MOLECULAR ANALYSIS OF ETHYLENE SIGNAL TRANSDUCTION IN TOMATO

A Dissertation

by

LORI C. ADAMS-PHILLIPS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2003

Major Subject: Genetics

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ABSTRACT

Molecular Analysis of Ethylene Signal Transduction in Tomato.

(December 2003)

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Co-Chairs of Advisory Committee: Dr. James. J. Giovannoni
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The plant hormone ethylene plays an important role in plant growth, development, and physiology. One of the critical components of the ethylene signal transduction pathway, *ctr1* (constitutive triple response), was identified using a particularly useful seedling screen that takes advantage of the profound effects ethylene has on etiolated seedlings, known as triple response. *CTR1* is one of six Arabidopsis *MAPKKK*s that are related to the Raf kinases, and acts as a negative regulator of ethylene response. In this study, isolation and characterization of a family of *CTR1-like* genes in tomato is reported. Based on amino acid alignments and phylogenetic analysis, the tomato *CTR1-like* (*LeCTR*) genes are more similar to Arabidopsis *CTR1* (*AtCTR1*) than any other *MAPKKK* sequences in the Arabidopsis genome. The capacity of the *LeCTR* genes to function as negative regulators in ethylene signal transduction was tested through complementation of the Arabidopsis *ctr1-8* mutant. Quantitative real-time PCR was carried out to generate an expression profile for the *CTR1-like* gene family during different stages of development marked by increased ethylene biosynthesis,

including fruit ripening. The possibility of a multi-gene family of *CTR1-like* genes in other species besides tomato was examined through mining of EST and genomic sequence databases.

Based on nucleotide and amino acid identity, *At4g24480* is most similar to *AtCTR1* and could potentially represent a *CTR1-like* gene in Arabidopsis. Arabidopsis plants carrying a T-DNA insert in the *At4g24480* locus were examined for abnormal ethylene response phenotypes including sensitivity to other hormones, signal molecules and abiotic stresses. Two mutant alleles, *ctr1-1* and *ctr1-8*, containing mutations that disrupt kinase activity and receptor association, respectively, were examined for sensitivity to these same treatments in an effort to better characterize ethylene hormone and non-hormone interactions. They also served as controls to determine if At4g24480 indeed possessed CTR1-like function.

Arabidopsis and tomato represent species with very distinct fruit ripening/maturation programs. The critical dependence on ethylene for fruit ripening in tomato might have resulted in alteration or modification of the ethylene signal transduction pathway relative to Arabidopsis. Plans to characterize individual functions of the *LeCTR* genes through over-expression and reduced expression in tomato are outlined.

I dedicate this dissertation to my husband, Bryan Phillips,

who was with me through it all and believed in my ability to succeed

even when I thought it wasn't possible.

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CHAPTER I

INTRODUCTION

Plant hormones govern a range of developmental processes in plants and act as signal molecules to elicit responses to internal and external stimuli. The plant hormone ethylene plays an important role in plant growth, development, and physiology including the promotion of seed germination, inhibition of stem and root elongation and leaf expansion, flower formation, root hair development and root nodulation, abscission, senescence and fruit ripening (Abeles *et al*., 1992; Mattoo and Suttle, 1991). The ability of plants to perceive and respond to challenges in their environment is also critical to their survival. Ethylene synthesis can be induced by, and impact responses to, environmental stresses such as wounding, hypoxia and pathogen attack (Abeles *et al*., 1992).

From an agricultural perspective, not only is ethylene a consideration in disease resistance and stress tolerance, proper management of external ethylene plays a large role in postharvest handling procedures for a variety of fruits and vegetables. Just a few of the applications include: stimulation of flowering of pineapples and some flowering bulbs; promotion of fruit ripening; shuck loosening in walnut and pecan; degreening of citrus; and defoliation of cotton (Reid, 2002). It has been estimated that postharvest

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This dissertation follows the style and format of *The Plant Journal*.

losses in fresh fruit and vegetables is 5 to 25% in developed countries and 20 to 50% in developing countries (Kader, 2002). This is most certainly due in part to various un-desirable effects of ethylene such as: promotion of sprouting in potato; isocoumarin formation in carrots; abscission of leaves, flowers and fruits in ornamental plants; accelerated senescence through loss of chlorophyll in spinach, fresh herbs and broccoli; and decreased shelf-life and over-ripening in numerous fruits (Reid, 2002).

In recent years, considerable attention has been placed on the enhancement of the nutritional value of crops as basic nutritional needs for much of the world's population remain unmet (DellaPenna, 1999). Plants provide minerals and vitamins which humans cannot produce including nonessential micronutrients that have been linked to the promotion of good health. Tomato fruits are a rich source of beta carotene, folate, potassium, vitamin C, vitamin E, flavonoids, and lycopene. During the process of fruit ripening, changes in texture, color, flavor and aroma occur in addition to alteration in levels of vitamins and antioxidants (Jimenez *et al*., 2002; Ronen *et al*., 1999).

Various facets of fruit ripening are stimulated by ethylene, though it certainly is not the only contributing component (Vrebalov *et al*., 2002). For example, fruits can be classified into two groups, climacteric and non-climacteric, on the basis of their respiration and ethylene rates. Fruits including tomato, banana, and apple undergo climacteric fruit ripening, characterized by a developmentally regulated, autocatalytic increase in ethylene production and associated rise in respiration. Non-climacteric fruit such as citrus, strawberry and grape, do not exhibit a dramatic change in respiration and ethylene remains at a very low level. Such fruits do not require ethylene for fruit

ripening even though they may respond to ethylene. For instance, ethylene induces mRNA and pigment accumulation in the flavedo of orange and is used extensively in post-harvest practices in the de-greening of citrus. A greater understanding of the contributions of ethylene regarding fruit ripening, including a better understanding of interactions with other hormones and developmental factors, would facilitate the design of specific genetic tools to modify fruit and vegetable crops for enhanced quality, yield and nutritional value.

