has revolutionized the ethylene

biology field by allowing researchers to screen for mutants in ethylene biosynthesis and signaling.

The Ethylene Receptor Family — Arabidopsis and Tomato

Much of the initial ethylene perception and signal transduction research was done in *Arabidopsis thaliana* and thus we have exploited the Arabidopsis system to identify the orthologous genes in tomato. The Arabidopsis ethylene receptor *ETR1* was the first phytohormone receptor cloned in plants and was isolated from a mutagenized population that was screened for plants deficient in the triple response (Bleecker et al. 1988; Guzman and Ecker 1990). Ethylene insensitive mutants grow tall and spindly even in the presence of ethylene while constitutive ethylene response mutants will show a triple response in the absence of ethylene. *etr1-1* was isolated as an ethylene insensitive mutant in one of these screens and was later cloned and shown to encode an ethylene receptor with homology to bacterial two-component sensors (Chang et al. 1993). In subsequent work a total of five receptors were cloned from Arabidopsis.

The ethylene signal transduction pathway in Arabidopsis is believed to be relatively linear but we are unsure if all of the elements have been identified. Epistatic analysis has allowed researchers to putatively order the components starting with the receptors. The next component is the Raf-like Ser/Thr protein kinase, CTR1, which has been shown to physically interact with the receptors (Clark et al., 1998; Gao et al., 2003; Huang et al., 2003). CTR1 has significant homology to MAPKKKs and although no MAPKK or MAPK have been found to be involved in ethylene signal transduction, their involvement in this pathway cannot be ruled out. EIN2, a protein showing homology to Nramp metal transporters, is the next member of the pathway. The role and activity of this protein in the pathway is unknown but it is absolutely necessary since knockouts show complete ethylene insensitivity in every assay tested. The end of the ethylene signal transduction pathway is composed of the transcription factors EIN3 and ERF1. EIN3 loss-

of-function (LOF) mutants show partial ethylene insensitivity, which is probably due to redundancy within a gene family containing at least three members (Chao et al., 1997). In the absence of ethylene the EIN3 protein is targeted for degradation in the 26S proteasome by a pair of F-box proteins, EIN3-binding factors 1/2 (EBF1/2). Upon ethylene binding this repression is released and EIN3 binds to the promoter of ERF1 activating its transcription and ERF1 is involved in regulating the transcription of ethylene responsive genes. ERF1 over-expressors show a slight constitutive ethylene response suggesting that there are other important players in the transcriptional control of ethylene responsive genes (Berrocal-Lobo et al. 2002).

ETR1 was not only the first phytohormone receptor to be cloned in plants but was also the first eukaryotic protein with homology to a histidine kinase (Chang et al. 1993). These receptors are endoplasmic reticulum-localized proteins that have copper-mediated ethylene binding and are present *in vivo* as dimers (Chen et al., 2002; Ma et al., 2006; Schaller and Bleecker 1995; Schaller et al. 1995). The Arabidopsis receptor proteins (ETR1, ETR2, ERS1, ERS2 and EIN4) can be separated into three structurally different regions.

The sensor domain is composed of three putative transmembrane (TM) sequences in ETR1 and ERS1 and four domains in ETR2, ERS2 and EIN4, with the first TM sequence representing a cleavable ER-targeting peptide. These transmembrane sequences are where the copper-mediated ethylene binding takes place. This region also contains all of the known mutations that cause ethylene insensitivity, likely due to an inability to bind ethylene or transmit the signal through a conformational change. The amino acids necessary for dimerization are present in this region and homodimerization has been proven *in vivo* but heterodimerization has not been demonstrated (Schaller et al. 1995).

The next domain present in this family is a region that shows homology to histidine kinases (Dutta et al., 1999). Histidine kinase domains contain five highly conserved sub-domains, N, G_1, F, G_2 and the histidine (H) that is autophosphorylated. The ETR1 and ERS1 proteins contain all five of these sub-domains while the other three members lack at least one sub-domain. The ETR1 protein is the only member of the family that exhibits HK activity in vitro, but the conserved histidine is not necessary for protein function based on the ability for a mutant lacking this residue to rescue a receptor mutant (Gamble et al. 1998; Gamble et al. 2002, Wang et al. 2003). The other family members all exhibit Ser/Thr kinase activity based on *in vitro* kinase assays (Moussatche and Klee, 2005). This lack of histidine kinase activity in these family members fits well with the finding that most of the other family members do not contain all of the conserved regions in the histidine domain. All kinase assays completed so far have been done *in vitro* and there has been no kinase activity directly linked to ethylene signal transduction *in vivo*.

The third and final domain found in these proteins is the receiver, located at the C-terminus of the protein. This region shows homology to the output domains from bacterial two-component sensors and contains an aspartate that is active in phosphorelay in these bacterial pathways. The ERS1 and ERS2 proteins lack this domain while the other family members contain it, suggesting that it may play a role in some family member-specific functions.

Using sequence and exon/intron organization comparisons, ETR1 and ERS1 have been classified as Subfamily 1 receptors while ETR2, ERS2 and EIN4 have been classified Subfamily 2 receptors. Considering the degree of divergence within the family, there may be specific functions for each of the family members. The evidence suggests that the receptors may not be completely redundant, although most genetic evidence suggests functional overlap.

Mutant analysis of the Arabidopsis ethylene receptor family has allowed a better understanding of the receptor's role in transducing the ethylene signal. All of the initial receptor mutants cloned were semidominant, insensitive mutants. Single gene LOF mutants have no obvious phenotypes which is most likely due to functional redundancy within the family. Based on all of the genetic data available the receptors appear to function as negative regulators of the ethylene response (Hua and Meyerowitz 1998). The default state of the receptor is one in which the receptor actively suppresses ethylene responses in the absence of the hormone and ethylene binding removes this suppression. The double mutant *etr1/ers1* and triple or quadruple mutants show constitutive ethylene responses even in the absence of increased ethylene biosynthesis (Wang et al., 2003), presumably because basal ethylene levels are able to inactivate the remaining receptors. This model suggests that a decrease in receptor content will increase ethylene responsiveness while an increase in receptor levels will decrease tissue responsiveness. This simplified model does not appear to tell the entire story because it presumes that all of the receptors contribute equally to the signal and recent work has suggested this may not be true. Overexpression of a Subfamily 2 member was unable to rescue the constitutive ethylene response phenotype of the double Subfamily 1 mutant, suggesting some family member-specific functions (Wang et al., 2003). Work done in our lab has found that the system in tomato may be quite different from that of Arabidopsis.

The tomato ethylene receptor family is composed of six members, *LeETR1-6* with *LeETR3* corresponding to the *NR* gene (Fig. 1, Zhou et al. 1996a; Zhou et al. 1996b; Lashbrook et al. 1998;Tieman and Klee 1999). All receptor family members have been shown to bind ethylene with the exception of LeETR6 because it was not available at the time of analysis (O'Malley *et al*., 2005). The first of the tomato ethylene receptor genes to be cloned was *NR*. This gene was

isolated from a mutant that shows semidominant ethylene insensitivity which prevents floral wilting and abscission, alters leaf senescence and prevents fruit ripening (Wilkinson et al. 1995). The basic structures of the receptors are similar to those of the Arabidopsis family but within the tomato family the sequences are quite divergent with less than 50% identity at the extremes (Figure 1). The transmembrane domains show the highest levels of sequence similarity owing to the importance of this domain in the transmission of the signal. LeETR1, 2 and NR have three putative transmembrane domains while LeETR4, 5 and 6 have four putative transmembrane domains. The NR protein is the only member of this family that lacks the C-terminal receiver domain (Figure 1). LeETR4, 5 and 6 resemble the Subfamily 2 receptors found in Arabidopsis in that they are missing at least one of the conserved sub-domains in the HK domain and contain the fourth transmembrane sequence (Figure 1-1). Each of the receptors has a distinct expression pattern throughout fruit development, with *NR*, *ETR4* and *ETR6* being ethylene inducible (current work). *NR* and *ETR4* are both pathogen inducible, with the increase in expression being a function of the increase in ethylene production found during a disease response (Ciardi et al., 2000). An increase in receptor expression is likely an important factor in reducing the amount of damage that occurs as a result of this increase in ethylene production.

The basic model for ethylene response states that the receptors act as negative regulators of ethylene response and that higher receptor expression reduces sensitivity and lower expression increases sensitivity. This model explains why multiple gene knockouts in Arabidopsis show a constitutive response. While much of the available data fit this model it does not address the importance of ethylene dissociation from the receptor or protein turnover. The K_d of ethylene dissociation was measured in yeast-expressed AtETR1 and was found to be approximately 12 hours. This is likely to be an underestimate since it did not factor in protein turnover (Schaller

and Bleeker, 1995). There is no evidence to suggest that ethylene is able to dissociate from the receptor, suggesting this association may be permanent. Isoform-specific antibodies have been generated for a number of the Arabidopsis receptors and the tomato NR protein but no work has been done to study *in vivo* turnover rates or ethylene's effect on receptor turnover. This type of evidence will be necessary to draw any conclusions about the receptor's importance in a plant's response to ethylene.

The current model suggests that the only way that a plant can reduce its response to ethylene is by synthesis of new receptors. Less receptor leads to more sensitive tissue and more receptor leads to less sensitive tissue. Previous work has shown that the current data do fit the receptor model. Plants overexpressing *NR* have been found to be less sensitive to ethylene in triple response assays and pathogen studies (Ciardi et al. 2000). *LeETR4* antisense lines with significantly reduced expression show phenotypes consistent with a constitutive ethylene response. Phenotypes of these lines include epinastic growth, premature flower senescence and abscission and for fruit, a reduction in the time from anthesis to breaker and from breaker to red ripe (Tieman et al 2000). The effect on time from anthesis to breaker is quite significant with a decrease of as much as 11 days compared to WT controls. *LeETR4* antisense lines also have an altered response to pathogen infection because an increase in *ETR4* expression is one way in which the plant reduces the amount of tissue damage. These lines display an accelerated hypersensitive response in response to infection with an incompatible pathogen with greater ethylene production and hastened expression of pathogenesis-related genes (Ciardi et al. 2000). Antisense suppression of *LeETR1*, *2* and *NR* have no observable phenotype but this result is likely due to redundancy within the system. The *NR* antisense lines show an unusual phenotype in that with the reduction of *NR* expression levels there is a concomitant increase in *ETR4*

expression and this may explain why the *NR* antisense lines do not show any constitutive ethylene response phenotypes. This phenomenon has been termed functional compensation and appears to be a built-in system that allows the increase in expression of one family member when another has been reduced (Tieman et al 2000).

The expression level of each of the tomato receptors has been monitored in response to multiple ethylene-related phenomenons and at least one receptor is up-regulated in each of the responses. On the other hand, a reduction in receptor expression has never been seen even though it would increase the tissue's responsiveness to ethylene. So it seems that as soon as a plant starts producing ethylene more receptor is produced thus attenuating the response. While this may seem counterproductive it is not uncommon for a phytohormone in plants to be attenuated as soon as it's induced (Rashotte et al., 2003). Increased response to ethylene can be very detrimental to plant tissues and since ethylene slows the growth of a plant, it could have long term effects. The expression levels of all the receptors remain low throughout immature fruit development and show a sharp increase at the onset of ripening, the time at which ethylene production is at its highest. So it seems that at the point when ethylene is having its greatest effect on plant development, receptor levels are at their very highest. It has been known for some time that ethylene is intimately involved in the timing of fruit ripening and our research seeks a better understanding of its role. Based on our previous research and that of others we believe that if ethylene production rates during fruit development exceed the level of receptor synthesis then there would be a de-repression of the system that would lead to an increase in sensitivity to the hormone. At some point in development, when the fruit are ripening competent, sensitivity to ethylene would rise past a threshold level where ripening could be initiated. Based on this model the reduction of receptor levels in transgenic plants should reduce time to ripening and based on

our previous results this is true, *ETR4* antisense plants ripen faster than controls (Tieman et al 2000).

Protein Degradation Through the 26S Proteasome

Protein degradation is an important regulatory mechanism that has been adopted by many organisms. It has emerged as a mechanism of control as important as gene expression in controlling cellular processes. Protein degradation has been implicated in the control of signaling cascades, defense against viral infection, breakdown of cellular regulators and arguably its most important role is the removal of abnormal proteins (Jabben et al. 1989, Glotzer et al. 1991, Scheffner et al. 1993). The degradation of proteins generally falls into two classes: (1) relocation of proteins to degradative organelles such as the lysosome or vacuole and (2) targeting the proteins for degradation by the 26S proteasome. These two pathways are the principal modes of degradation for both soluble and membrane bound proteins, albeit less is known about how membrane-bound proteins are degraded. While relocation to degradative organelles is an important type of protein degradation the focus of this review will be on the role of the 26S proteasome in protein degradation.

The 26S proteasome is one of the most important proteolytic systems in plants and our understanding of this system has grown considerably in the past decade. This system utilizes the 76-amino acid protein ubiquitin (Ub) as a reusable tag to target specific proteins to the multi-subunit 26S proteasome for proteolysis. The attachment of Ub occurs at lysine residues on the target protein and often occurs as a polyubiquitin chain of Ub monomers. Upon proteolysis in the proteasome the Ub monomers are released to be used in another round of targeting. Ubiquitination of target proteins occurs in a three-step conjugation cascade and can occur on proteins in the cytoplasm, nucleus, integral membrane proteins and ER resident proteins that are retro-translocated across the ER membrane.

The ubiquitin attachment cascade occurs in a three-step process designated E1, E2 and E3. The E1 component of the cascade is an ubiquitin-activating enzyme that binds ubiquitin at a conserved cysteine. This enzyme is constitutively expressed and has little impact on target specificity. The Arabidopsis genome encodes two E1 isoforms (Hatfield et al., 1997). The E2, or ubiquitin-conjugating enzyme, is encoded by at least 37 family members in Arabidopsis (Vierstra, 1996). This enzyme shuttles the ubiquitin moiety between the E1 and E3 proteins (Pickart, 2001). The size of this family suggests that different E2s may be involved in regulating specific pathways, although no specific functions have been assigned to any plant E2s. The specificity of individual E2s likely occurs through their interaction with specific E3s. In addition, the E2s are not all specific to ubiquitin but are also used for conjugating ubiquitin-like proteins including NEDD, RUB and SUMO (Li et al., 2006). The E3, or ubiquitin-protein ligases, is the component of the cascade that specifically recognizes proteins for ubiquitination. Because of the specificity of this protein/complex it is encoded by several large families of genes, with more than 1300 members in Arabidopsis (Vierstra, 2003). Four different types of E3 ligases have been identified in plants: HECT, RING/U-box, SCF and APC (Smalle and Vierstra, 2004). HECT E3 ligases are composed of a large single polypeptide (often >100kDa), with seven family members present in the Arabidopsis genome (Downes et al., 2003). Little is known of the functions of plant HECTs, although one is known to be important for trichome development. Like the HECT family, each RING/U-box family member is a single polypeptide that acts to bring together the E2-Ub and target substrate. This group of proteins is each encoded by a large family of proteins with 480 RING finger-containing and 64 U-box containing proteins, respectively, in Arabidopsis (Azevedo et al., 2001; Kosarev et al., 2002). This type of E3 ligase has been implicated in a diverse number of cellular processes in plants, including, auxin signaling, photomorphogenesis,

self incompatibility and removal of abnormal proteins (Smalle and Vierstra, 2004). The SCF type of E3 ligases are composed of a complex of four different polypeptides. This type of E3 ligase acts in a similar manner to that of RING/U-box proteins in that they bring together the E2-Ub and the target substrate. Plants have the ability to synthesize a vast number of SCF type E3 ligases. The Arabidopsis genome contains two RBX1 subunits, five cullin subunits, 21 SKP-like proteins and almost 700 F-box proteins (Farras et al., 2001; Gagne et al., 2002; Shen et al., 2002) . The F-box proteins provide the target specificity for this complex and constitute one the largest gene superfamilies in the Arabidopsis genome. The APC type of E3 ligases is the most complex type of E3, being composed of eleven subunits. Most of these subunits are encoded by single genes in Arabidopsis and thus it is likely that they only form a small number of APC type E3s (Capron et al., 2003). The APC was first identified as being important for the regulation of mitosis through degradation of mitotic cyclins in yeast; it has been subsequently shown to have a similar function in plant cells (Blilou et al., 2002).

The 26S proteasome is an ATP-dependent proteolytic complex that is composed of 31 subunits organized in two major subcomplexes. The 20S core protease (CP) is the portion of the complex that houses the proteolytic activity, alone it is an ATP- and Ub-independent protease. The CP has hydrolyzing, trypsin-like and chymotrypsin-like activity allowing it to degrade a broad range of peptide bonds (Voges et al., 1999). The 19S regulatory particle (RP) can bind to both ends of the CP and is the portion of the complex that recognizes the Ubs attached to targeted proteins (Voges et al., 1999). The RP performs a number of additional functions including unfolding the target protein, Ub removal, opening the gate to the CP core and directing the target protein into the CP lumen (Smalle and Vierstra, 2004). Regulation of the activity and specificity of the proteasome is thought to be affected by a number of factors including

association with additional proteins and substitutions or modifications to complex subunits. The Arabidopsis genome encodes two isoforms of nearly all proteasome subunits. Transcriptional control of the complex subunits in yeast is facilitated by a single transcription factor, Rpn4, that is negatively regulated at the protein level by the 26S proteasome itself.

The role of the 26S proteasome in regulating many signal transduction pathways has been confirmed in plants. The proteasome has been implicated in the action of all plant hormones. In addition, it is important for a plant's response to both abiotic and biotic stimuli. Its role in auxin and ethylene signaling are arguably the best characterized roles in hormone signaling. The auxin signal transduction pathway is negatively regulated by a family of proteins (AUX/IAAs) that bind and inhibit the functions of a family of transcription factors, the auxin response factors (ARFs). Upon auxin binding the AUX/IAAs are targeted for degradation, thus releasing the transcription factors to initiate expression of auxin responsive genes. The use of mutants and proteasome inhibitors has confirmed this pathway and has facilitated the identification of the auxin receptor as the F-Box protein, TIR1. TIR1, and TIR1-like proteins, specifically target the AUX/IAAs for polyubiquitination and is an interesting example of the importance of the 26S proteasome in regulating hormone pathways (Dharmasiri et al., 2005).

The ethylene signal transduction pathway is also regulated by the proteasome, which modulates transcription factor activity/abundance. The F-Box proteins EBF1/2 target the EIN3, and EIN3-like, transcription factors for degradation in the absence of ethylene. Upon ethylene binding, this repression is removed and the transcription factor is able to activate transcription of primary ethylene responsive genes. In the case of EBF1/2, each has a different role in response to ethylene, with EBF1 being more important during early ethylene response and EBF2 more

important later during the response and in the resumption of growth after ethylene removal (Binder et al., 2007).