Ethylene biosynthesis

The synthesis of ethylene begins with methionine and proceeds via S-adenosylmethionine (SAM) to the cyclic, non-amino acid intermediate, aminocylcopropane-1carboxylic acid (ACC) involving a reaction catalyzed by ACC synthase (ACS) (Adams and Yang, 1979) (Figure 1). The by-product of this reaction including the methylthio group is recycled through the Yang cycle (named after S.F. Yang who was instrumental in elucidating this pathway) (Miyazaki and Yang, 1987). Conversion of ACC to ethylene is carried out by ACC oxidase (ACO) (Hamilton *et al*., 1990; Hamilton *et al*., 1991). Interestingly, SAM is also utilized in the synthesis of certain polyamines via SAM decarboxylase (Figure 1). Tomato fruit that are engineered to overexpress SAM decarboxylase produce significantly higher amounts of ethylene providing direct evidence that the level of SAM is not rate limiting for either pathway (Mehta *et al*., 2002).

Both ACC synthase and ACC oxidase are encoded by multi-gene families in

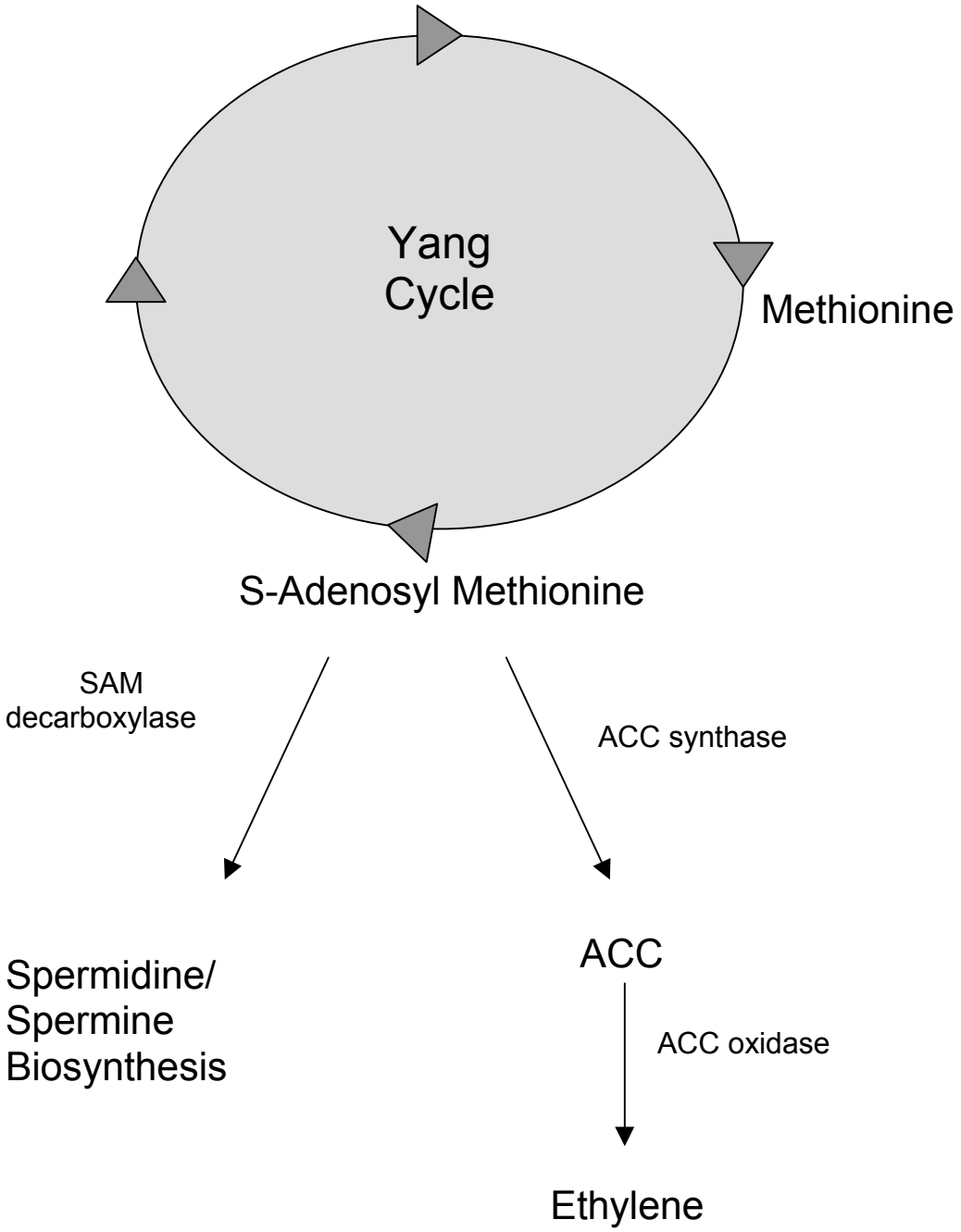


Figure 1. Ethylene biosynthesis pathway.
For simplicity, chemical structures are not shown but can be found in Bleecker and Kende, 2000. The triangles in the Yang Cycle represent the recycled methylthio group.