The importance of the proteasome in response to abiotic stimuli is best characterized by its role in regulating light signaling. PhyA, a red/far red absorbing photoreceptor, is rapidly ubiquitinated and turned over following photoconversion to the Pfr form. In addition to the regulation of photoreceptor protein levels, regulation of transcription factors is also performed by the proteasome. In the absence of a light source the RING-E3 COP1 is present in the nucleus where it targets a number of transcription factors for degradation. Upon illumination COP1 is removed from the nucleus and the transcription of light responsive genes occurs. These examples represent an extremely small percentage of the pathways in which the proteasome has been implicated and there are many more that have not been characterized.

While much is known about the degradation of soluble proteins by the proteasome, relatively little is known about integral membrane protein degradation, especially in plants. What is known about this pathway has been elucidated in yeast and to a lesser extent in humans. A major regulatory and house keeping pathway that involves degradation of proteins in the endoplasmic reticulum (ER) or integrated into the ER membrane and has been termed ER-associated degradation (ERAD) has been uncovered. ERAD is responsible for targeting misfolded ER proteins that are retro-translocated back across the ER membrane and also targeting misfolded integral membrane proteins that are subsequently extracted from the membrane and degraded by the proteasome (Meusser et al., 2005). It has been hypothesized that different targeting complexes may be present in cells that target membrane proteins with misfolded cytosolic domains, internal membrane domains or ER luminal domains (Carvalho et

al., 2006). These complexes contain a number of different subunits but each contains a membrane-bound E3 ligase that attaches the ubiquitin monomers to the substrate.

A number of membrane-bound ERAD substrates have been identified but an interesting example is that of the inositol 1,4,5-triphosphate (IP_3) receptor. Activation of a G protein-coupled receptor (GPCR) increases phospholipase C activity that generates diacylglycerol and the second messenger IP_3. IP_3 moves through the cytoplasm to IP_3 receptors located in the ER membrane, which activate channels that mobilize internal reserves of Ca^{2+}. A persistent activation of GPCRs leads to a down-regulation of IP_3 receptors in order to prevent any deleterious effects of continually elevated cytosolic Ca^{2+}. This down regulation requires the 26S proteasome and it has been shown that IP_3 binding induces ubiquitination of the IP_3 receptor, leading to degradation (Zhu and Wojcikiewicz, 2000). In addition, a binding-defective mutant receptor was shown to be resistant to ubiquitination and this resistance is not caused by the removal of potential ubiquitination sites. It was hypothesized that ligand binding causes a conformational change that exposes a signal leading to ubiquitination (Zhu and Wojcikiewicz, 2000).

The 26S proteasome has emerged as an essential part of a cell's repertoire for maintaining cellular integrity and regulating a myriad of different pathways. Its involvement in both plant development and responses to environmental stimuli implies an important evolutionary advantage allowing these sessile organisms to flourish in many different environments.

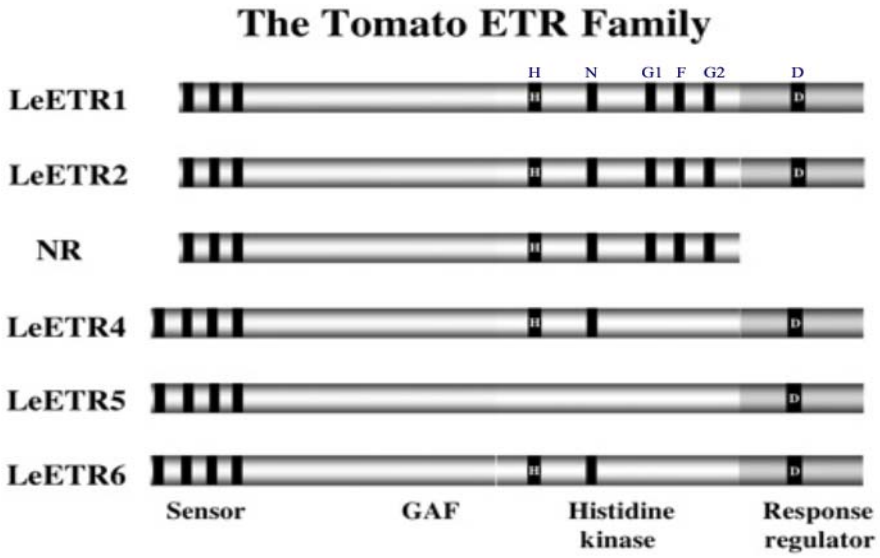


Figure 1-1. Schematic representation of tomato ethylene receptor family. Black bars in sensor domain represent putative transmembrane domains. Black boxes in histidine kinase domain represent conserved sub-domains while black box in response regulator represents conserved aspartate involved in phosphorelay. (Klee, 2004)

CHAPTER 2
ETHYLENE RECEPTOR DEGRADATION CONTROLS THE TIMING OF RIPENING IN TOMATO

Introduction

The plant hormone ethylene is a gaseous molecule that regulates multiple processes including germination, organ senescence, stress responses and fruit ripening (Abeles et al., 1992). The role of ethylene in fruit ripening has been intensively studied in a number of species, but most notably tomato, which has emerged as an important model for the study of fleshy fruit development. Ethylene plays a critical role in determining the timing of ripening and thus provides an attractive point to control fruit ripening through genetic modification.

Climacteric fruits such as tomato are characterized by an increase in respiration and a concomitant increase in ethylene biosynthesis just prior to the initiation of ripening. Ethylene is essential for normal fruit ripening in these species and blockage of either ethylene production or perception leads to improper ripening. In tomato fruits, ethylene has profoundly different effects depending on the stage of development. There is a distinct developmental switch that occurs upon fruit maturation (Giovannoni, 2001). Although applied ethylene does not initiate ripening in immature fruits, it does significantly hasten the onset of subsequent ripening (Yang, 1987); the more ethylene to which an immature fruit is exposed, the earlier it ripens. Similar effects have been observed in banana where Burg and Burg (1962) demonstrated that treatment of immature green banana fruits shortened the time to ripening relative to untreated controls. The mechanism by which fruits measure cumulative ethylene exposure is unknown.

Genetic analysis in tomato and Arabidopsis has shown that ethylene receptors act as negative regulators of the ethylene response pathway (Hua and Meyerowitz, 1998; Tieman et al., 2000). In the absence of the hormone, receptors actively suppress ethylene responses. Upon ethylene binding, that suppression is removed and the response occurs. In tomato there are six

known ethylene receptors (*LeETR1,2,4-6* and *NR*) (Wilkinson et al, 1995; Zhou et al., 1996; Lashbrook et al., 1998; Tieman and Klee, 1999). Functional analyses have indicated that some Arabidopsis family members have a more important role in ethylene signaling than others. Further, no single loss-of-function mutation has a major effect on ethylene responses, indicating a degree of functional redundancy. However a completely different picture emerges in tomato where loss of a single subfamily II receptor, LeETR4, results in increased ethylene sensitivity. Antisense *LeETR4* plants show phenotypes consistent with a constitutive ethylene response including significantly earlier fruit ripening (Tieman et al., 2000). This mutant phenotype can be restored to wild type by over-expression of the Subfamily I receptor, *NR*. No ethylene-associated developmental effects have been observed in lines with reduced expression of *NR* (Tieman et al., 2000), *LeETR1*, *LeETR2* or *LeETR5* (Tieman and Klee, unpublished results).

The receptor signaling model states that the receptors are acting as negative regulators of ethylene response. Experimentally it has been shown that reduction of receptor content increases ethylene sensitivity (Hua and Meyerowitz, 1998; Tieman et al., 2000; Cancel and Larsen, 2002; Hall and Bleecker, 2003) while increased receptor content has the opposite effect (Ciardi et al., 2000). We have previously shown that *NR* and *LeETR4* transcripts are up-regulated in ripening fruits (Wilkinson et al., 1995; Tieman et al., 2000). Since fruit ripening is dependent upon ethylene action, it seems illogical to increase receptor content and thus decrease ethylene responses. To better understand the role of the tomato ethylene receptor family during fruit development we have characterized the behavior of both the receptor RNAs and proteins during fruit development. Contrary to the RNA data, protein blot analysis showed that receptor protein levels are at their highest during immature fruit development and significantly decline at the onset of ripening. This paradox is explained by observations that ethylene treatment induces a

rapid degradation of receptor proteins. Here, we present data indicating an important role for *LeETR4* and *LeETR6* in modulating the timing of ripening. Reduced levels of these receptors mediated by either antisense RNA or protein degradation results in earlier fruit ripening.

Results

A Subset of the Receptor Family Shows Ripening-associated Expression and Is Ethylene-inducible in Fruit

Expression of all six ethylene receptor genes was assayed throughout fruit development to assess stage-specific expression. Quantitative RT-PCR (qRT-PCR) analysis of each receptor transcript showed low expression of all receptors throughout immature fruit development but upon maturation there was a significant increase in *NR*, *ETR4* and *ETR*6 transcripts (Fig. 2-1). This ripening-associated increase in expression constituted a 10-fold increase in total receptor mRNA content by the breaker stage. Since the receptors are negative regulators of ethylene responses, the observed increases in mRNA levels during an ethylene-dependent process seems counter-intuitive as an increase in receptors would make the fruit less sensitive to ethylene.

Ripening-associated gene expression can be the consequence of increased ethylene production. Previous analysis has shown that *ETR4* and *NR* are in fact ethylene-inducible in leaf tissue (Ciardi *et al*., 2000). To determine if the receptor gene family is regulated by ethylene in fruit tissue, individual fruits were treated with 50 ppm ethylene for 15 h. Expression analysis of each receptor showed a 9-, 10- and 7-fold increase in *NR*, *ETR4* and *ETR6*, respectively (Fig. 2-2). Expression of ETR1, ETR2 and ETR5 changed little in response to the ethylene treatment. Based on this analysis it appears that expression of *NR*, *ETR4* and *ETR6* is the consequence of the climacteric increase in ethylene production at the onset of ripening.

LeETR6 Antisense Lines Show Phenotypes Consistent with a Constitutive Ethylene Response

Single gene knockouts of ethylene receptors in Arabidopsis show no obvious phenotypes and only the subfamily I double mutant (Hall and Bleecker, 2003; Qu et al., 2007) or triple and quadruple mutants (Hua and Meyerowitz, 1998) show any ethylene-related phenotypes. As previously shown by Tieman *et al*. (2000) this is not the situation in tomato as lines having significantly reduced *LeETR4* expression show ethylene hypersensitive phenotypes. When *LeETR6* antisense lines were generated, we found similar phenotypes to those seen in *LeETR4* antisense lines, including a reduction of time to ripening by as much as seven days (Table 2-1). Additional ethylene-related phenotypes include epinastic leaf growth and premature flower senescence (Fig. 2-3). These results indicate gene-specific reductions in expression of either *LeETR4* or *LeETR6* but not the other four receptors (data not shown) results in a hypersensitivity to ethylene, including premature fruit maturation and ripening.

Receptor Protein Levels Are Distinctly Different From Transcript Levels During Fruit Development

A wealth of recent work has demonstrated that post-translational control is an important component of hormone pathway regulation. In order to uncover any potential post-translational regulation of ethylene receptors, antibodies against NR, ETR4 and ETR6 were produced. Tissues were collected for a comprehensive study of mRNA and protein expression during fruit development. Measurement of receptor mRNA expression showed an increase in transcript levels at the onset of ripening and these levels often remained high until fruits were completely red (Fig. 2-4A). Microsomal membranes were isolated to enrich for the receptor proteins and were used for protein quantification. Analysis of protein levels throughout fruit development revealed an unexpected result; levels were highest during immature fruit development and significantly declined at the onset of ripening (Fig. 2-4B). Data from cv. Flora-Dade are

presented, although identical results were obtained in the Pearson and Micro-Tom cultivars. This reduction in protein occurred despite increased RNA content (Fig. 2-4C). The results indicate that RNA levels are not predictive of receptor protein content nor the signaling state of the tissue. Rather, there must be an additional level of control of ethylene perception. Because the drop in receptor content coincided with the onset of autocatalytic ethylene synthesis, we subsequently examined whether ethylene binding induces receptor turnover.

Treatment of Leaf and Fruit Tissue with Ethylene Causes a Rapid Degradation of Receptor Proteins That Likely Occurs Through a Proteasome-dependent Pathway

To determine whether ethylene binding induces receptor degradation, immature fruits and vegetative tissues were exposed to exogenous ethylene. Ethylene treatment of fruits resulted in 4, 5 and 8-fold increases in *NR*, *ETR4* and *ETR6* mRNA, respectively (Fig. 2-5A). Concomitant with this increase in transcripts there were reductions of 60%, 60% and 40% in NR, ETR4 and ETR6 proteins, respectively, within 2 h and this reduction was sustained throughout the treatment (Fig. 2-5B). Removal of ethylene after 8 h of treatment lowered transcripts to pre-treatment levels but receptor proteins remained lower even 24 h after treatment ceased (Fig. 2-5A). Ethylene-mediated receptor degradation was also observed in vegetative tissues. Treatment of seedlings with 50 ppm ethylene for 2 h resulted in 10-, 5- and 13-fold increases in *NR*, *ETR4* and *ETR6* mRNA, respectively. Similar to the data collected from immature fruit there was 60%, 40% and 50% reduction in NR, ETR4 and ETR6 protein levels, respectively (Fig. 2-6B). Taken together, the results indicate that ethylene exposure in both vegetative and reproductive tissues results in an immediate drop in receptor protein levels that is independent of transcript levels.

The 26S proteasome-dependent degradation pathway has emerged as a key point of regulation in many phytohormone signaling pathways (Guo and Ecker, 2003; Dill *et al*., 2004; Gagne *et al*., 2004; Dharmasiri *et. al*., 2005; Kepinski and Leyser, 2005). To determine if this pathway

is responsible for the turnover of ethylene receptors, seedlings were treated with the proteasome inhibitor MG132 prior to ethylene treatment. Following ethylene treatment, levels of each protein actually increased, likely because of ethylene-induced increases in transcription/translation (Fig. 2-6B). Very little is known about mechanisms of ER-associated protein degradation in any system (Meusser et al., 2006). Presumably ubiquitinated proteins are rapidly extracted from the membrane and degraded by the cytoplasmic 26S proteasome complex. We did not observe larger ubiquitinated forms of immuno-reactive receptors in the microsomal membrane fractions. Even after several-fold concentration, no receptors could be detected in the soluble fraction (data not shown). Nonetheless, the MG132 results are consistent with a ubiquitin-mediated receptor degradation.

In order to demonstrate that ethylene binding is necessary for degradation, seedlings were pre-treated with the ethylene action inhibitor 1-methylcyclopropene (1-MCP) prior to ethylene treatment. 1-MCP is a competitive inhibitor of ethylene and its attachment to the receptor is essentially irreversible (Sisler, 2006). If ethylene binding is essential for the degradation of the receptor, 1-MCP should stabilize the protein. Pretreatment of tomato seedlings with 1-MCP prevented the ethylene-induced receptor degradation (Fig. 2-6B) as well as the ethylene-induced increase in mRNA (Fig. 2-6A), indicating that ethylene binding is essential for receptor degradation. To further confirm that ethylene binding is necessary for protein degradation we utilized the semi-dominant *Nr* mutant that has a greatly reduced ethylene response. The mutant Nr protein is unable to bind ethylene when heterologously expressed in yeast (Klee and Bleecker, unpublished data). Treatment of *Nr* seedlings with 50 ppm ethylene for 2 h caused a 50% and 62% decrease in ETR4 and ETR6 proteins (Fig. 2-6B), respectively, but caused significantly less change in the level of NR protein. Taken together, the results are consistent

with enhanced receptor degradation following ethylene binding. However, we cannot completely exclude the existence of an ethylene-induced receptor degradation machinery.

Receptor Levels in Developing Fruit Determine the Timing of Ripening

To determine whether ethylene-induced receptor depletion is the cause of the early ripening phenotype seen in ethylene treated fruit, immature fruits were exposed to ethylene while still attached to the plant and then allowed to ripen. Protein and mRNA samples were collected throughout the duration of the experiment to correlate lower protein levels with reduced time to ripening. Treated fruits ripened on average three days prior to untreated fruits (Table 2-2). Receptor protein levels were lowered upon treatment with ethylene at 15 days post anthesis (DPA) and remained lower than untreated controls throughout fruit development, indicating that lower receptor levels correlate with earlier ripening (Fig. 2-7). Transcript data show that the fruits responded to the ethylene treatment and upon removal of the ethylene, transcripts returned to pre-treatment levels (Fig. 2-8).

Discussion

Upon maturation, tomato fruits undergo a developmental transition that is defined by their response to ethylene (Lincoln *et al*., 1987). A number of system 1 and/or system 2-associated genes have been identified in fruits. The E4 and E8 genes are excellent examples with E4 being ethylene inducible throughout fruit development (both in response to system 1 and system 2 ethylene) and E8 only being ethylene-inducible in mature fruit (system 2 specific). While much is known concerning the role of ethylene during ripening its function during the immature phase of fruit development is less well understood. When mature fruits are exposed to ethylene, a ripening program is initiated. While treatment of immature fruits does not initiate ripening it does hasten the onset of ripening; the more the fruit is exposed to ethylene, the earlier it ripens (Burg and Burg, 1962; Yang, 1987). How the fruit measures cumulative ethylene exposure is not

known. We have provided evidence indicating a specialized role for two receptors, ETR4 and ETR6, in modulating ethylene responses, including fruit maturation. Reduced level of these receptors mediated by either antisense RNA or ethylene-mediated protein degradation results in earlier fruit ripening. Ethylene exposure also resulted in a parallel depletion of the other ethylene-inducible receptor protein, NR. Our results are consistent with a model in which ethylene receptor content is a major determinant of when fruits initiate the ripening program. Since the receptors are negative regulators of ethylene signaling, depletion would lead to a progressive increase in hormone sensitivity. When a particular threshold sensitivity is reached, ripening would commence. Alternatively, receptors may act as a brake on ripening initiation. It must be noted that there are other elements independent of ethylene that also must be in place for ripening to initiate; most notably the RIN transcription factor (Vrebalov et al., 2002).

Receptor gene expression is low and constitutive throughout immature fruit development with little difference between any of the family members (Fig. 1). At the onset of ripening there is an increase in expression of *NR*, *ETR4* and *ETR6* that results in a 10-fold increase in total receptor mRNA content. In contrast to mRNA expression, protein levels are at their highest in immature fruits and show a significant decrease at the onset of ripening and remain low (Fig. 2-4B) as a consequence of ethylene exposure. Ethylene binding likely causes a conformational change in the receptors that makes them susceptible to degradation. In this context it is interesting to note the model of Arabidopsis receptor signaling presented by Wang et al. (2006). These authors provide genetic evidence supportive of a transitional state in which a receptor continues to actively suppress downstream ethylene responses after ethylene is bound. This intermediate state subsequently transitions to a receptor-inactive state. Our results suggest that the "transmitter-off" state may actually be receptor degradation. It would be most interesting to

determine whether the mutations that define this transition state stabilize the protein. This receptor degradation is dependent upon the action of the 26S proteasome. At least in some cases, ubiquitination is associated with phosphorylation state (Hochstrasser, 1996). Although the ethylene receptors are considered to be ancestral histidine kinases, many do not possess histidine kinase activity (Moussatche and Klee, 2004). However, all of the receptors are functional kinases; those that do not have histidine kinase activity are serine kinases. In light of the degradation of receptors following ethylene binding, it is possible that the phosphorylation state of the receptor may mediate ubiquitin binding. Although ligand-induced receptor degradation has not been reported for plant hormones, it has been observed in animals where growth hormone (GH) signaling is mediated by receptor levels (Flores-Morales et al. 2006). The GH receptor, like ethylene receptors, is a membrane-associated protein in which hormone binding also increases ubiquitin-mediated turnover (Govers et al. 1999).

The ethylene receptor family in tomato, like Arabidopsis, is split into two groups with *LeETR1*, *LeETR2* and *NR* belonging to subfamily I and *LeETR4-6* belonging to subfamily II. The Arabidopsis results indicate that there is a distinct difference between subfamily I and II members. With the exception of a subfamily I double mutant (*etr1ers1*), single and double gene knockouts in Arabidopsis show no obvious phenotypes. This is likely due to functional redundancy within the gene family. Over-expression of a subfamily II member in an *etr1ers1* double mutant cannot rescue the ethylene-hypersensitive phenotype (Wang et al. 2003). In a reciprocal experiment over-expression of a subfamily I member in a subfamily II triple mutant was sufficient to rescue the ethylene response phenotype. Together these data indicate that the subfamily I receptors are more important than the subfamily II receptors in determining competency to respond to ethylene. The Arabidopsis paradigm does not hold for tomato (Fig. 2-

3, Tieman *et al*. 2000). Plants with reduced expression of either *LeETR4* or *LeETR6*, both subfamily II members, show phenotypes that are consistent with an exaggerated ethylene response, including epinastic growth, premature flower senescence and early fruit ripening. Over-expression of *NR* in a *LeETR4* antisense line is able to rescue the ethylene response phenotype, indicating functional redundancy between subfamily I and II members (Tieman et al. 2000). Apparently there is a large degree of plasticity within the ethylene signaling pathway and different plants have adapted the signaling components as appropriate for their situation.

Plant hormones are involved in most developmental processes and are critical for abiotic and biotic stress responses. Plants can regulate hormone action through synthesis, catabolism or perception. We have shown that a significant part of the regulation of ethylene responses involves ligand-mediated receptor degradation. Frequently ethylene responses, particularly those related to stresses, are transitory. In order to shut down an ethylene response, synthesis of new receptors is essential. Our results with ethylene exposure to immature fruits indicate that receptor degradation is apparently an important level of developmental control. Our results also indicate that conclusions concerning receptor functions based on RNA levels must be interpreted cautiously. Whether ethylene-mediated receptor turnover and replenishment are important for other ethylene-mediated processes remains to be determined.

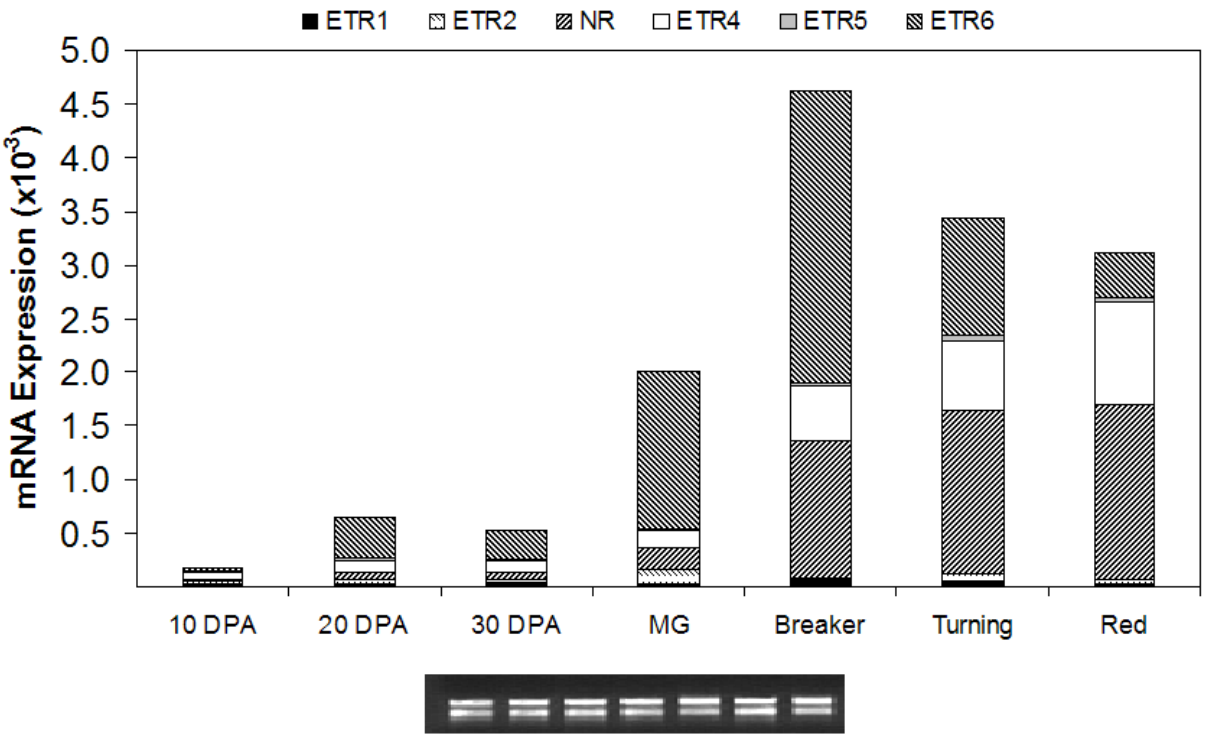


Figure 2-1 Ethylene receptor family mRNA levels during fruit development. qRT-PCR analysis of each receptor transcript in fruit tissue from different stages of fruit development. DPA, days post anthesis; MG, mature green; Breaker, first external color change; Turning, ~30% red color. Expression levels are presented as percentage of total RNA.

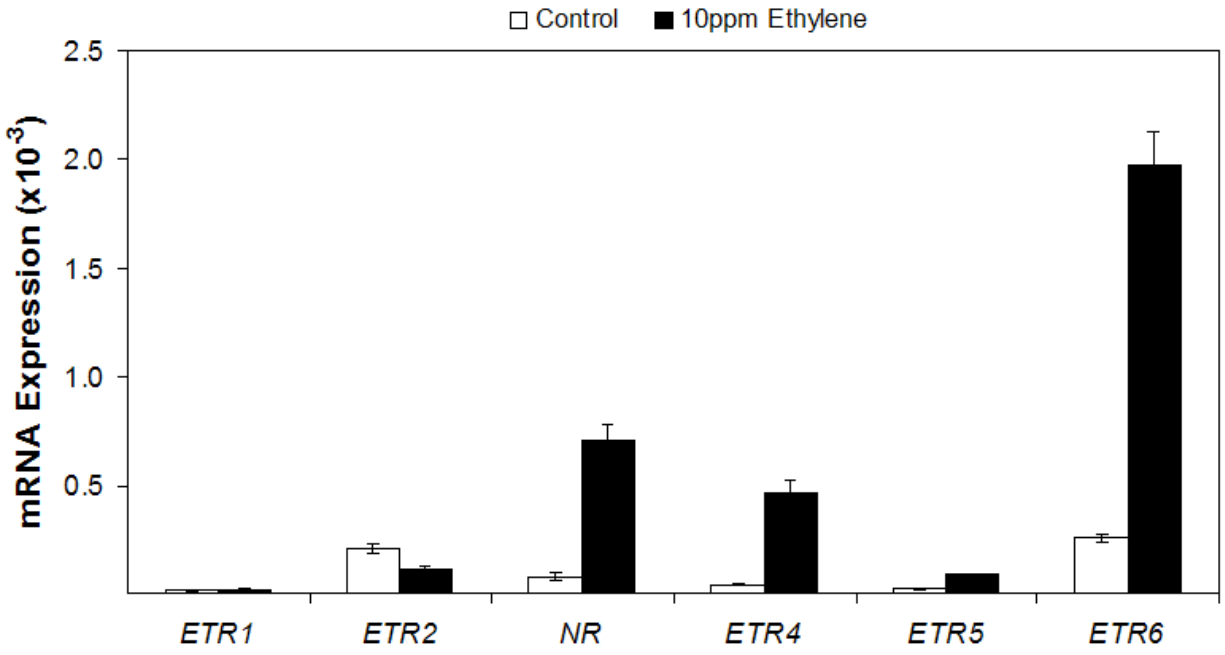


Figure 2-2 Ethylene-inducibility of each receptor mRNA in 20 days post anthesis, immature fruit. qRT-PCR analysis of expression of each receptor in response to 10ppm ethylene as a percentage of total RNA (±SE).

Figure 2-3 Constitutive ethylene response phenotypes of *LeETR6* antisense lines. Epinastic leaf growth (A) and early flower senescence (B) of *LeETR6* antisense lines. Equivalent aged wild type flowers are shown for comparison (C).

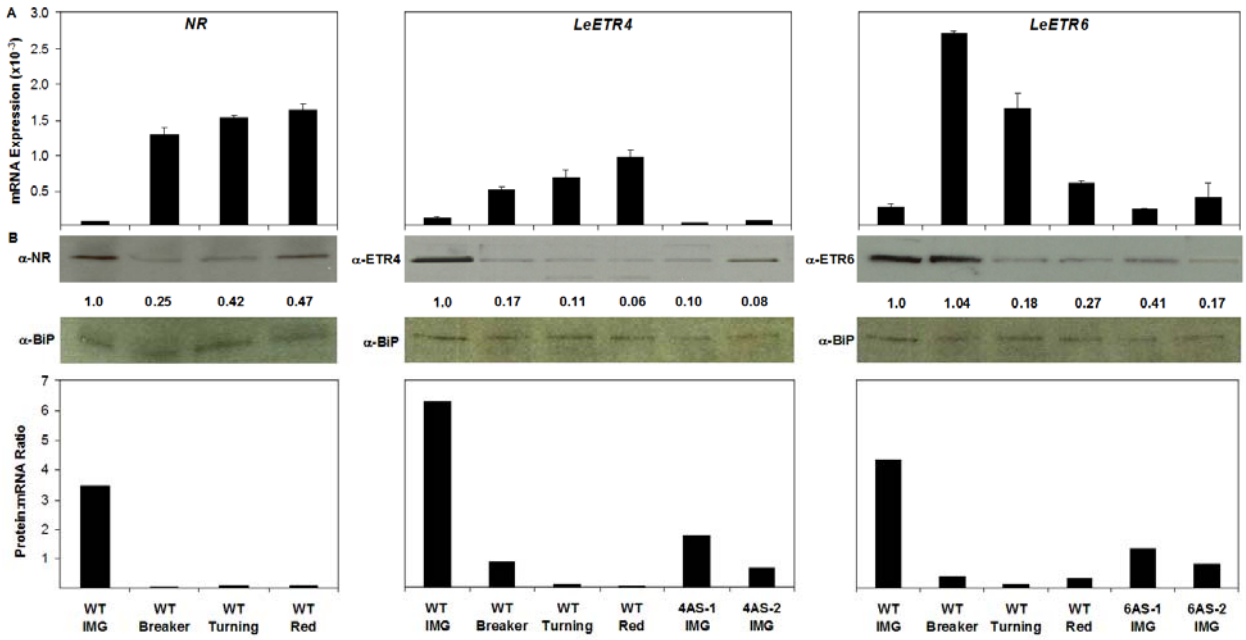


Figure 2-4 Receptor gene expression and protein levels show distinct differences during fruit development. qRT-PCR analysis of gene expression expressed as percentage of total RNA (±SE) (A) and protein blot analysis (B) throughout fruit development in *L. esculentum* cv. Flora-Dade (WT). Levels of RNA and protein are also shown for independent *LeETR4* (4AS-1, 4AS-2) and *LeETR6* (6AS-1, 6AS-2) antisense lines. Values below each receptor protein blot represent the amount of protein in each lane relative to the IMG stage. BiP antibody was used as a loading control and used to normalize protein values. C. Ratio of protein to mRNA. IMG: Immature green stage. Protein quantification was determined by densitometric analysis of Western blots using the NCBI software ImageJ.

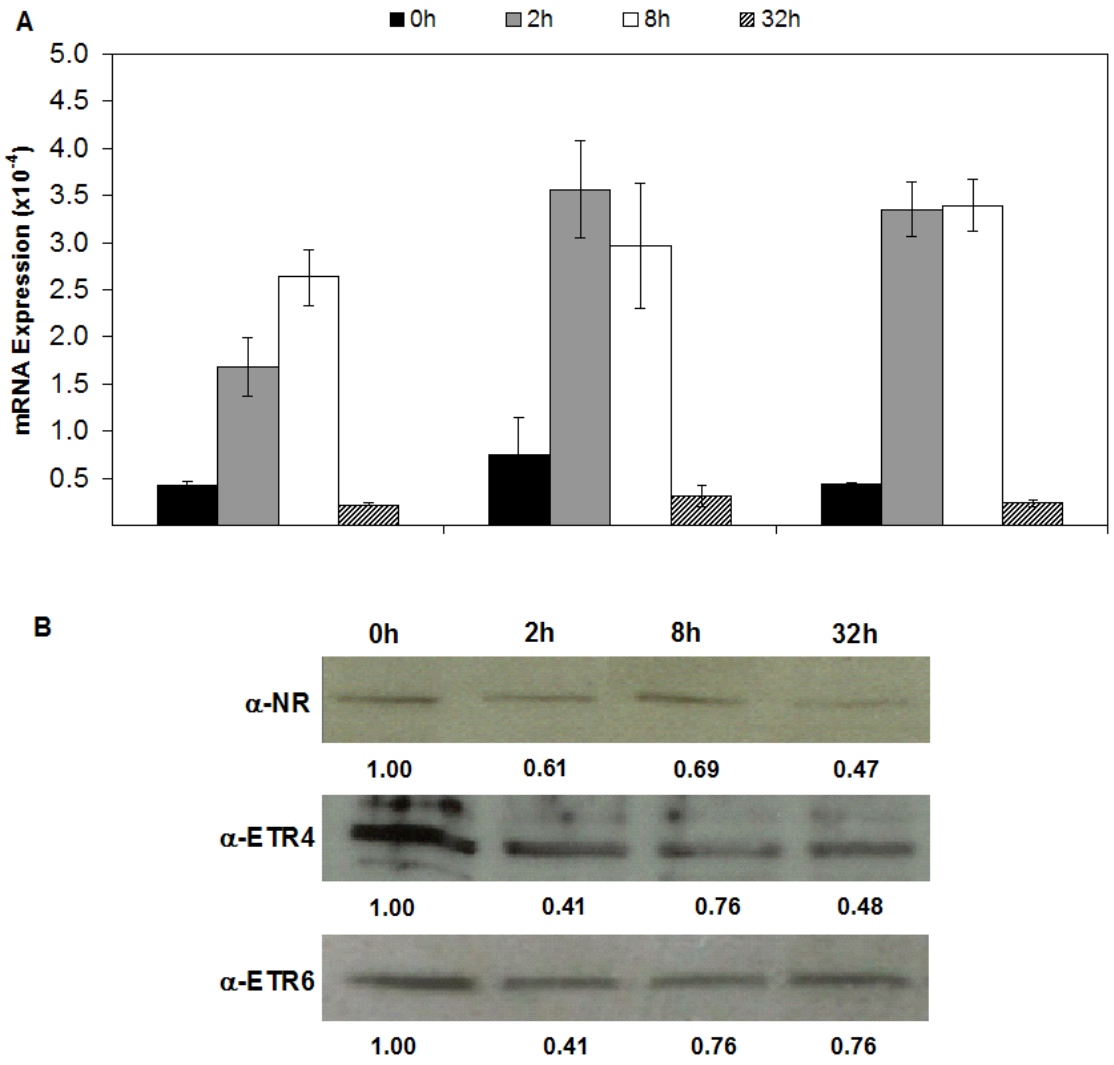


Figure 2-5 Ethylene binding induces degradation of receptors in detached immature fruits. Fruits were exposed to 10 ppm ethylene for 8 h. 32h time point represents fruit that were treated for 8 h and left in air for a further 24 h. qRT-PCR analysis of gene expression (A) and protein blot analysis (B) of ethylene-treated immature fruits. Values below protein blots represent the amount of protein in each lane relative to the 0 h time point. BiP antibody was used as a loading control and used to normalize protein values. Data represent the results of two independent experiments (±SE).