numerous plant species including *Arabidopsis thaliana*, rice, mung bean, tomato, and carnation (Barry *et al*., 1996; reviewed in Johnson and Ecker, 1998). The expression of ACS and ACO genes is highly regulated, displaying distinct patterns of expression in various tissues at different stages of development and in response to abiotic and biotic stresses (Barry *et al*., 1996; Barry *et al*., 2000). In addition, there is evidence to suggest regulation of these genes can occur beyond the level of gene expression. For example, the LeACS2 protein from tomato is post-translationally modified through phosphorylation in response to wounding (Tatsuki and Mori, 2001). One model in Arabidopsis predicts the binding of a hypothetical inhibitor, possibly encoded by *ETO1* (ethylene overproducer), to *ACS5* (the Arabidopsis gene which corresponds to *LeACS2*) that could prevent activity of ACS5 until it is released through phosphorylation (Wang *et al*., 2002). It is likely, based on conservation of the phosphorylated serine residue, that other ACS genes undergo the same general form of negative regulation which would account for the rapid change (within seconds) in ACS activity in response to wounding, bypassing the requirement for ACS gene transcription (Wang *et al*., 2002).

Signal transduction in Arabidopsis

Much of what is known regarding the steps involved in ethylene perception and signal transduction has been realized through studies of the model plant species *Arabidopsis thaliana*. One of the most valuable mutant screens in Arabidopsis for elucidating mechanisms of hormone signal transduction is based upon alteration of the seedling triple response to ethylene. “Triple response” refers to the morphological changes that

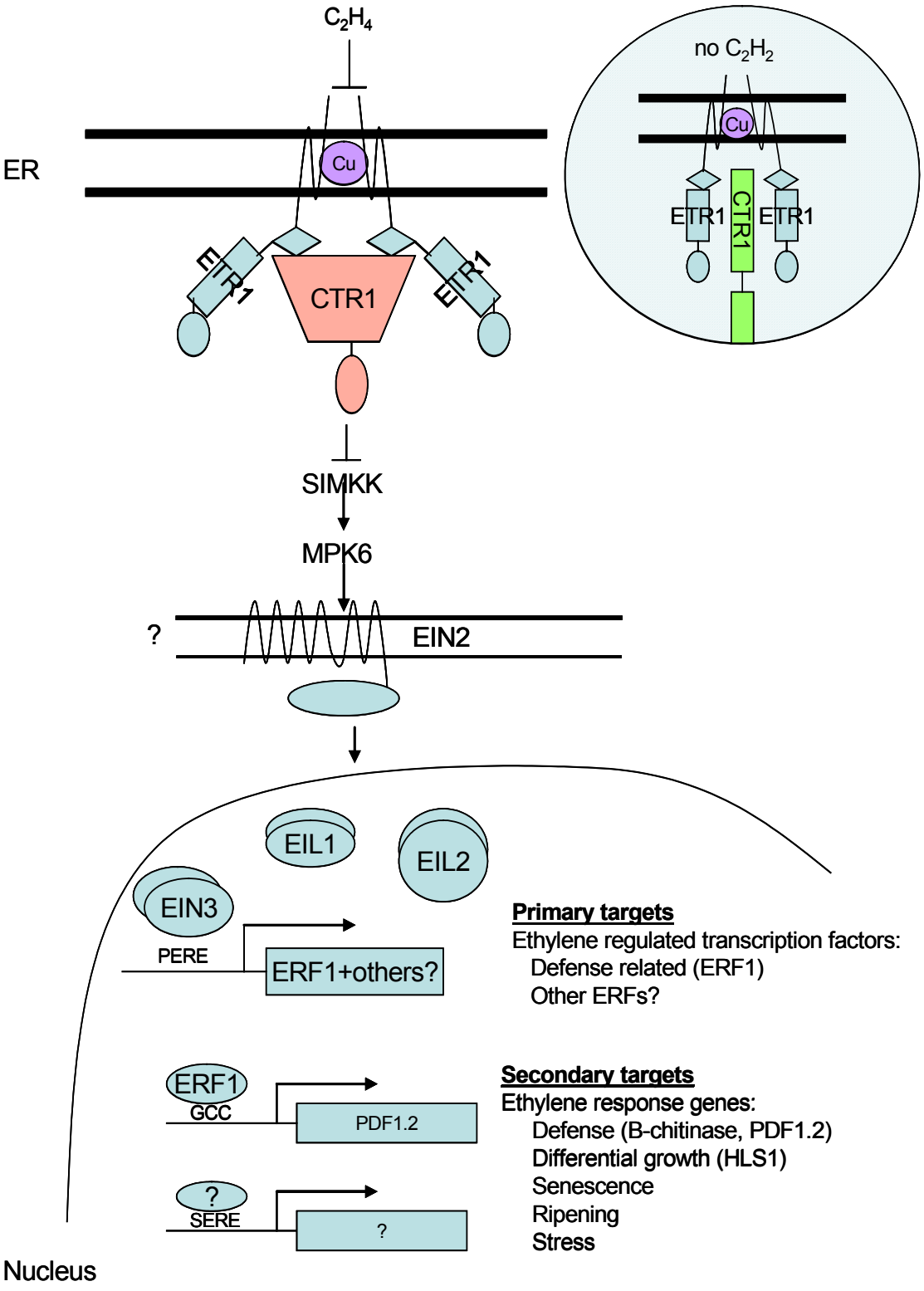
seedlings undergo when they are grown in the dark in the presence of ethylene: exaggerated apical hook formation, inhibition of root and hypocotyl elongation, and swelling of the hypocotyl (Guzman and Ecker, 1990). This screen has been utilized to identify the majority of plant ethylene signal transduction mutants identified to date (Bleecker *et al*., 1988; Ecker, 1995; Kieber, 1997). Specifically, mutants have been isolated based on their sensitivity or insensitivity to the presence or absence of ethylene and many of the corresponding genes have been cloned. The result has been the discovery of various components of the signal transduction pathway from ethylene receptors to downstream transcription factors and emergence of an ordered path of ethylene signaling (Figure 2) (Bleecker and Kende, 2000; Chang and Shockey, 1999; Ecker, 1995; Stepanova and Ecker, 2000).