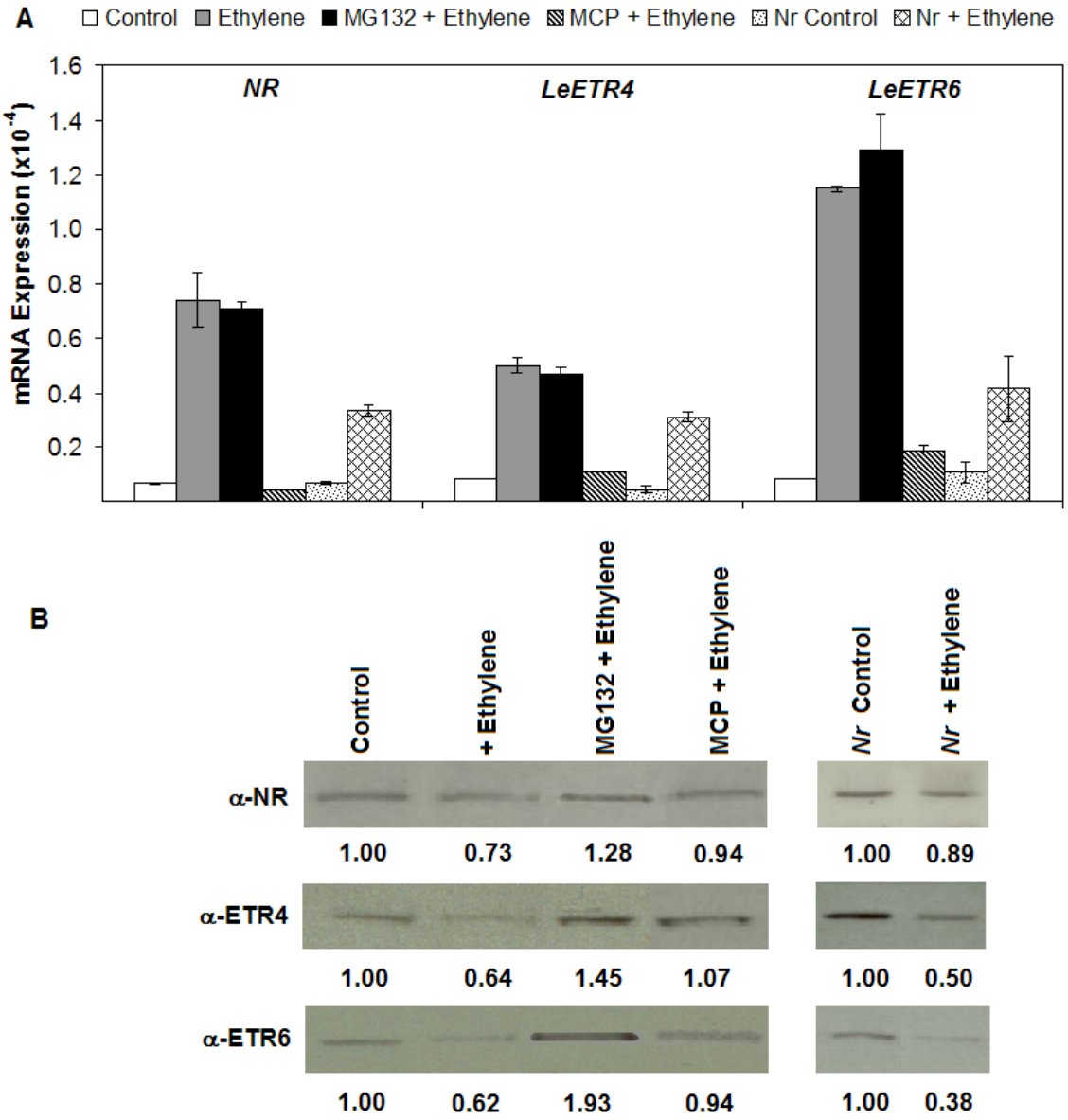


Figure 2-6 Ethylene binding induces degradation of receptor proteins in vegetative tissue. qRT-PCR analysis of gene expression (A) and protein blot analysis (B) of *L. esculentum* cv. Micro-Tom and Never-ripe (Nr) seedlings after treatment with 50 ppm ethylene for 2 h. Data represent the results of two independent experiments (±SE). Values below protein blots represent the amount of protein in each lane relative to the 0 h time point. BiP antibody was used as a loading control and used to normalize protein values.

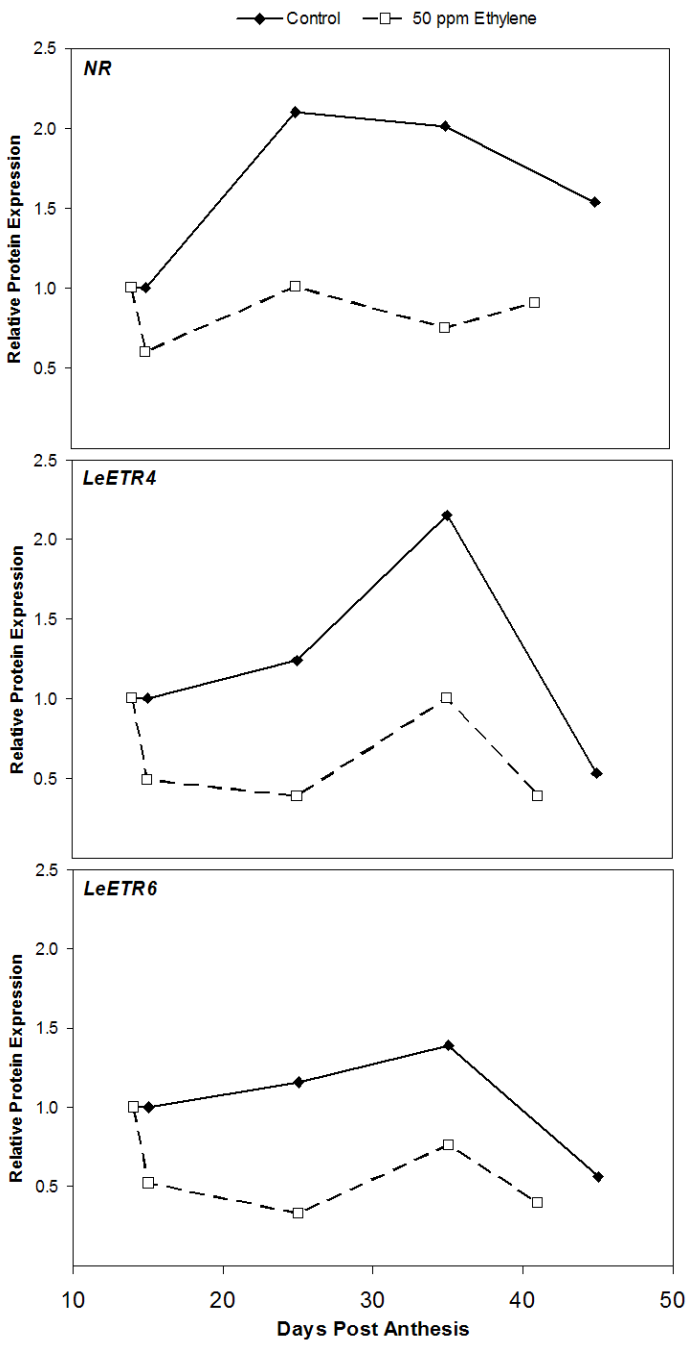


Figure 2-7 Ethylene treatment induces turnover of receptor leading to early ripening fruit. 15 days post anthesis (DPA) fruit were treated with 50 ppm ethylene while attached to the plant. Relative protein expression of NR, ETR4 and ETR6 normalized to an internal control, BiP. Values are plotted relative to the pretreatment protein level.

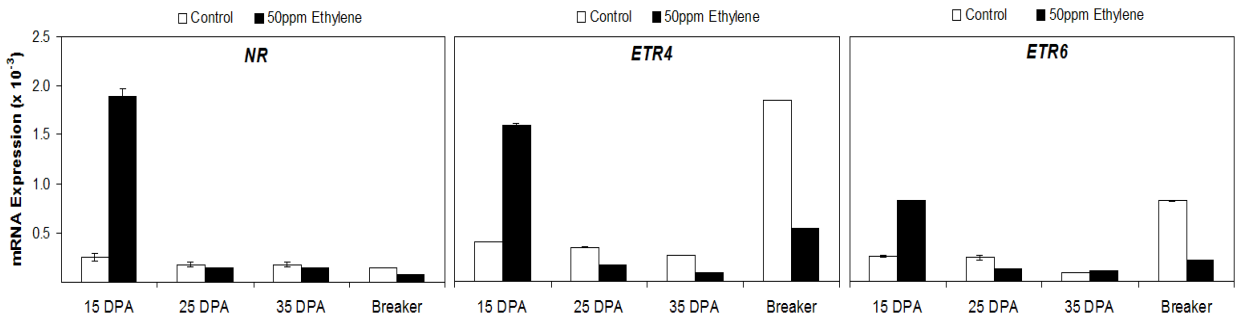


Figure 2-8 Ethylene treatment induces expression of receptor mRNAs in attached fruit. qRT-PCR analysis of expression of NR, ETR4 and ETR6 in response to 50ppm ethylene as a percentage of total RNA (±SE).

Table 2-1 Days from anthesis to breaker of *LeETR6* antisense lines

Line	Days	% Reduction *LeETR6* mRNA
WT	43.33 ± 0.71	
LeETR6AS-1	38.42 ± 0.90*	85.1 ± 2.4
LeETR6AS-2	37.00 ± 1.46*	75.6 ± 6.4
LeETR6AS-3	35.83 ± 0.78*	72.8 ± 5.8

Values represent mean of at least fifteen fruit for each line.
*p-value<0.001 based on Student's t-test.

Table 2-2 Days from anthesis to breaker of ethylene treated Microtom fruit

Treatment	Days
- Ethylene	45.33 ± 1.41
+ Ethylene	41.20 ± 0.80*

Values represent mean of at least ten fruit for each treatment. Experiment repeated with similar results.
*p-value<0.05 based on Student's t-test.

CHAPTER 3
FRUIT-SPECIFIC SUPPRESSION OF THE ETHYLENE RECEPTOR *LEETR4* RESULTS IN EARLY RIPENING FRUIT

Introduction

Tomato is the most economically important vegetable crop grown in the USA. Worldwide, ~70 million metric tons are produced each year. Short growing seasons in higher latitudes often reduce the number of cultivars a grower can use in outdoor cultivation. One mechanism to circumvent climate-related limitations is to grow early-maturing varieties. This offers a distinct advantage to growers, because the first fruit to market in a season can garner a higher price. As our knowledge of the molecular control of fruit ripening expands, biotechnology can provide useful tools for generating early ripening cultivars. While much effort has focused on delayed ripening, particularly as it relates to the ripening hormone ethylene, opportunities to hasten fruit development have been relatively neglected. We have developed a tissue-specific approach to enhance ethylene responses in tomato fruits by depletion of an ethylene receptor. Transgenic fruits mature 5-7 days earlier than controls with no deleterious effects on yield, fruit size or quality. This technology should be applicable to any fruit whose ripening is dependent on ethylene.

Ethylene is a phytohormone that controls or influences many aspects of plant growth and development (Abeles, 1992). Many of the developmental processes controlled by ethylene such as senescence, organ abscission and fruit ripening are critically important to agriculture. For example, climacteric fruits, such as tomato, banana and apple, require an increase in ethylene biosynthesis at maturity in order to ripen. Transgenic plants that are reduced in either synthesis or perception of ethylene exhibit delayed ripening (Oeller et al., 1991; Klee et al., 1991; Wilkinson et al., 1995; Hamilton et al., 1990). Conversely, it should be possible to speed up fruit maturation by increasing synthesis or perception of ethylene. Indeed, it has been known for many

years that ethylene application to immature tomato fruits does cause earlier onset of ripening (Yang, 1987). Because of the pleiotropic negative effects of excessive ethylene exposure on plant growth, simply increasing ethylene synthesis is not practical. Here, we describe an approach involving tissue specific depletion of an ethylene receptor resulting in early ripening fruit.

Receptors function as negative regulators of the ethylene response pathway (Hua and Meyerowitz, 1998; Tieman et al., 2000). In the absence of the hormone the receptor actively suppresses ethylene responses and ethylene binding removes this suppression. In practical terms, this means that ethylene sensitivity is inversely correlated with receptor levels; depletion of receptors effectively increases ethylene sensitivity because there are fewer receptors to inactivate. Recent work on the tomato ethylene receptor family has demonstrated that receptor levels during fruit development determine the timing of ripening (Kevany et al., 2007). Protein levels are at their highest during immature fruit development and significantly drop at the onset of ripening, facilitating ethylene-mediated ripening processes. Ethylene treatment of immature fruits causes receptor degradation and earlier fruit ripening (Kevany et al., 2007).

Results

LeETR4 RNAi Transgenic Plants Produce Early Ripening Fruit

Antisense-mediated reduction in either of two tomato ethylene receptors, *LeETR4* or *LeETR6*, results in premature ripening (Tieman et al., 2000; Kevany et al., 2007). However, these plants are severely affected in many aspects of growth and it is not clear that the early ripening is a direct effect of transgene expression. We postulated that fruit-specific suppression of the *LeETR4* receptor would result in early ripening without undesirable ethylene-related effects. In order to test the hypothesis a strategy was developed to specifically reduce *LeETR4* expression throughout fruit development. To achieve this goal we generated a construct

consisting of an *LeETR4* RNAi inverted repeat sequence fused to the promoter of *Tfm7*, a gene that is expressed specifically in immature fruits (Santino et al., 1997). Transgenic plants were generated by Agrobacterium-mediated transformation into the tomato cultivar Flora-Dade, a large fruited variety developed for Florida fresh tomato production. Transgenic lines that showed no vegetative expression of the silencing construct were identified and assayed in a greenhouse for time from anthesis to breaker stage (the first visible signs of ripening) in two successive seasons. Three lines that exhibited both a reduction in time from anthesis to breaker and a reduction of *LeETR4* transcript throughout fruit development were chosen for further characterization. Transgenic lines began ripening between 5 and 7 days earlier than controls (Figure 3-1). No significant effects were observed on time from breaker to fully ripe nor were there differences in color of ripe fruits (data not shown). As expected, *LeETR4* transcript levels were reduced by as much as 73% in immature fruit and 95% in ripening fruit (Figure 3-2A). While *Tfm7* expression has been reported to be immature fruit-specific the RNAi effect persisted into ripening fruit (Figure 3-2A). This gene-specific reduction in expression was not seen in non-target tissues such as leaves (Figure 3-2A). Expression analysis of the other family members showed no decrease in transcript levels in transgenic plants (Figure 3-3). Protein blot analysis confirmed that ETR4 protein levels were correspondingly reduced at all stages of fruit development relative to non-transgenic control fruit (Figure 3-2B).

Early Ripening Lines Show Altered Ripening Coordination

Performance of the transgenic plants was also assessed in the field using standard commercial practices. Harvests were conducted on a weekly basis in which all fruit that had begun to show external color development were picked and staged for their degree of ripeness. Transgenic plants had more ripening fruit in the first harvest than the control plants and

transgenic lines were stripped of between 77% and 86% of their fruit within the first two harvests (Figure 3-4).

Transgenic Fruits are Indistinguishable from Wild Type Fruits in Horticultural Traits

Early maturing varieties of fruits frequently lack the quality of slower ripening varieties. To achieve maximum value it would be advantageous if early ripening fruits maintain the size, yield and flavor qualities of later ripening cultivars. Altering the time to maturation could potentially impact synthesis of sugars, acids and volatile compounds associated with flavor. In addition, fruit size and yield could potentially be negatively affected by earlier maturation and harvest. To address these questions, tests were performed to assess quality and yield attributes.

Analyses of yield and fruit size were conducted in both greenhouse and field- grown plants. To assess yield, fruits were harvested at the onset of ripening and individually weighed. Average fruit size for two of the transgenic lines was slightly lower than control fruit but this difference was not statistically significant (Table 3-1 and Table 3-2). Total yield and the number of fruit per plant were not affected by the presence of the transgene (Table 3-1 and data not shown).

Tomato flavor is the sum of a complex interaction between taste and olfaction. Sugars and organic acids stimulate taste receptors while a set of volatile organic compounds (VOCs) stimulate olfactory receptors (Buttery et al., 1993; Buttery and Ling, 1993). In order to assess potential effects on flavor, total soluble solids, citric acid, malic acid and the 16 most important VOCs were measured (Table 3-1, Table 3-3 and Table 3-4). Similar results were obtained on both field-grown and greenhouse-grown materials. Although a very few statistically significant differences in citric acid and some VOCs were observed, they were not repeatable from season to season. All of these differences are well within the range of observed season-to-season

variations. Therefore, we concluded that the transgenic and control fruits are essentially equivalent.

Discussion

While the essential role of ethylene in mediating climacteric fruit ripening has been known for many years, its role during immature fruit development is only now being elucidated. Previous work has shown that ethylene treatment of immature tomatoes or bananas quantitatively reduces the time to the onset of ripening (Burg and Burg, 1962; Lyons and Pratt, 1964; McGlasson et al., 1975; Yang, 1987) but the mechanism by which fruits measure cumulative ethylene exposure has remained unknown until now. We have identified a potential mechanism by which plants use ethylene receptor levels to measure cumulative ethylene exposure (Kevany et al., 2007). Ethylene binding triggers a ubiquitin-dependent receptor protein degradation. If receptors are not replaced after ethylene-mediated degradation, as occurs in immature fruit (Kevany et al., 2007), the fruit will become more sensitive to subsequent ethylene exposure and ripen earlier. The precise, fruit specific targeting of *LeETR4*, described here, validates the model. These results define a critical role for LeETR4 in mediating ethylene responses. The special importance of this and another subfamily 2 receptor, LeETR6, to ethylene responses (Kevany et al., 2007) contrasts markedly with what is known about ethylene perception in Arabidopsis. In Arabidopsis, no single loss-of-function receptor mutant has an obvious effect on ethylene responses and the subfamily 1 receptors seem to have a more important role in ethylene signal transduction (Wang et al., 2003). These results taken together with results described in Kevany et al. (2007) more broadly demonstrate that plants have the capacity to regulate hormone responses by modulating receptor levels.

Tissue-specific modulation of ethylene sensitivity in transgenic plants has resulted in fruits with altered ripening without an agronomic penalty. A similar approach to precisely separate an

advantageous trait from pleitropic negative effects was employed by Davuluri et al., (2005) who used fruit-specific suppression of *DET1*, a photomorphogenesis regulatory gene, to increase both carotenoid and flavonoid content in transgenic tomatoes. Previous work on *DET1* had reported increases in these phytochemicals in loss-of-function mutants but global suppression of *DET1* led to a number of serious developmental defects that would prevent these plants from being used commercially.

We present here a crop improvement that should provide significant value to producers. Early season harvests of tomatoes and many other horticultural crops usually constitute a substantial percentage of a season's profits. The first fruit picked can be sold at a premium because supply is generally low and demand is high. We have generated transgenic lines in an elite background that ripen up to a week earlier than their control (Figure 3-1). These lines have none of the developmental defects associated with global receptor suppression (Tieman et al., 2000; Kevany et al., 2007) because of fruit-specific suppression of the gene (Figure 3-2A). This approach for engineering early ripening should be applicable to any climacteric fruit species.