In Arabidopsis, ethylene is perceived by a family of five ethylene receptors (ETR1, ETR2, ERS1, ERS2, EIN4) with similarity to bacterial two-component histidine kinase receptors (Hua *et al*., 1995; Hua and Meyerowitz, 1998; Sakai *et al*., 1998) (Figures 2 and 3). Bacterial two-component regulators typically consist of a sensor domain which receives signals and a transmitter domain that autophosphorylates on a histidine residue. These functions comprise the first component of two-component systems. The second component contains a response regulator protein with a receiver domain, which receives the phosphate on an aspartate residue from the transmitter, and an output domain, which mediates responses depending on the phosphorylation state of the receiver (Figure 3). In addition to participating in ethylene signal transduction, other proteins in plants resembling two-component proteins have been shown to play roles in

light signaling and cytokinin signaling (Elich and Chory, 1997; Kakimoto, 1996). Strong evidence suggests plant phytochromes evolved from an ancestral histidine kinase, Cph1 from *Synechocystis* (Elich and Chory, 1997; Pepper, 1998). While no ethylene receptor sequences have been found in any of the 70 fully sequenced microbial genomes, several sequences have been retrieved from two cyanobacteria genomes, *Synechocystis* and *Anabaena* (Mount and Chang, 2002). The slr121 protein from *Synechocystis* and two genes from *Anabaena* (*all*0182 and *alr*4715) share homology to the ethylene binding domain and histidine kinase domain found in the ethylene receptors from Arabidopsis (Mount and Chang, 2002). Interestingly, the slr1212 protein is capable of binding ethylene with high affinity (Rodriquez *et al*., 1999). Taken together, this evidence has lead to the conclusion that functional ethylene receptors have been inherited through the plastid lineage.

The ethylene receptors can be divided into two sub-families based on structural similarities: subfamily 1 includes ETR1 and ERS1, subfamily 2 includes ETR2, ERS2 and EIN4 (Bleecker, 1999) (Figure 3). All five of the ethylene receptors contain an N-terminal, membrane–associated sensor domain. This domain shows high-affinity binding to ethylene when expressed in yeast and was shown to form a membrane associated disulfide-linked dimer (Hall *et al*., 2000; Schaller and Bleecker 1995; Schaller *et al*., 1995). Ethylene binding also appears to be mediated through a copper cofactor delivered by RAN1 (Hirayama *et al*., 1999; Rodriquez *et al*., 1999) (Figure 2). The residues thought to be essential for histidine kinase activity are not completely conserved in subfamily II receptors begging the question for the role of histidine kinase activity in

Figure 2. Ethylene signal transduction pathway as defined in Arabidopsis.
Binding of ethylene to members of the receptor family (here represented by ETR1) is mediated by a single copper ion (Cu), delivered by RAN1 (not shown). Ethylene negatively regulates the signal transduction pathway upon binding the receptor, possibly through a conformational change in CTR1 that reduces its kinase activity (shown in red). Conversely, when there is no ethylene, CTR1 is active (shown to the right, in green) and can repress downstream ethylene responses. Upon inactivation of CTR1, SIMKK is relieved from inhibition and activates ethylene signaling through a cascade to downstream components including EIN2 and EIN3/EILs. EIN3/EILs initiate a transcription factor cascade through activation of primary transcription factors (ERF1) which in turn activate secondary ethylene response target genes.


C_2H_4
ER
Cu
ETR1
CTR1
ETR1
SIMKK
MPK6
?
EIN2
EIL1
EIL2
EIN3
PERE
ERF1+others?
ERF1
GCC
PDF1.2
?
SERE
?
Nucleus
no C_2H_2
Cu
ETR1
CTR1
ETR1
Primary targets
Ethylene regulated transcription factors:
Defense related (ERF1)
Other ERFs?
Secondary targets
Ethylene response genes:
Defense (B-chitinase, PDF1.2)
Differential growth (HLS1)
Senescence
Ripening
Stress