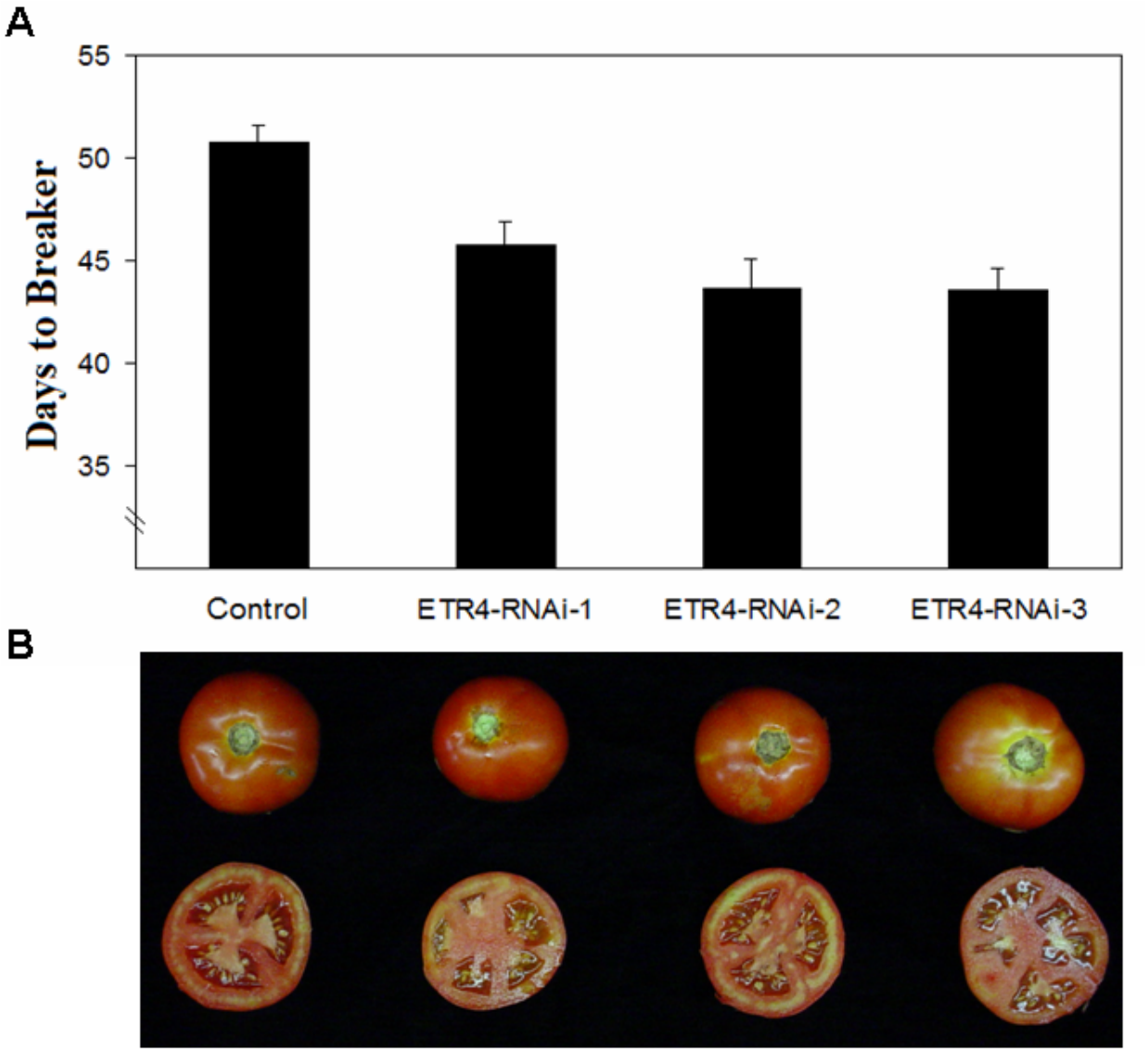


Figure 3-1 Fruit-specific *ETR4* RNAi Transgenic Lines Produce Early Ripening Fruit. (A) Days from anthesis to breaker were measured by tagging open flowers and recording the number of days until the first signs of color development. (B) Fruit from transgenic lines are similar in shape and color to control fruit.

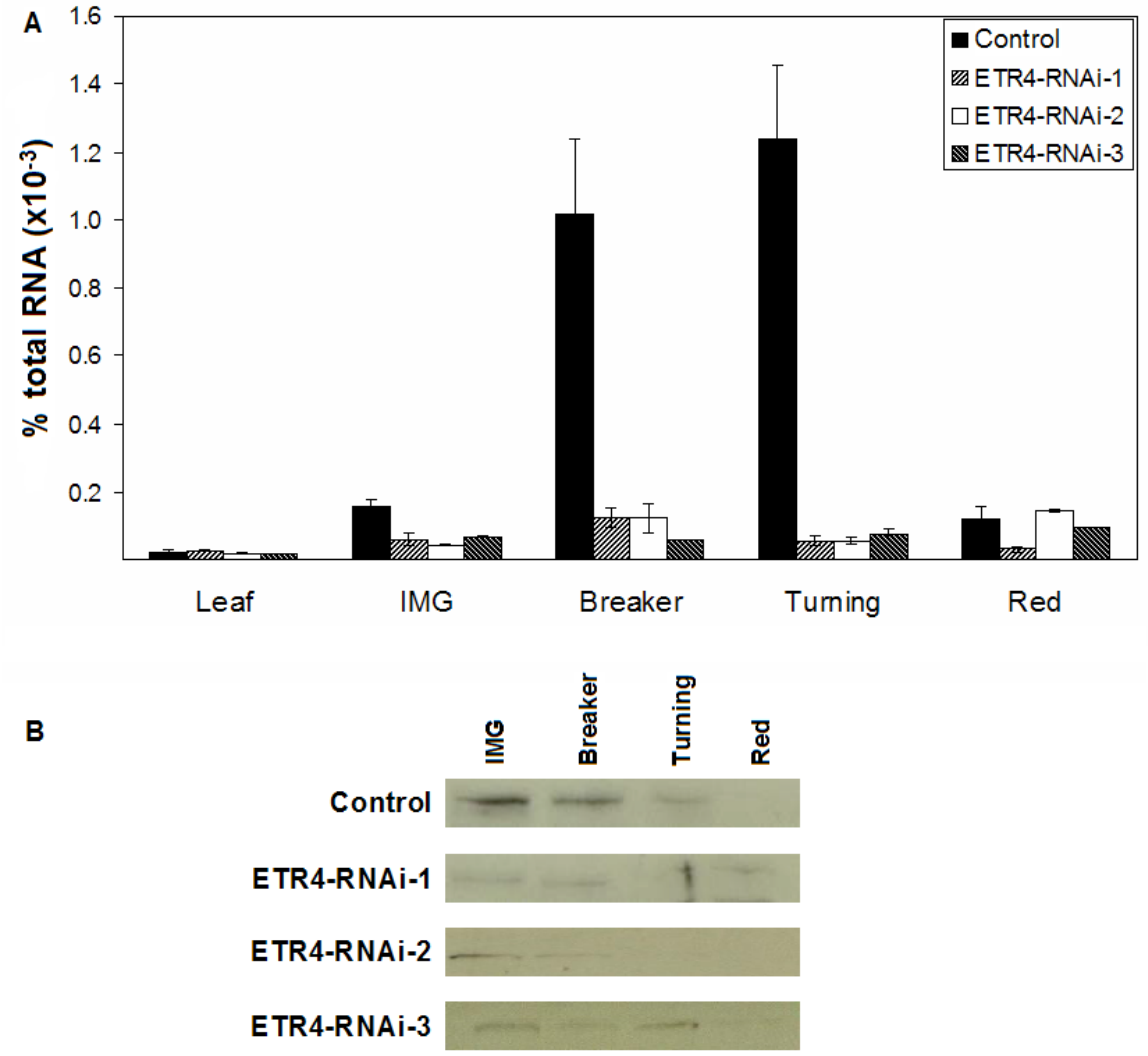
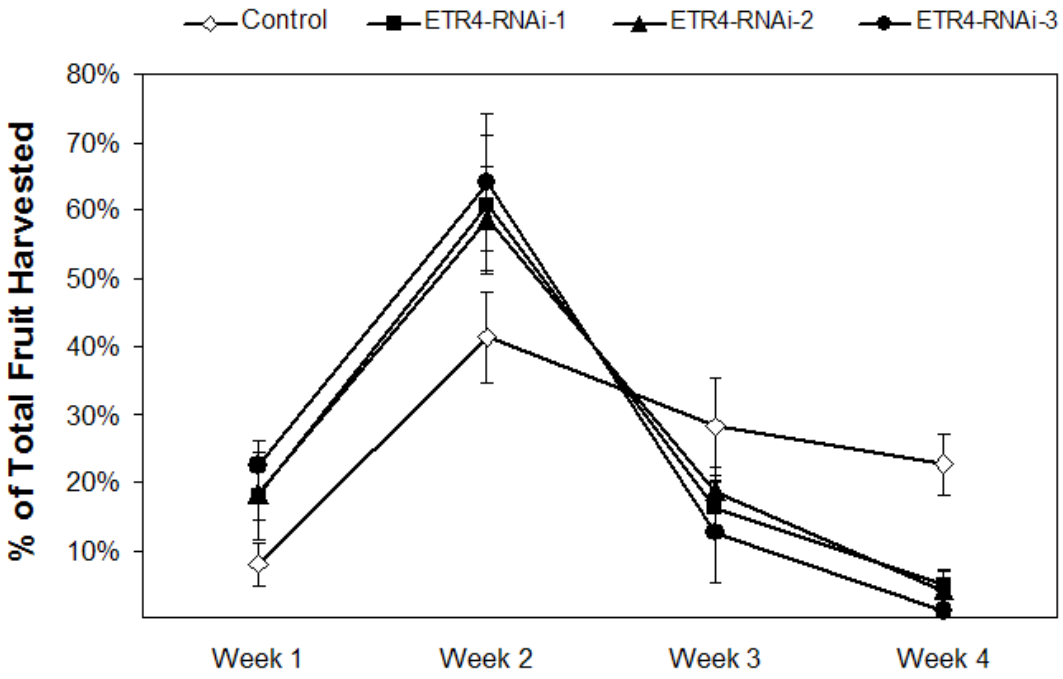


Figure 3-2 Suppression of *LeETR4* is Fruit-specific. (A) qRT-PCR analysis of *ETR4* transcript levels in leaf tissue and throughout fruit development in control and RNAi transgenic lines. (B) Protein blot analysis of ETR4 protein levels in control and transgenic lines. IMG, immature green; Breaker, first external color change; Turning, ~30% red color.



Cumulative Harvest as % of Total

Line	Week 1	Week 2	Week 3	Week 4
Control	7.8%	49.1%	77.4%	100.0%
ETR4-RNAi-1	18.1%	78.9%	95.2%	100.0%
ETR4-RNAi-2	18.3%	77.0%	95.8%	100.0%
ETR4-RNAi-3	22.3%	86.4%	99.0%	100.0%

Figure 3-3 *ETR4*-RNAi Transgenic Plants Have Altered Ripening Coordination. Fruits showing visible color development were harvested on a weekly basis. Values represent the percent of total fruit harvested each week ±SE.

Table 3-1 Weight, yield, brix, citric acid and malic acid from field grown fruits

	Weight		Yield/Plant				Citric Acid		Malic Acid	
Line	(g)	(n)	(kg)	(n)	°Brix	(n)	(mg/gfw)	(n)	(mg/gfw)	(n)
Control	135.5±3.1	218	4.51±0.4	9	4.1±0.2	10	2.76±0.04	5	0.22±0.02	5
RNAi-1	130.7±3.9	185	4.53±0.6	8	3.9±0.1	10	2.63±0.06	5	0.23±0.03	5
RNAi-2	131.4±3.1	262	4.49±0.4	12	3.8±0.1	10	2.69±0.14	5	0.23±0.02	5
RNAi-3	142.9±4.1	137	4.56±0.3	10	4.0±0.1	10	2.57±0.16	5	0.21±0.03	5

Table displays mean ±SE. n=number of fruit examined, or plants in case of yield study.

Table 3-2 Weight, yield, brix, citric acid and malic acid from greenhouse grown fruits

	Weight		Yield/Plant				Citric Acid		Malic Acid	
Line	(g)	(n)	(kg)	(n)	°Brix	(n)	(mg/gfw)	(n)	(mg/gfw)	(n)
Control	115.4±4.4	98	3.68±0.2	3	4.2±0.1	10	3.10±0.03	15	0.50±0.03	15
RNAi-1	103.7±3.3	64	3.58±0.5	2	4.2±0.1	10	2.92±0.10	15	0.52±0.04	15
RNAi-2	106.9±3.2	70	4.13±0.2	2	3.8±0.1*	10	3.01±0.04	15	0.48±0.03	15
RNAi-3	118.9±4.4	142	4.69±0.4	3	4.0±0.0	10	3.24±0.03*	15	0.48±0.03	15

Table displays mean ±SE. n=number of fruit examined, or plants in case of yield study.

*Statistically significant p-value<0.05 based on Student's t-test

Table 3-3 Volatile organic compounds from field grown fruits

Compound	Control	RNAi-1	RNAi-2	RNAi-3
cis-3-Hexenal	37.48±6.48	50.70±7.99	66.69±4.76*	34.76±5.53
β-Ionone	0.05±0.01	0.07±0.01	0.06±0.01	0.04±0.01
Hexanal	83.38±22.22	116.06±19.78	166.23±18.54*	59.51±4.08
β-Damascenone	0.02±0.00	0.03±0.01	0.02±0.00	0.02±0.00
1-Peneten-3-one	0.46±0.09	0.48±0.07	0.44±0.02	0.40±0.04
3-Methylbutanal	4.25±0.47	4.19±0.42	4.49±0.44	4.62±0.63
trans-2-Hexenal	1.06±0.17	1.27±0.29	1.63±0.06*	0.86±0.08
2-Isobutylthiazole	4.82±1.16	5.74±0.99	5.66±0.65	3.79±0.42
1-Nitro-2-phenylethane	1.60±0.33	1.20±0.43	1.92±0.37	1.12±0.40
trans-2-Heptenal	0.15±0.03	0.17±0.03	0.20±0.04	0.13±0.02
Phenylacetaldehyde	0.45±0.06	0.50±0.1	0.51±0.15	0.37±0.01
5-Methyl-5-hepten-2-one	3.40±0.87	3.81±0.80	4.57±0.86	3.02±0.29
cis-3-Hexenol	50.62±8.52	64.78±3.63	69.19±8.37	41.60±4.54
2-Phenylethanol	1.86±0.39	1.89±0.55	2.61±0.67	1.49±0.04
3-Methylbutanol	20.16±3.27	18.16±4.05	17.19±4.05	16.56±3.40
Methyl salicylate	0.13±0.01	0.19±0.05	0.17±0.05	0.14±0.04

Values are ng g^{-1} FW h^{-1} and table displays mean ±SE with n=6.

* Statistically significant p-value<0.05 based on Student's t test.

Table 3-4 Volatile organic compounds from greenhouse grown fruits

Compound	Control	RNAi-1	RNAi-2	RNAi-3
cis-3-Hexenal	105.01±26.92	105.41±29.49	115.46±19.79	122.32±50.31
β-Ionone	0.07±0.01	0.08±0.03	0.10±0.02	0.08±0.00
Hexanal	97.50±15.17	131.17±30.44	118.18±23.15	183.49±11.45*
β-Damascenone	0.02±0.01	0.02±0.01	0.03±0.01	0.02±0.00
1-Peneten-3-one	0.47±0.02	0.53±0.22	0.59±0.15	0.37±0.05
3-Methylbutanal	7.51±1.00	7.72±1.11	8.01±0.81	5.82±0.43
trans-2-Hexenal	2.15±0.53	2.44±0.67	2.26±0.50	2.63±0.76
2-Isobutylthiazole	2.55±0.54	2.38±0.65	2.33±0.40	2.70±0.57
1-Nitro-2-phenylethane	0.07±0.01	0.10±0.00	0.07±0.00	0.10±0.01
trans-2-Heptenal	0.25±0.07	0.36±0.13	0.33±0.06	0.29±0.06
Phenylacetaldehyde	0.27±0.02	0.23±0.06	0.30±0.03	0.42±0.04*
5-Methyl-5-hepten-2-one	3.66±0.63	5.07±1.64	3.51±0.49	4.34±1.48
cis-3-Hexenol	53.70±6.73	57.12±11.99	50.53±6.44	59.99±1.47
2-Phenylethanol	1.04±0.29	1.20±0.03	1.32±0.19	1.67±0.03*
3-Methylbutanol	47.15±3.01	48.20±11.74	43.55±8.62	46.35±11.81
Methyl salicylate	0.17±0.06	0.23±0.07	0.11±0.03	0.34±0.14

Values are ng g^{-1} FW h^{-1} and table displays mean ±SE with n=4.

* Statistically significant p-value<0.05 based on Student's t test.

CHAPTER 4
IDENTIFICATION OF QTLS THAT MODIFY TIME TO RIPENING AND RIPENING-ASSOCIATED ETHYLENE PRODUCTION

Introduction

The use of wild germplasm has become an important method for crop improvement by today's plant breeders. Genetic diversity in today's domesticated varieties is narrow and land races that could provide traits necessary for crop improvement are being lost every year. The development of introgression lines (ILs) that each contain a single chromosome segment introgressed into an otherwise uniform background has allowed for the identification of many monogenic traits and quantitative trait loci (QTLs) (Frary et al 2003; Doganlar and Tanksley 2000; Fridman et al 2002). In tomato, a number of introgression lines have been developed from crosses with wild relatives, including *L. pennellii* (Eshed and Zamir 1995), *L. hirsutum;* Monforte and Tanksley 2000), and *L. peruvianum*. These libraries are useful in identifying QTLs because any phenotypic variation can be associated with the introgressed segment. The entire library can be screened for a particular phenotype and individual lines can be isolated. Once these lines are identified the introgressed segment can be further reduced into sub-ILs by subsequent back crossing. This permits further refinement of the QTL location and potentially, map-based cloning. ILs have been used to identify QTLs responsible for changes in yield, quality and stress responses (Fridman et al 2004; Zamir 2001).

Tomato is the most economically important vegetable crop grown worldwide, providing significant incentive for crop improvement research. Short growing seasons in higher latitudes often reduce the number of varieties a grower can use or can force them to use greenhouses that require a significant investment. Identification of loci that control the time it takes a fruit to reach maturity could offer a tremendous opportunity for breeders. Early ripening loci could be selectively bred into elite varieties that would be otherwise impossible to grow at higher

latitudes. In addition to traditional breeding transgenic approaches are being developed to provide options for growers but here I will focus on the traditional method.

Regulation of ethylene biosynthesis at the molecular level is a poorly understood process. Work done in Arabidopsis led to the identification of proteins that regulate the activity of the key biosynthetic enzyme ACC synthase (ACS). The ETO1 and ETO-like proteins posttranslationally regulate the stability of ACS by targeting it to the 26S proteasome. Loss-of-function and dominant gain-of-functions mutants were isolated by screening mutagenized populations for plants exhibiting a triple response in the absence of exogenous ethylene. Where *ctr1* mutants exhibit this phenotype because of loss of signaling capability in the absence of ethylene, *eto* mutants produce significantly more ethylene than controls because of enhanced ACS stability. An obvious difference between Arabidopsis and tomato is that tomato fruit go through a developmental switch that results in a significant increase in ethylene production. While we understand that a developmental switch occurs that triggers the expression of particular ACS and ACO isoforms an understanding of the regulation of the expression and activity of these enzymes is lacking in tomato.

While we will assess early ripening and increased ethylene biosynthesis separately, there is a significant possibility that a locus that leads to increased ethylene production could also lead to early ripening. While increased ethylene production leading to early ripening could prove to be easier to understand it could prove less useful in terms of breeding early ripening lines because excessive ethylene production could lead to undesirable effects.