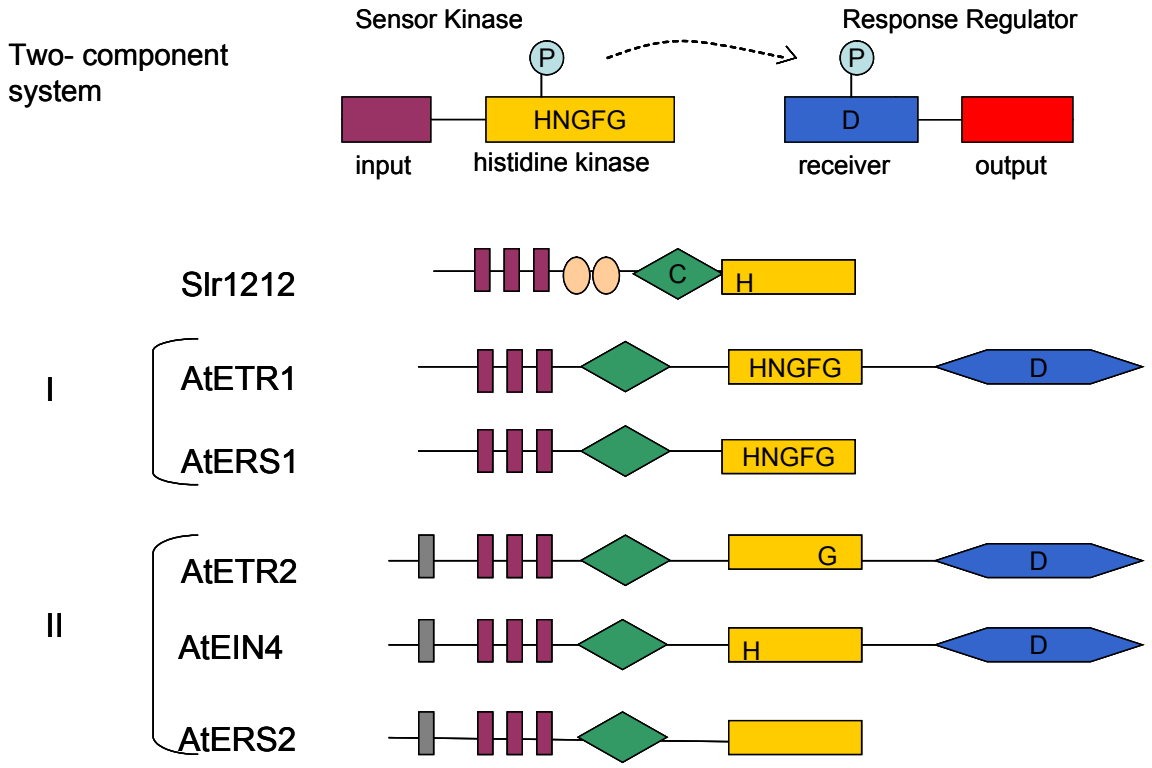


Figure 3. Similarity of ethylene receptors to bacterial two-component regulators.
Shown at the top is a schematic of the prototypical two-component system in bacteria. Underneath are diagrams of the ethylene receptors found in *Synechosystis* (Slr121) and Arabidopsis (shown grouped together by family). The three purple rectangles correspond to the ethylene binding domain; the gray rectangles represent a hydrophobic N-terminal extension characteristic of Subfamily 2 receptors whose function is not well understood. The GAF domain is shown as a green diamond, the histidine kinase including the 5 conserved domains found in functional histidine kinases (HNGFG) is shown in yellow, and the receiver domain including the aspartate residue (D) is shown in blue. Pink circles represent PAS domains and the C inside the GAF domain represents a GAF-like chromophore-binding domain.

receptor signaling. Indeed, it has been recently demonstrated through rescue of *ers1*; *etr1* double mutants with a histidine kinase-inactivated form of ETR1 that histidine kinase phosphotransfer is not required for receptor signal transmission (Wang *et al*., 2003). While dominant gain-of-function mutations in the ethylene receptors confer ethylene insensitivity, double, triple and quadruple loss-of-function mutants in these genes result in constitutive ethylene response phenotypes indicating their function as redundant negative regulators of ethylene signaling (Hua and Meyerowitz, 1998; Wang *et al*., 2003).

Downstream of the receptors is CTR1, which acts as a negative regulator of ethylene responses (Figure 2). Loss-of-function mutations in *CTR1* result in global constitutive activation of all ethylene responses examined including: constitutive seedling triple response, delayed opening of the apical hook and expansion of cotyledons in light, smaller adult rosette leaves and inflorescences, delayed bolting, infertile flowers, less-extensive root system, reduction in epidermal leaf cell size, and constitutive activation of basic-chitinase (an ethylene-regulated pathogenesis-related gene) (Kieber *et al*., 1993). Only one gene with CTR1 function has been isolated to date in Arabidopsis and tests for epistasis with available receptor mutants suggest the product of this single gene is involved in signaling from all members of the receptor family (Hua and Meyerowitz, 1998). CTR1 has been shown to possess intrinsic serine/threonine protein kinase activity, which is required to suppress ethylene responses (Huang *et al*., 2003).

Insights into the mechanism of action for receptor to CTR1 signaling reveal several lines of evidence suggesting CTR1 interacts directly with the receptors as part of

a signaling complex (Figure 3). The N-terminal domain of CTR1 was shown to associate physically with subfamily 1 receptors ETR1 and ERS1 and the subfamily 2 receptor ETR2 via yeast two-hybrid analysis (Cancel and Larsen, 2002; Clark *et al*., 1998). More recently, CTR1 was found to localize to the endoplasmic reticulum (ER), which coincides with ETR1 localization to the ER (Chen *et al*., 2002; Gao *et al*., 2003). ETR1 was co-purified with CTR1 C-myc tagged protein and site directed mutations in crucial ETR1 histidine kinase residues did not eliminate the interaction between ETR1 and CTR1, indicating that histidine kinase activity was not required for interaction (Gao *et al*., 2003). A mutation in *ctr1-8* that alters a conserved glycine residue found in a conserved motif of the CTR1 protein apparently disrupts interaction of CTR1 with the receptor (since this mutation does not disrupt the kinase activity but still results in constitutive activation of ethylene responses) (Huang *et al*., 2003). *ctr1-1* and *ctr1-4* proteins harboring mutations that disrupt the kinase activity still associate with the ER membrane while *ctr1-8* protein does not, suggesting that though kinase activity of CTR1 is required, correct sub-cellular localization is also required to repress ethylene responses (Gao *et al*., 2003). Most single loss-of-function receptor mutants had little effect on the level of membrane-associated CTR1 while double and triple mutants substantially reduced the levels of CTR1 bound to the membrane (Gao *et al*., 2003). This corresponds to the physiological effects of these mutants upon ethylene responses, as described earlier. The only exception to this was in the *etr1-7* mutant where 2-4 fold higher levels of CTR1 were recovered in the membrane fraction than in wild-type controls (Gao *et al*., 2003). This mutant is the only single loss-of-function receptor mutant that exhibits a