In an effort to identify QTLs associated with ripening modification and ethylene production, a screen was performed on a set of ILs derived from the *L. hirsutum* genome. *L. hirsutum* was chosen to conduct this experiment because it is an unusual relative of the cultivated

tomato. *L. hirsutum* produces small green fruit that never show any signs of ripening such as softening, carotenoid accumulation or volatile production. Maturity can only be assayed by the measurement of ethylene production rates and fruit do not reach maturity until approximately 70 days post-anthesis (Grumet et al, 1981). Once fruit reach maturity, there is a sharp increase in ethylene production that peaks at between 2000-4000 uL kg^{-1} day^{-1}, roughly ten times that of cultivated tomato varieties. These unusual phenotypes suggest the presence of loci that may influence ripening and ethylene synthesis in unusual ways.

Results

In a preliminary experiment ethylene emissions of fruit grown in the field were measured and a line (LA 3945) that produces up to four times the amount of ethylene produced by the control at the red stage was identified. This phenotype was confirmed with greenhouse-grown fruit (Figure 4-1). In an effort to conduct a more comprehensive analysis, 35 different lines, each containing a different segment of the *L. hirsutum* genome, along with both isogenic parents were grown in triplicate in the greenhouse. A randomized complete block design was utilized as an experimental design in order to control for variation within the greenhouse. Flowers of each line were tagged at anthesis to determine the number of days from anthesis to breaker (Figure 4-2). Statistical analysis using Dunnett's test identified three lines (3935, 3958 and 3968) that had reduced time to ripening with a p-value<0.05. Fruit from the same plants were collected at the breaker and red ripe stages to measure ethylene emission rates (Figure 4-3 & 4-4). Statistical analysis using Dunnett's t test identified four (3922, 3935, 3944 and 4005) and three lines (3922, 3934 and 3969) with increased ethylene emission in breaker and red fruit, respectively. In addition to the lines identified by statistical analysis we included a few lines for each trait assayed that were close to the p-value<0.05 cut-off. Figure 4-5 is a representation of the approximate locations of each introgressed segment in the tomato genome. This map was used as

a guide to develop a library of markers from the sequence information available in the SOL Genomics Network database. A postdoctoral researcher in our lab has taken over this project and will use the markers to fine map the exact locations of these pieces. The map also contains the locations of all known ethylene receptors and ACC-synthase (ACS) isoforms because they are possible candidates for these QTLs.

In order to replicate the results of the first experiment we grew the identified lines in a greenhouse to assess the ripening trait and in the field to assess ethylene emission. Again, each line was grown in triplicate along with both isogenic parents. In addition to the selected lines additional lines from the collection that overlap the introgressed *L. hirsutum* segments were included in the analysis to better map the location of each QTL. Greenhouse data for the ripening lines is presented in Figure 4-6. Of the original lines selected, only those that had previously showed a statistically significant change in ripening (3935, 3958 and 3968) repeated a reduction that was again statistically significant. The other lines (3921, 3955 and 3964) were assayed again because they were close to making our cutoff of 0.05. The fact that these lines did not show a significant reduction in the following season strongly supports our confidence in the statistical analysis of the data from the first season. Interestingly line 3921 did not itself show a reduction in the second season but three overlapping lines 3922, 3923 and 3924 were found to be significantly lower than the control. Due to the labor intensive nature of measuring ripening time, only lines 3935, 3958 and 3968 will be further characterized.

In order to gain a better understanding of the increase in ethylene emission, field grown fruit were harvested at four stages and ethylene emissions were measured. Figure 4-7 shows the complexity of the trait, with some lines being statistically higher at some stages and not at others. IL 3922 and two overlapping ILs had a higher level of ethylene emission during early ripening

(i.e. breaker) but returned to WT levels by the red stage. IL 3935 and its overlapping lines were low early in ripening but statistically higher at the pink and red stages. In accordance with early studies, line 3945 had the highest ethylene emissions of any of the lines tested, with more than 2-fold higher rates at the pink stage. Additional lines overlapping 3945 had higher ethylene emission at each ripening stage tested. IL 3969 had higher emission rates at the breaker and pink stages but returned to WT levels by the red stage. The complex nature of this phenotype has made analysis more difficult. While we were principally interested in ethylene emissions in fruit we were interested to determine if the increases were limited to the fruit. Ethylene emissions of young leaves were assayed (Figure 4-8). No statistically significant differences were seen for any of the lines suggesting that the increases were confined to ripening fruit tissue. Interestingly the *L. hirsutum* isogenic parent (1777) showed the lowest leaf ethylene emissions and this was confirmed by repeating this experiment. Due to the fact that many of the introgressed pieces were quite large, backcrosses were made to the isogenic *L. esculentum* parent for all lines that repeated in the second season. All of the data displayed from the second season (Figures 4-6 and Figure 4-7) were collected and analyzed by Dr. Valeriano Dal Cin, a postdoctoral researcher in our lab. He has also isolated homozygous recombinants from the backcrosses performed during the second season and is currently analyzing the progeny of those recombinants.

Due to our interest in receptor function we were intrigued to see that the IL that consistently emitted more ethylene (3945) contains a chromosomal segment that potentially encodes the *L. hirsutum* ortholog of *LeETR4*. In order to determine which allele is present in 3945, the intron of this gene from the 3945 line was cloned and compared to both the *L. esculentum* and *L. hirsutum* sequences (Figure 4-9). The IL was confirmed to contain the *L. hirsutum* allele. In order to understand whether this allele showed any differential expression I

performed qRT-PCR on RNA collected from vegetative and reproductive tissues (Figure 4-10). While there are some differences at particular stages the basic trend of expression is similar between the control and IL. In addition to an analysis of developmental expression both parents and 3945 seedlings were exposed to ethylene in order to determine if the ethylene inducibility of the *L. hirsutum* allele was altered (Figure 4-11). No significant difference was observed between any of the genotypes. An additional time to ripening experiment showed no statistical difference between the *L. esculentum* parent and 3945. Subsequent marker analysis of the introgression region at 3945 found that both 3944 and 4005 overlap 3945 and all three have increased ethylene emission. This region of the introgressed segment is not the area that contains *LhETR4*. These additional data suggest that the *L. hirsutum* allele of *LeETR4* is likely not the cause of the increased ethylene phenotype.

Discussion

Marker assisted breeding is an important technique used to address fundamental problems in plant biology and crop improvement. It is particularly important with the current public attitudes toward genetically modified organisms. Breeders are increasingly going to require the identification of markers that are linked to agronomically important traits. A close relationship between researchers and breeders will allow for efficient introduction of newly identified traits into existing varieties.

While a number of different resources are available for mapping traits of interest in tomato, the development of introgression populations using different wild relatives has greatly enhanced this process. These populations facilitate sorting an entire genome down to a small, known segment that can be assayed for a particular phenotype. In addition, once the tomato genome sequencing project is complete it will be relatively straightforward to screen a population and then search a particular genomic location for candidate genes.

While a great deal of research has been done on the ethylene biosynthetic pathway the only proteins that have been identified that act to modulate this pathway are the ETO proteins. This modulation is accomplished by inhibiting activity of the ACS protein and targeting it for degradation via the 26S proteasome. Identification of additional regulators of ethylene synthesis will broaden our understanding of the factors affecting synthesis, whether they be positive or negative. We present here a strategy to identify QTLs that control ripening-associated ethylene production by screening *L. hirsutum* ILs. The choice of the *L. hirsutum* introgression population was originally made because of the unusual ability of this species to produce high levels of ethylene during ripening (Grumet et al., 1981). We have identified six introgression lines that produce significantly more ethylene during at least one stage of fruit ripening (Figures 4-3, 4-4 and 4-7). This increased ethylene emission is restricted to ripening fruit as no difference was seen in leaf ethylene biosynthesis rates in selected introgression lines (Figure 4-8).

A significant amount of research surrounding fruit ripening has been completed in the past century but we still do not understand how fruits regulate ripening at the molecular level. In climacteric fruits it is clear that there are two important levels of regulation, developmental cues and ethylene synthesis. Work done on tomato in the past five years has begun to unravel this phenomenon at both levels with the identification of the *RIN* and *NOR* genes as well as the ethylene receptor family (Vrebalov et al., 2002; Kevany et al., 2007). While these findings are important steps, fruit development is a complex and there are likely many additional regulators of this process. Due to the unusual nature of ripening, or lack of ripening, in the wild tomato species *L. hirsutum,* we hypothesized it may allow for the identification of some additional factors regulating fruit development and specifically the onset of ripening. In addition to the ILs with increased ethylene emission our screen identified three lines that showed significantly

reduced time from anthesis to breaker that was confirmed in two experiments (Figures 4-2 and 4-6).

Future work will involve finer mapping of the *L. hirsutum* QTLs. Backcrosses have been performed for each ripening and ethylene line and recombinants are being identified by a post-doctoral researcher in our lab. A library of cleaved amplified polymorphic sequence (CAPS) markers has been generated to precisely map each introgressed segment. Eventually these loci will be cloned by a map-based approach or by use of the tomato genome sequence and will increase our knowledge of both these processes.

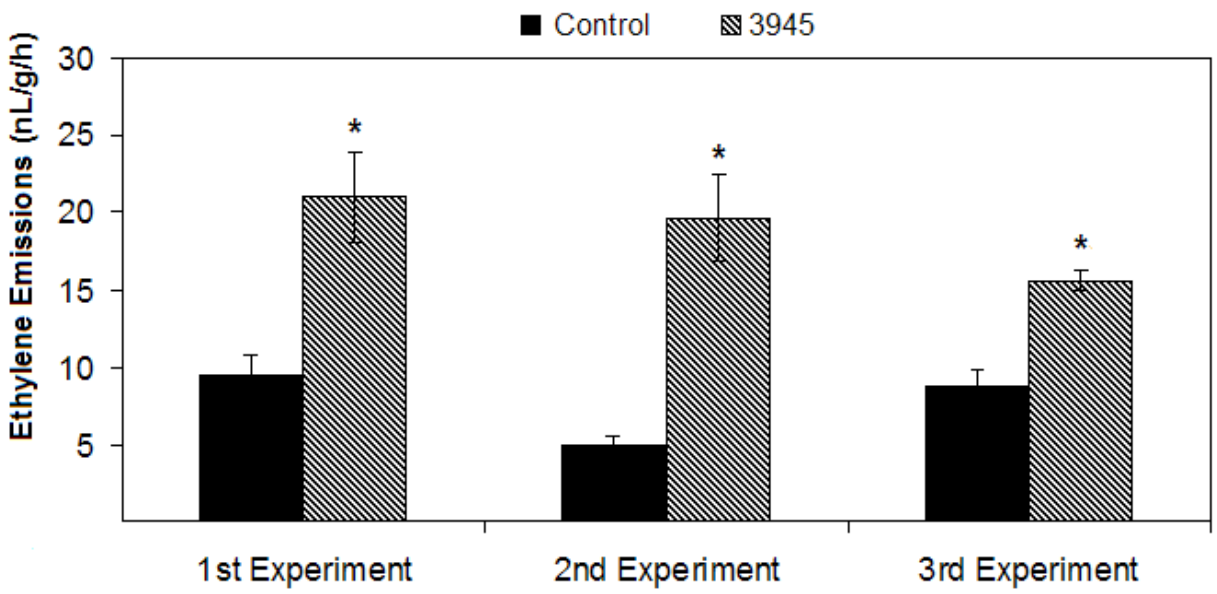


Figure 4-1 Ethylene emissions of fully ripe fruit from *L. hirsutum* IL 3945. Red fruit were sealed in 500 mL jars for ~1 h and ethylene emission was measured by gas chromatography. Values represent mean ±SE. *Statistically significant values p-value<0.05 based on Dunnett's t test.

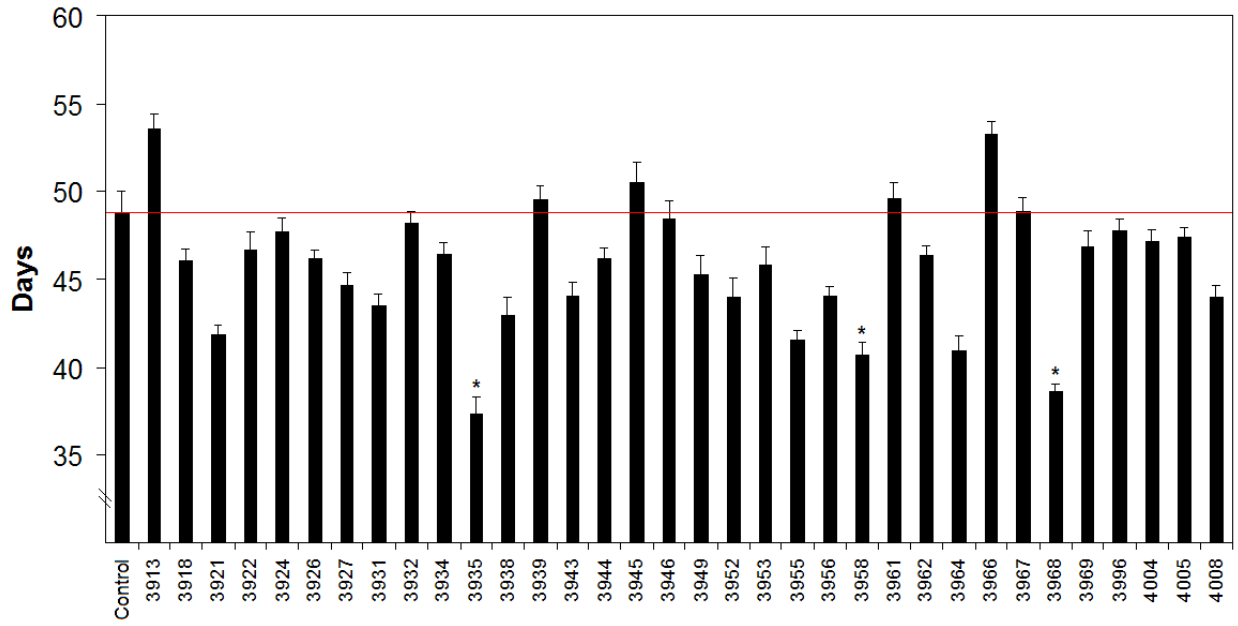


Figure 4-2 Days from anthesis to breaker of tagged fruits from *L. hirsutum* ILs. Open flowers were tagged at anthesis and the number of days to breaker were recorded. *Statistically significant values p-value<0.05 based on Student's t test.

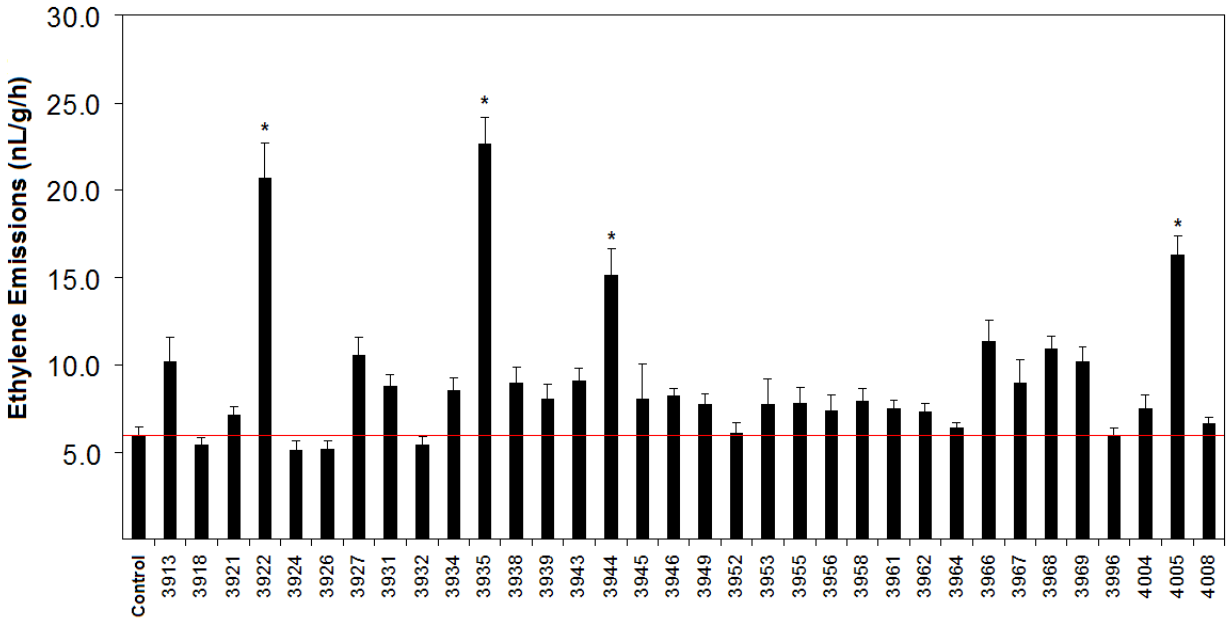


Figure 4-3 Ethylene emissions of breaker fruit from *L. hirsutum* ILs. Breaker fruit were sealed in 500 mL jars for ~1 h and ethylene emission was measured by gas chromatography. *Statistically significant values p-value<0.05 based on Dunnett's t test.

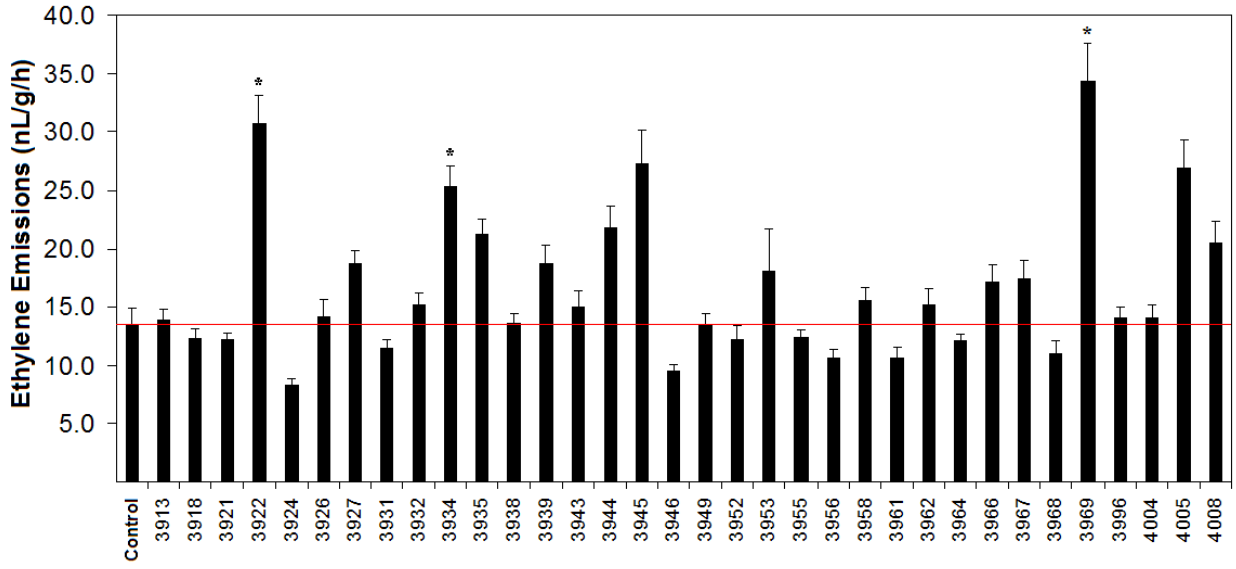


Figure 4-4 Ethylene emissions of fully ripe fruit from *L. hirsutum* ILs. Red fruit were sealed in 500 mL jars for ~1 h and ethylene emission was measured by gas chromatography. *Statistically significant values p-value<0.05 based on Dunnett's t test.

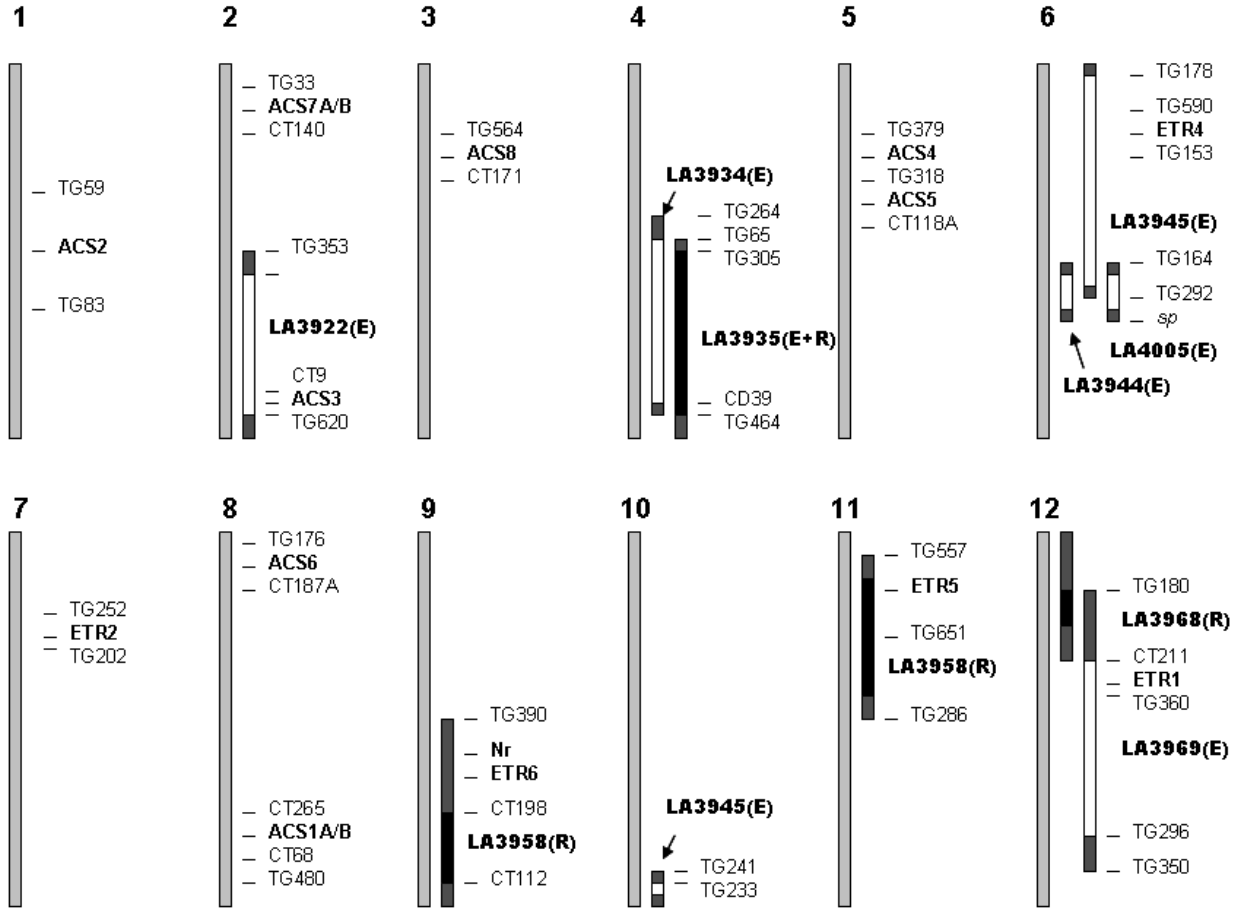


Figure 4-5 Genomic map showing locations of introgressed regions that contain putative ripening-associated QTLs. Black regions represent locations of QTLs controlling ripening phenotype. White regions represent locations of QTLs controlling increased ethylene emission phenotype. Dark grey regions represent portions of introgressed pieces that are ambiguous based on original mapping done with population. Map also contains locations of all known ethylene receptors and ACC synthase isoforms.

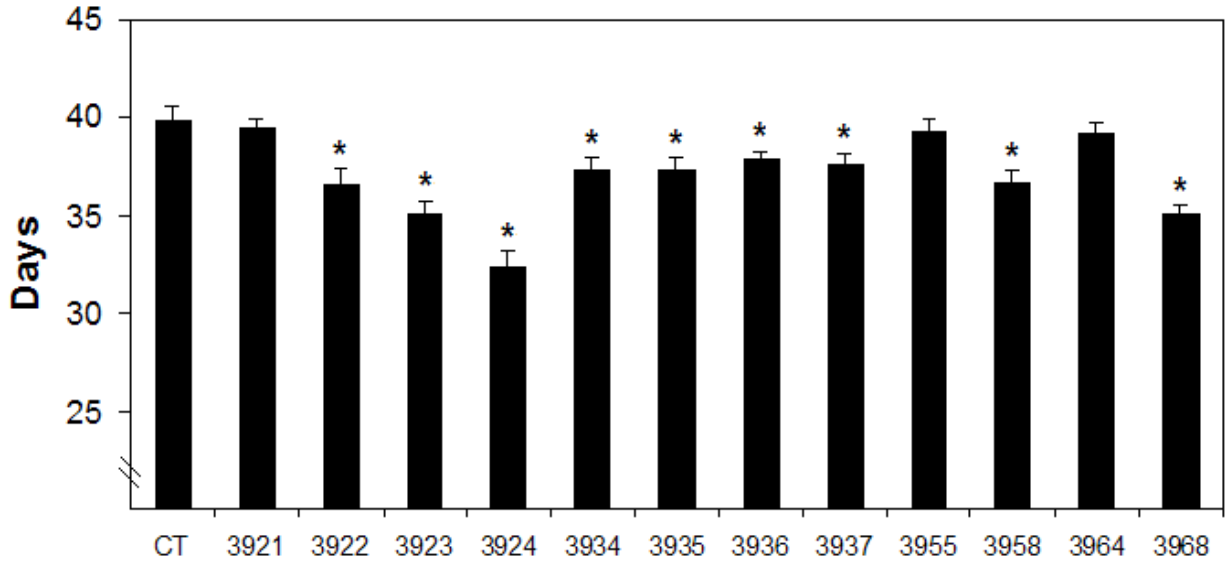


Figure 4-6 Days from anthesis to breaker of tagged fruits from *L. hirsutum* ILs. Open flowers were tagged at anthesis and the number of days to breaker were recorded. *Statistically significant values p-value<0.05 based on Student's t test. CT, control.

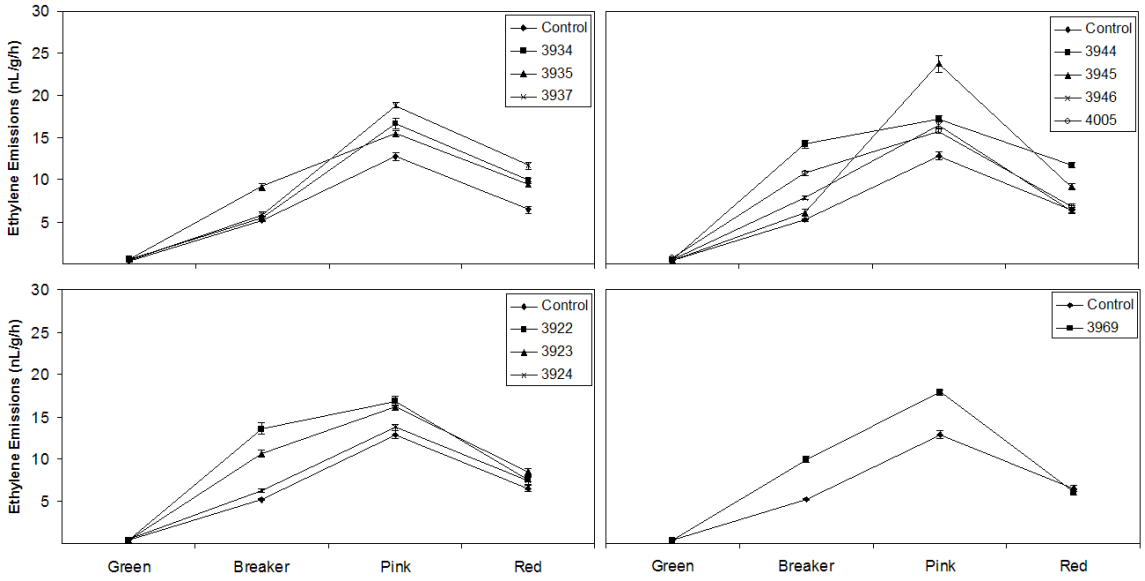


Figure 4-7 Ethylene emissions of fruits from field-grown *L. hirsutum* ILs. Fruit at indicated stages were sealed in 500 mL for ~1 h and ethylene emission was measured by gas chromatography. Breaker, first external signs of ripening; pink, ~70% color development.

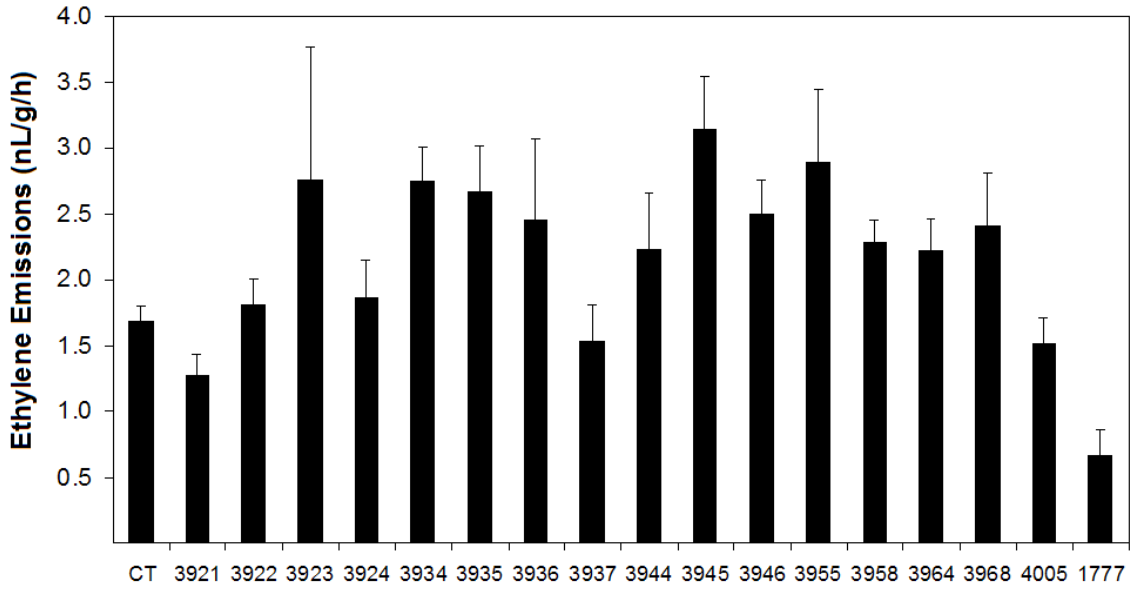


Figure 4-8 Ethylene emissions of leaves from *L. hirsutum* ILs. Young leaves were harvested and immediately placed in 5 mL plastic tubes, but were left uncapped for ~1/2 h to permit release of wound-induced ethylene. After sealing, tubes were left for ~ 3 h and then ethylene emission was analyzed by gas chromatography. CT, control.

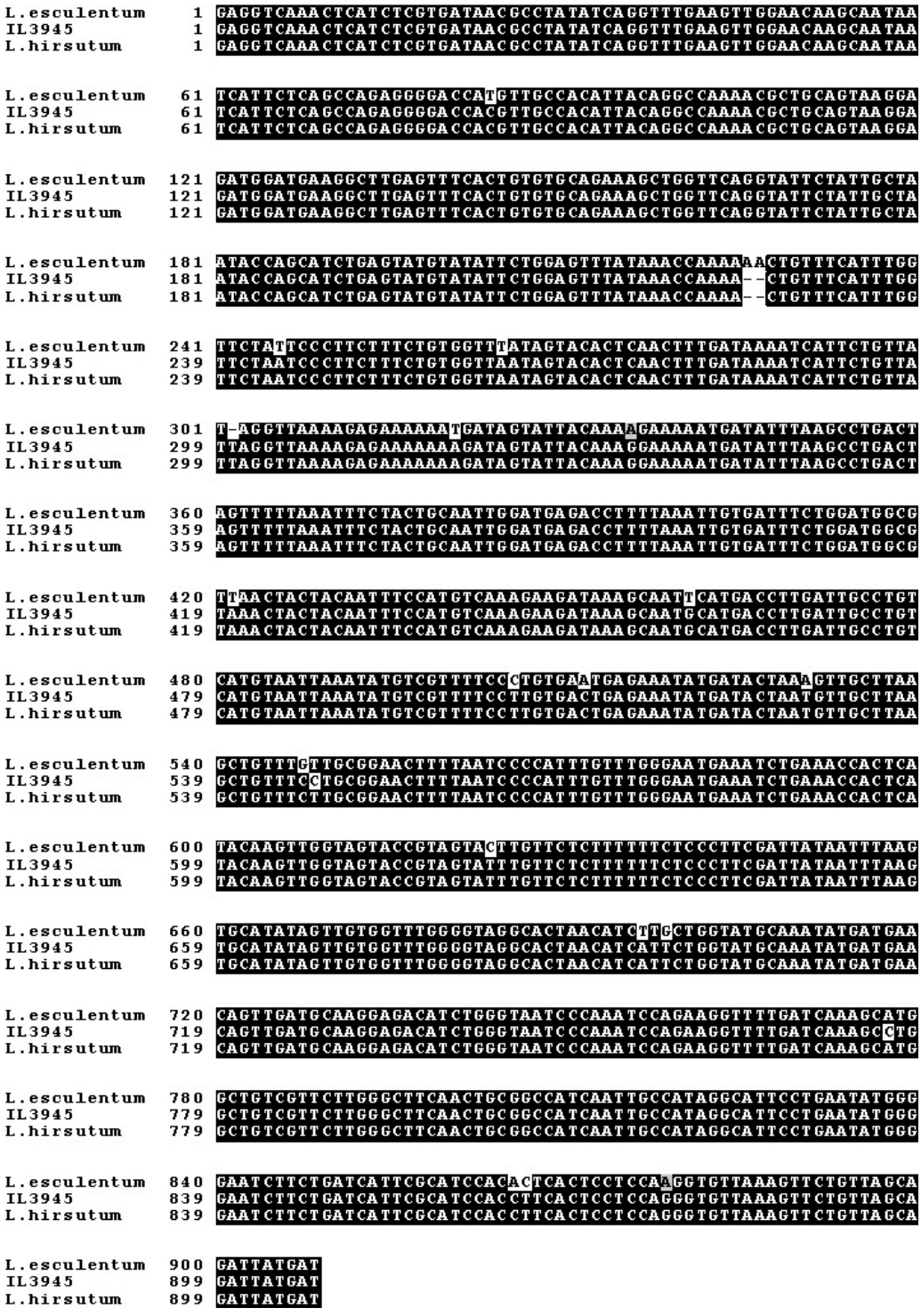


Figure 4-9 Nucleotide alignment of ETR4 genomic sequence. Sequence isolated from *L. esculentum*, *L. hirsutum* and IL 3945. Alignment was done using ClustalW and presented using Shade Box software.

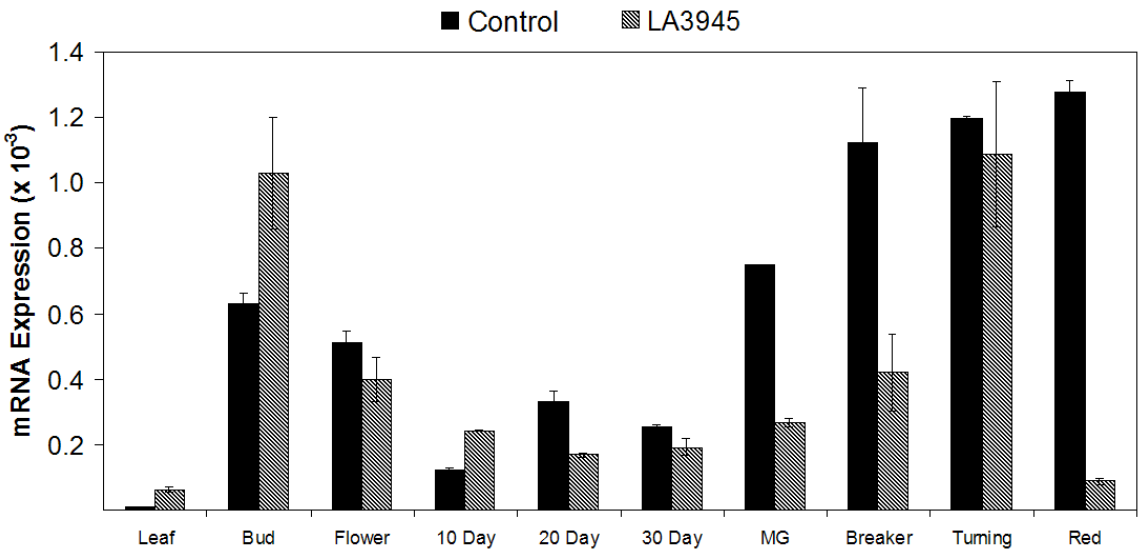


Figure 4-10 mRNA expression of *LeETR4* in WT and the *L. hirsutum* IL 3945. qRT-PCR analysis of transcript levels in leaf and reproductive tissues. Values represent mean ±SE and presented as % of total RNA.