partial ethylene response phenotype in hypocotyls and shows enhanced sensitivity to ethylene in both hypocotyls and roots (Cancel and Larsen, 2002; Hua and Meyerowitz, 1998). It is possible that ETR1 may play a specific role in activation of CTR1 that cannot be substituted for by other members of the receptor family (Cancel and Larsen, 2002; Gao *et al*., 2003). It should also be noted that the double loss-of-function mutant *etr1*;*ers*, exhibits constitutive ethylene response and can only be rescued by subfamily 1 receptors (and not by subfamily 2 receptors) further supporting the idea that subfamily 1 receptors (of which ETR1 is a member) play a unique and necessary role in ethylene signaling in *Arabidopsis* (Wang *et al*., 2003).

As *CTR1* shows high sequence similarity to members of the *Raf* family of MAPKKKs (Map kinase kinase kinase), it has been speculated that the ethylene signal is propagated through a MAPKKK cascade to downstream targets (Figure 3). It was recently demonstrated that over-expression of SIMKK (an ethylene inducible MAPKK) resulted in a constitutive triple response seedling phenotype and enhanced gene expression of several ethylene-induced genes including *MPK6*, an ethylene-inducible MAPK. In addition, *MPK6* expression was shown to be constitutively activated in *ctr1* mutants suggesting a role for this gene in addition to *SIMKK* in ethylene signaling (Ouaked *et al*., 2003).

Epistasis analysis places EIN2 downstream of CTR1 in the ethylene signaling pathway (Figure 3) (Roman *et al*., 1995). EIN2 encodes a protein with 12 putative transmembrane domains with similarity to the Nramp family of metal ion carriers in the amino terminal portion of the protein (Alonso *et al*., 1999). The carboxy terminus is

novel except for a coiled-coil motif predicted to be involved in protein: protein interactions. Over-expression of the carboxy terminal but not full length EIN2 resulted in constitutive activation of ethylene phenotypes in *ein2* mutant plants, suggesting a role for the N-terminal domain in regulating EIN2 response to ethylene.

EIN2 operates upstream of EIN3 and the EIL (EIN3-like) family of nuclear localized proteins in ethylene signaling (Chao *et al*.,1997; Solano *et al*., 1998) (Figure 3). EIN3 appears to be a global regulator of ethylene responses as overexpression of EIN3 or EIL1 in wild-type or *ein2* mutants confers constitutive ethylene response in all stages of development (Chao *et al*., 1997). Homodimers of EIN3, EIL1 and EIL2 are able to bind to a defined target site designated as a primary ethylene response element (PERE) in the promoter region of the transcription factor, ERF-1 (Ethylene-Response-Factor1) (Solano *et al*., 1998).

ERF1 is important in the regulation of ethylene response genes including *B-chitinase* and *PDF1.2* (Figure 3). *ERF1* is part of a large multigene family in Arabidopsis and genes encoding ERF (also known as EREBP) proteins have been found in both dicots and monocots but not in yeast or other fungi (Ohme-Takagi *et al*., 2000). These ERFs bind the 'GCC' box found in promoters of ethylene and pathogen-inducible genes (Ohme-Tagaki and Shinishi, 1995). Five ERF genes were isolated from Arabidopsis and shown to respond differentially to ethylene, wounding, cold, high salinity and drought (Fujimoto *et al*., 2000). Functional analysis revealed that the ERF genes could act as both activators and repressors of GCC box-dependent transcription (Fujimoto *et al*., 2000) which adds a further level of complexity. However, proof that

these ERFs other than ERF1 function in the ethylene response pathway awaits further experimental evidence.

Not all ethylene-inducible GCC box containing genes are activated by ERF1 since it was shown that even though *HOOKLESS1* (*HLS1*) contains a GCC box, it is not induced in ERF1 over-expressing plants (Solano *et al*., 1998). This activation of a subset of responses is exhibited at the phenotypic level as well, as etiolated seedlings that overexpress ERF1 show inhibition of hypocotyl and root cell elongation, but lack an exaggerated apical hook (Solano *et al*., 1998). The GCC box motif is not found in regulatory regions of fruit ripening genes and flower petal senescence genes (Ohme-Tagaki *et al*., 2000). Thus, it is likely that the GCC box motif may represent a secondary ethylene response element (SERE) bound to by a subset of ERFs while distinct cis-elements are likely to be involved in regulation of other ethylene-associated processes such as fruit ripening and senescence (Figure 3).

Identification of other targets of EIN3 and ERF1 will facilitate answering the seminal question of how the ethylene signal is interpreted and channeled in order to produce an appropriate ethylene response. In this regard, further genetic screens will be necessary in order to identify additional ethylene signal transduction components. For example, applying a variation on the "classical" triple response screen using low doses of ethylene, two weak ethylene insensitive (*wei*) mutants were recently discovered. *wei2* and *wei3* do not map to other previously known ethylene-related mutants/genes and epistasis analysis places them downstream in the pathway from CTR1 (Alonso *et al*., 2003*b*). Phenotypes displayed by *wei2* and *wei3* are restricted to the roots and while this

is characteristic of mutants also affected in the response to auxin (Lehman *et al*., 1996; Roman *et al*., 1995), these two mutants show normal sensitivity to auxin (Alonso *et al*., 2003*b*). Since *wei2* and *wei3* are downstream mutants specifically altered in their response to ethylene, it is possible that they could function at steps connecting the general ethylene-response pathway to the process of auxin-mediated growth.

Cross-talk with other hormones and signaling molecules

One way in which multiple hormones interact to modulate plant development is through induction of biosynthesis of one hormone by another or through post-transcriptional/translational modification of the genes involved in biosynthesis. This is most certainly the case regarding the regulation of ethylene biosynthesis as evidence for induction of ACC synthase gene expression by application of another hormone is abundant in the literature. Several representative examples include: auxin regulation of ethylene biosynthesis through induction of *ACS4* in Arabidopsis and *ACS1* and *ACS2* in pea (Abel *et al*., 1995; Peck and Kende, 1998), cytokinin elevation of ethylene biosynthesis through post-transcriptional modification of ACS5 (Vogel *et al*., 1998), and brassinosteriod enhanced *ACS7* gene expression in mung bean (Yi *et al*., 1999).

Interactions between ethylene and other plant hormones are also being uncovered as mutations that were initially identified for alterations in response to one hormone often turn out to influence the sensitivity to another hormone or signaling molecule. For example, the *eir1* (*ethylene insensitive roots*) mutant that shows ethylene-insensitivity only in the roots (Roman *et al*., 1995) turned out to have a defect in an auxin transport

protein (Luschnig *et al*., 1998). Likewise, the expression of the *HLS1* (*hookless1*) gene is regulated by ethylene but encodes a putative acetyltransferase that presumably controls auxin transport (Lehman *et al*., 1996). New *ein2* mutant alleles have turned up in screens for resistance to inhibition of auxin inhibitors (Fujita and Syono, 1996) and resistance to low levels of cytokinin (Cary *et al*., 1995). Additionally, new mutant *ctr1* and *ein2* alleles were recovered in screens for enhancers and suppressors, respectively, of the ABA-resistant seed germination mutant *abi1-1* (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000). Unexpectedly, while *ein2* showed increased seed ABA responsiveness, it exhibited reduced ABA responsiveness in the roots (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000). Screens for sucrose sensitivity resulted in identification of a sugar-insensitive mutant (*sis1)* which was found to be allelic to *ctr1* (Gibson *et al*., 2001) and characterization of the glucose-insensitive mutant *gin1* revealed that this mutant could be phenocopied in wild-type plants through application of exogenous ethylene (Zhou *et al*., 1998). It cannot be ruled out that abnormal ethylene sensitivity indirectly results in the phenomena observed in these hormone and sugar sensitivity assays. However, one example of how two separate linear signal transduction pathways could be communicating at the molecular level is illustrated below.

Using *ein2* and *coi1* mutants deficient in ethylene and jasmonate responses, respectively, it was shown that activation of both ethylene and jasmonate pathways is required for induction of the plant defensin gene *PDF1.2* in Arabidopsis and that these hormones act synergistically to induce *PDF1.2* expression (Penninckx, *et al*., 1998). More recently, it has been shown that ERF1, a likely activator of PDF1.2, acts as a

downstream component in both ethylene and jasmonate signaling pathways (Lorenzo *et al*., 2003). Not only is *ERF1* expression upregulated by both jasmonate and ethylene, ERF1 over-expression is sufficient to restore *PDF1.2* expression in *coi1* mutants.

There appear to be a multitude of positive and negative interactions between different plant hormones and ethylene depending on the tissue and developmental stage of the plant. Thus, the type of response to a given stress or developmental event will likely depend upon the positive or negative interaction that is established between ethylene and other hormonal signaling pathways. In establishing where "cross-talk" actually exists, it will be important to determine that the components of the two signaling pathways are expressed in the same cell and physically interact under normal physiological conditions (Wang *et al*., 2002).

Fruit ripening

Ripening of fleshy fruit is a highly coordinated process influenced by light, hormones, temperature and genotype. This process involves changes in color, aroma, texture, and flavor to produce a fruit that is attractive to seed dispersing organisms. Dehiscent (or non-fleshly) fruit such as the silique from Arabidopsis rely heavily on elements of the environment to aid in seed dispersal. The overall goal is the same in both cases and there are most certainly common regulatory mechanisms underlying maturation of both types of fruit (Ferrandiz *et al*., 1999; Giovannoni, 2001). Nevertheless, we can only fully understand similarities and differences that exist as a result of these specific mechanisms that have evolved to aid in seed dispersal, through careful analysis of both

systems of fruit maturation. Ripening of fleshy fruits has received considerable attention due the large component of the human diet it represents and the research presented here focuses on ripening of fleshy fruit using tomato as a model system.