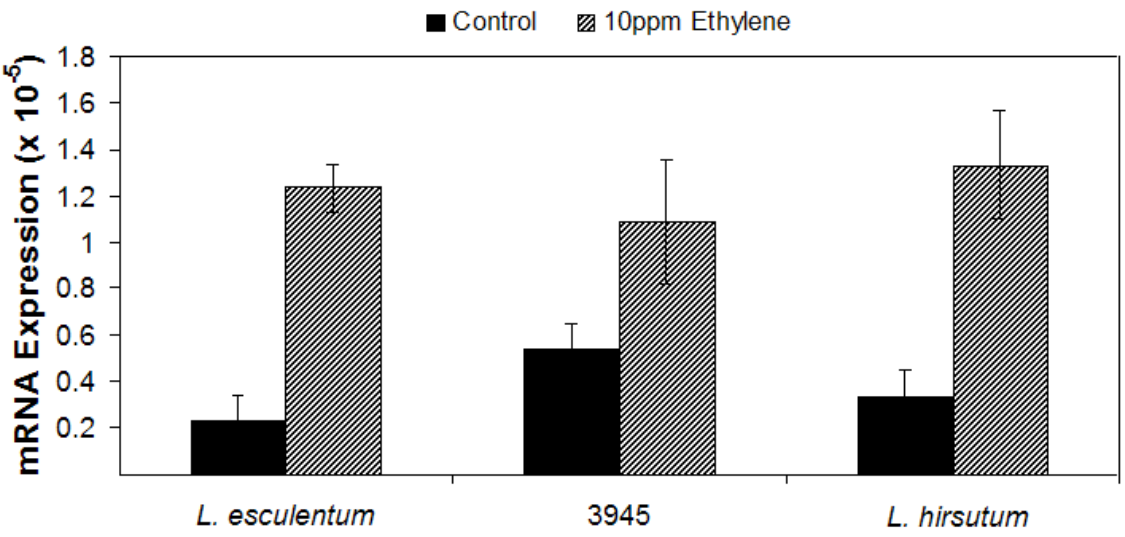


Figure 4-11 mRNA expression of *LeETR4* in seedlings of WT, *L. hirsutum* and IL 3945. qRT-PCR analysis of transcript levels in leaf tissue treated with 10ppm ethylene. Values represent mean ±SE and presented as % of total RNA.

CHAPTER 5
CONCLUSION

The solanaceous species *Lycopersicon esculentum*, tomato, has emerged as the model for studying fleshy fruit development. Because tomato is a climacteric species it is also the species of choice for studying ethylene's involvement in fruit development. Ethylene is essential for normal fruit ripening in these species and blockage of either ethylene production or perception leads to improper ripening. In tomato fruits, ethylene has profoundly different effects depending on the stage of development with a distinct developmental switch that occurs upon fruit maturation. Treatment of mature fruits results in the initiation of a ripening program. While treatment of immature fruits does not initiate ripening, it does significantly hasten the onset of subsequent ripening (Yang, 1987). Our understanding of how fruits measure this ethylene exposure has not been previously determined. The primary objective of this project was to identify the mechanism controlling this phenomenon and to gain a better understanding of the factors that control ripening in general.

Previous work in our lab showed that reduced accumulation of a single receptor, *LeETR4*, resulted in fruits that ripen significantly earlier than control fruits (Tieman et al., 2000). In this study *LeETR6* reduced expression lines exhibited similar effects to those of *LeETR4* (Table 2-1; Kevany et al., 2007). While these results are consistent with the model that the receptors act as negative regulators of ethylene signaling, they do not address the question of how fruits measure cumulative ethylene exposure. Analysis of receptor mRNA expression during fruit development indicated a significant increase in receptor mRNAs at the onset of ripening coincident with the increase in ethylene biosynthesis (Figure 2-1, Figure 2-2). Contrary to the mRNA expression data, protein blot analysis of NR, ETR4 and ETR6 showed receptor protein levels highest in immature fruit with a significant decrease at the onset of ripening (Figure 2-4). While these data

are contradictory to the mRNA expression data, they are consistent with a model in which ethylene binding affects receptor protein stability. In an attempt to validate this hypothesis, we exposed fruit and vegetative tissues to ethylene and observed that receptor proteins are rapidly degraded in response to ethylene and that this likely occurs through the 26S proteasome-dependent pathway (Figure 2-5, Figure 2-6). While ligand binding-induced degradation of receptors has been described in mammalian and yeast systems, this work is the first example in plants. These results led to a hypothesis that reduced levels of receptor proteins, due to ethylene exposure, control the early ripening in ethylene treated immature fruit. To test this hypothesis, we treated immature fruits, while still attached to the plant, with ethylene and measured protein levels throughout fruit development. Treated fruits had reduced receptor protein levels after ethylene treatment and these fruits ripened earlier than untreated controls (Table 2-2, Figure 2-7). Together these data are consistent with our model that ethylene exposure leads to a degradation of receptor proteins and that ethylene receptor levels modulate the timing of ripening.

While reduction of receptor levels results in early ripening fruit, systemic reduction also causes severe developmental effects that would prevent the use of this method for crop improvement (Figure 2-3). A technique to reduce the time from fruit set to the onset of ripening could allow for an increase in the number of varieties available to farmers in higher latitudes. To generate early ripening lines, we developed a fruit-specific RNAi construct to reduce *LeETR4* levels only in the fruit. Fruit-specific suppression of *LeETR4* resulted in fruits that ripened up to 7 days early (Figure 3-1). While early ripening fruit would be advantageous they must also retain the same quality as traditional varieties. To test fruit quality I measured average fruit size, yield, soluble solids, malic and citric acid content as well as the most important tomato flavor volatile organic compounds. There was little or no difference between transgenic and control fruits. In

addition to providing a unique method of crop improvement these data also validate our model that receptor levels in the fruit control the timing of ripening.

While biotechnology has provided us with many tools for gene discovery and crop improvement, current public concerns have limited the marketing of transgenic foods. In an effort to identify additional factors that regulate the timing of ripening we undertook a genetic approach. A screen of a *L. hirsutum* introgression population was conducted because of the unusual ripening characteristics and high ethylene biosynthesis levels of this species. Individual lines were screened for reduced time from anthesis to breaker and for increased ripening-associated ethylene synthesis. Three lines with a reduction of time to breaker were identified and the results were repeatable across seasons. Seven lines that had increased ethylene emissions at the breaker or red stages were identified. Due to the large segments of the *L. hirsutum* genome that are found in these lines they had to be backcrossed to the *L. esculentum* parent to better map the loci controlling ethylene emissions. Recombinants that may provide material for a map-based cloning approach for gene discovery have been identified.

The work presented here has significantly increased our understanding of how ethylene regulates ripening in climacteric fruits. While ethylene is not required for the ripening of non-climacteric fruits, it can have significant effects on fruit development in these species. Ethylene can cause damage to the fruits of many different species and our understanding of receptor function could greatly enhance our ability to limit these losses. Ethylene-related losses in underdeveloped countries often account for a significant proportion of the postharvest losses and are an opportunity for our research to have a serious impact.

CHAPTER 6
MATERIALS AND METHODS

Plant Materials and Growth Conditions

L. hirsutum cv. Flora-Dade, *LeETR4*-AS, *LeETR6*-AS and TFM7-*ETR4*-RNAi lines were grown in a greenhouse set at approximately 27^{o}C. Individual plants were grown in 3 gal pots that were watered twice a day and supplemented with slow release fertilizer. Time to ripening data was collected by tagging open flowers and recording the number of days from anthesis to breaker. *L. hirsutum* cv. Micro-Tom and *Nr* plants were grown in a growth chamber under standard conditions (16 h day/8 h night). Field plants were grown in randomized, replicated plots in Live Oak, FL. Plants were grown using standard commercial practices in raised plastic mulched beds.

Development of Transgenic Plants

LeETR4-AS and *LeETR6-AS* lines were generated by cloning the full-length *LeETR4* or *LeETR6* coding region into a vector in the antisense orientation under the control of the Figwort Mosaic Virus 35S promoter (Richins *et al*., 1987) and followed by the *Agrobacterium tumefaciens* nopaline synthase (*nos*) 3' terminator. The transgene was introduced into cv. Flora-Dade by the method of McCormick *et al*. (1986), with kanamycin resistance as a selectable marker. Transgenic lines with a reduction of >70% of *LeETR6* transcript were identified (Table 1). The specificity of the transgene was determined by quantification of every receptor mRNA from leaf tissue. In each case there was no effect on RNA levels of any other receptor.

LeETR4 fruit-specific RNAi lines were generated using method outlined by Dexter et al. (2006). Briefly, two overlapping fragments of coding region were PCR amplified from tomato fruit cDNAs, one 400 bp and 200 bp in length, primer sequences found in Table 6-1. The two PCR products were ligated end to end and subsequently ligated into an EcoRI site in the

pMON999 vector that contained the TFM7 fruit specific promoter. The cassette containing the promoter, RNAi fragment and *nos* terminator were excised from the vector and ligated into the pHK plant expression vector. The transgene was introduced into cv. Flora-Dade by Agrobacterium-mediated transformation according to McCormick et al. (1986), with kanamyacin resistance as a selectable marker.

Pharmacological Treatments

Ethylene treatments of plant material were done in sealed 38 L tanks. Treatments were performed using either 10 or 50 ppm, as indicated, concentrations in tanks was monitored by gas chromatography. These levels are both within the linear response range for *NR* and *LeETR4* ethylene inducibility (Ciardi et al. 2000). Proteasome inhibitor studies were performed by spraying seedlings with an 80 μM MG132 solution (8% DMSO) 4 h prior to 2 h ethylene treatment. Control seedlings were sprayed with an 8% DMSO solution. 1-MCP treatment of seedlings was performed at 1 ppm in a sealed 38 L tank for 16 h prior to 2 h ethylene treatment. Control seedlings were sealed in identical tanks for the same duration of time. All microsomal membrane preparations were performed immediately after treatment ended.

Recombinant Protein Expression and Antibody Production

Coding regions of *LeETR4* (a.a. 532-684) and *LeETR6* (a.a. 522-688) were amplified with primer pairs ETR4-PF, ETR4-PR, ETR6-PF and ETR6-FR (Table 6-1) from fruit cDNAs generated with the Clontech One-step cDNA Synthesis kit. PCR products were digested with BamHI and BglII and cloned into the Invitrogen pTrcXHisA vector and subsequently transformed into the BL21(DE3) (Invitrogen) *E. coli* strain for recombinant protein expression. 100 mL cultures were grown at 30^{o}C and induced with 1 mM IPTG for 4 h. Cells were spun down at 8,000 x g, resuspended in 10mL of lysis buffer (8 M urea) and pulse sonicated for 1 min. Lysate was spun down at 8,000 x g and supernatant was purified with Ni-NTA affinity column

as directed. Recombinant protein was submitted to Cocalico Biologicals (Reamstown, PA) for antibody production in rabbits using their standard protocol. Antiserum was received and used to probe both antigens to determine antiserum specificity for its respective antigen.

RNA Expression Analysis

Total RNA extractions were performed using the Qiagen RNeasy Mini Kit with subsequent DNase treatment to remove any contaminating DNA. RNA was quantified by spectroscopy and visually analyzed on ethidium bromide-stained gels to assure equal concentrations of all RNAs. Quantitative RT-PCR assays were performed using the Applied Biosystems Taqman One-step RT-PCR kit in an Applied Biosystems GeneAmp 5700 Sequence Detection System as described (Tieman et al. 2001). PCR conditions were as follows, Step 1: 48^{o}C for 30 min, Step 2: 95^{o}C for 10 min and Step 3: 95^{o}C 15 sec and 60^{o}C for 1 min (40X). Primer and probe pairs for each gene assayed can be found in Table 6-1. Levels of *LeETR* RNAs were quantified using RNAs synthesized by *in vitro* transcription from plasmids containing the coding region of each gene using a Maxiscript *in vitro* transcription kit (Ambion, Austin TX USA). Total μg of *in vitro*-transcribed RNA were determined and the *in vitro* transcription product used for a standard curve in real-time RT-PCR analysis. Results are reported as % *LeETR* RNA in total RNA.

Microsomal Membrane Isolation and Protein Blot Analysis

Microsomal membrane fractions were isolated from fruit or seedlings with a homogenization buffer containing 30 mM Tris (pH 8.2), 150 mM NaCl, 10 mM EDTA, and 20% (v/v) glycerol with protease inhibitors (1 mM PMSF, 10 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL chymostatin) as described (Schaller *et al*., 1995). Tissue was homogenized at 4^{o}C using a polytron and then centrifuged at 8,500 x g for 15 min at 4^{o}C. The supernatant was

strained through cheesecloth then centrifuged at 100,000 x g for 30 min at 4°C and the subsequent membrane pellet was resuspended in 10 mM Tris (pH 7.5), 5 mM EDTA, and 10% (w/w) sucrose with protease inhibitors and stored at -80 °C. Protein concentrations were determined using the Bio-Rad Protein Assay reagent with BSA used for a standard curve. 20 μg of total protein was run out for each sample on a 12% Tris-HCl gel and proteins were transferred to a nitrocellulose membrane using the Bio-Rad Mini Trans-Blot cell. Membranes were blocked overnight in 10% Carnation milk/Tris Buffered Saline-Tween (TBST) at 4°C. Membranes were washed 2x5 min in TBST and then incubated with primary anti-ETR4 (1:2000) or anti-ETR6 (1:5000) antibody diluted in 5% Carnation milk/TBST for 1 h. Membranes were subsequently washed 3x10 min in TBST and then incubated with peroxidase conjugated goat anti-rabbit (1:5000) secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland) diluted in 5% Carnation milk/TBST for 45 min. Membranes were finally washed 3x10 min in TBST. Visualization of signal was performed using the Amersham ECL Detection reagents before being exposed to film. Quantification of bands was accomplished by using the NCBI imaging software ImageJ (http://rsb.info.nih.gov/ij/). Values were normalized to an anti-BiP (endoplasmic reticulum immunoglobulin binding protein) antibody (generously provided by Alan Bennett, Univ. of California, Davis) which was used as an ER-localized loading control.

Acid and Soluble Solids Analysis

Individual tomato fruit were homogenized in a blender for 30 s and frozen at –80 °C until acid analysis. Samples were thawed, centrifuged at 16 000 *g* for 5 min. The supernatant was analyzed for citric and malic acid content using citric acid and malic acid analysis kits (R-Biopharm, Marshall, MI) according to the manufacturer's instructions. Soluble solids are expressed as °Brix which is a measurement of the mass ratio of dissolved sucrose to water in a

liquid. Individual fruit were homogenized in a blender for 30 s. 1 mL of the homogenate was centrifuged at 16,000 x g for 2 min. ~75 uL of supernatant was applied to a handheld refractometer.

Volatile Analysis

Ripe tomato fruit from each line and its corresponding control collected from the field were harvested and volatiles from pooled fruits were collected on the day after harvest. Fruits collected from plants grown in the greenhouse were analyzed for fruit volatiles immediately after harvest. Tomato fruit volatiles were collected from chopped fruit with nonyl acetate as an internal standard as described by Schmelz *et al*. (2003). Chopped fruit was enclosed in glass tubes, air filtered through a hydrocarbon trap (Agilent, Palo Alto, CA) flowed through the tubes for 1 h with collection of the volatile compounds on a Super Q column. Volatiles collected on the Super Q column were eluted with methylene chloride after the addition of nonyl acetate as an internal standard. Volatiles were separated on an Agilent (Palo Alto, CA) DB-5 column and analysed on an Agilent 6890N gas chromatograph with retention times compared to known standards (Sigma Aldrich, St Louis, MO). Volatile levels were calculated as ng g^{-1} FW h^{-1} collection. Identities of volatile peaks were confirmed by GCMS as described by Schmelz *et al*. (2001).

Table 6-1 Oligonucleotide Primers and Probes

ETR4-PF	CC**GGATCC**CGTGATAACGCCTATATCAGG	BamHI
ETR4-PR	CC**AGATCT**GACGATTTGGAATGAGGATAC	BglII
ETR6-PF	CC**GGATCC**CCGAGATCAAACTCATCCAATG	BamHI
ETR6-PR	CC**AGATCT**GCCATCTAAATCAGGCAGATG	BglII
ETR4-RNAi-F1	GG**AGATCT**GGCATTCCTGAATATGGGG	BglII
ETR4-RNAi-R1	CC**GGCGCGCC**GAGGATACAGCAGGGCTAAG	AscI
ETR4-RNAi-F2	CC**GGATCC**GGCATTCCTGAATATGGGG	BamHI
ETR4-RNAi-R2	GG**GGCGCGCC**CATCATTCTACTTCCCCGTAGC	AscI
ETR1-TaqF	TTCAAGGATTAAAGGTTTTGGTGAT	
ETR1-TaqR	ATCACATCCAAGGTGTGTAAGCA	
ETR1-Probe	FAM-ATGAGAATGGTGTTAGCAGGATGGTAACCAAA-BHQ	
ETR2-TaqF	GCCGTCAGTGTACATGAGAAATTT	
ETR2-TaqR	AGTTTTCTTTTGTCACTTGGTCAGTGT	
ETR2-Probe	FAM-AGAGGCCACTTATTGTGGCACTAACTGGG-BHQ	
NR-TaqF	AGGGAACCACTGTCACGTTTG	
NR-TaqR	CTCTGGGAGGCATAGGTAGCA	
NR-Probe	FAM-AGTGAAACTCGGAATCTGTCACCATCCAA-BHQ	
ETR4-TaqF	GGTAATCCCAAATCCAGAAGGTTT	
ETR4-TaqR	CAATTGATGGCCGCAGTTG	
ETR4-Probe	FAM-AAAGCATGGCTGTCGTTCTTGGGCT-BHQ	
ETR5-TaqF	AGTCATCTTTTAGGAAACGCATGTT	
ETR5-TaqR	AGGAGTACATGAAGGCCTCTGAA	
ETR5-Probe	FAM-AATACAGAAATCCTTTGGAGCAACCG-BHQ	
ETR6-TaqF	ATTCCAAAGGCAGCCGTTAA	
ETR6-TaqR	GGATGTGGATATGTGGGATTAGAAG	
ETR6-Probe	FAM-CTCCACATATTCGGACATGCCTAAGGGA-TAMRA	

* Nucleotides in bold face represent restriction sites

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BIOGRAPHICAL SKETCH

Brian Michael Kevany was born in Cleveland, Ohio on September 28, 1980. When he was one he and his mother, father and older brother Thomas moved to North Olmsted, Ohio where his younger brother Daniel was born and where Brian spent his entire childhood. As a young boy Brian enjoyed discovering things in his backyard and playing golf, baseball and hockey, with hockey being a sport he played year round. When he was in high school he got a job at a local nursery and really enjoyed learning about plants. After high school Brian attended Michigan State University where he majored in horticulture specializing in biotechnology. While at MSU he worked as an undergraduate researcher in the Postharvest Physiology lab of Dr. David Dilley under the tutelage of Dr. John Golding. Dr. Golding allowed Brian to become intimately involved in the projects in the lab and fostered a great interest in plant research. After graduation, Brian joined the Plant Molecular and Cellular Biology Ph.D. program at the University of Florida as a pre-doctoral Alumni Fellow. While at UF he worked in the lab of Dr. Harry Klee studying the importance of the tomato ethylene receptor family during tomato fruit development. Upon completion of his Ph.D. degree, Brian will enter the lab of Dr. Michael Thomas in the Department of Bacteriology at the University of Wisconsin-Madison as a postdoctoral researcher.