Tomato as a model system

Tomato is an ideal model system for studying fruit ripening in climacteric fruits in particular. In practical terms, not only does tomato represent an important crop species, it also is diploid, has a relatively small genome (0.9pg/haploid genome), is self-pollinating although manual cross-hybridization is efficient, can be grown year-round in greenhouses, and has a fairly short generation period (~45-100 days depending on variety and season). Years of breeding have resulted in the collection of a valuable germplasm resource representing genes that influence various aspects of fruit development and ripening (see Giovannoni, 2001 and references therein). A series of introgression lines of a wild tomato species (*Lycopersicon pennellii*) into cultivated tomato (*L. esculentum*) have been developed resulting in 76 lines spanning the tomato genome (Eshed and Zamir, 1994). A host of molecular tools have been developed to facilitate positional cloning including YAC and BAC libraries (see Giovannoni, 2001 and references therein). A map has been generated with nearly 2000 markers in place encompassing the genome, which is continually being updated and can be viewed on the *Solanaceae* Genome Network website (http://www.sgn.cornell.edu) (Tanskley *et al*., 1992). There also are many resources available at the level of gene expression. The National Science Foundation sponsored development of a tomato EST database resulting in the creation of 23 cDNA libraries from various tissues, followed by single-pass 5'

sequence of 2,000-10,000 clones from each library which are publicly available through TIGR (The Institute for Genome Research) (http://www.tigr.org/tdb/lgi). Additionally, a tomato cDNA microarray has been developed with the purpose of answering questions about fruit development and ripening (Moore *et al*., 2002). Furthermore, the Tomato Expression Database (http://ted.bti.cornell.edu) has been initiated which allows public accessibility to normalized and replicated microarray data with links to view expression data for an individual gene or larger data-set (Fei and Giovannoni, unpublished). Data obtained from tomato should prove useful for making inferences and developing hypotheses relative to other members of the *Solanaceae* including eggplant, pepper, tobacco, petunia and potato. Comparative mapping studies indicate that conservation in gene content and order exists to varying degrees within the *Solanaceae*. Potato and tomato differ by only 5 chromosomal rearrangements (Tanskley *et al*., 1992) pepper and tomato share conserved linkage blocks and equivalent genic contents (Livingstone *et al*., 1999), and a significant portion of QTL for domestication traits in eggplant are conserved in tomato, pepper, and potato (Donganlar, 2002). In seeking to comprehend the complex process of fruit ripening, current areas of research include the identification of developmental cues which mediate fruit ripening, dissecting the role of ethylene in fruit ripening, and modification of fruit quality and nutrition.

Developmental and non-ethylene mediated control of fruit ripening

Naturally occurring mutants have been instrumental in dissecting ethylene and non-ethylene mediated control of vegetative growth and fruit development (Gray *et al*., 1994). One of the most useful and well-studied fruit ripening mutants is *ripening*-

inhibitor (*rin*). *rin* fruit do not undergo the usual increase in respiration and ethylene production during normal ripening, carotenoid accumulation is delayed and greatly reduced as the transition from chloroplasts to chromoplats is protracted and unsynchronized, and fruit softening is also inhibited (Tigchelaar *et al*., 1978). *rin* does appear to be sensitive to ethylene in dark-grown seedlings (Lanahan *et al*., 1994), and during the processes of floral abscission, and petal and leaf senescence (Vrebalov *et al*., 2002). Additionally, mutant fruit possess the capability to produce ethylene similar to wild-type fruit in response to wounding or blossom end rot (Tigchelaar *et al*., 1978) indicating that *rin* represents a fruit specific ripening defect. While *rin* fruit do not ripen in response to exogenous ethylene, induction of some ethylene-responsive genes occurs (Lincoln and Fisher, 1988). Taken together, these results indicate that RIN is likely to act in ethylene-independent regulatory cascades during early stages of fruit ripening. *RIN* was eventually cloned and sequence identity reveals that it is a member of the MADS-box family of transcriptional regulators (Vrebalov *et al*., 2002). Plant MADS-box genes are usually associated with floral development in Arabidopsis and isolation of *RIN* (designated hereafter as *LeMADS-RIN*) has revealed a novel function for plant MADS-box genes in fruit development. In addition to developmental factors, other hormones in addition to ethylene such as auxin, brassinosteroid, and cytokinin are likely to influence ripening though they are less-well characterized in this regard (Cohen, 1996; Martineau *et al*., 1994; Vidya and Rao, 2002).

Role of ethylene in fruit ripening

Economically important fruits such as tomato, apple, pear, melon, squash, peach, avocado, and many other so called "climacteric" fruit show increased synthesis and dependence upon ethylene for induction and completion of fruit ripening. Ethylene has indeed been shown to regulate expression of numerous genes related to ripening including: ACC synthases (Barry *et al*., 2000) and oxidases (Barry *et al*., 1996); *E4* (methionine sulphoxide reductase) and *E8* (dioxygenase) (Lincoln *et al*., 1987); *PSY* (phytoene synthase) (Bird *et al*., 1991); and *Tomlox A*, *B*, *C* (lipoxygenases), *PG* (polygalacturonse), and *LeEXP1* (expansin) (see Alexander and Grierson. 2002 and references therein; Zegzouti *et al*., 1999). The physiological importance of ethylene for fruit ripening has been demonstrated through analysis of tomato plants altered in their expression of genes involved in ethylene biosynthesis and perception, resulting in the inhibition of ripening and other ethylene associated responses (Klee, 1993; Lanahan *et al*., 1994; Oeller *et al*., 1991).

It has been proposed that there are two systems which operate to regulate ethylene production in plants (Barry *et al*., 2000; reviewed in Lelievre *et al*., 1997). System I is ethylene auto-inhibitory, functions during normal vegetative growth, and is responsible for basal levels of ethylene present in all tissues. In System 2, ethylene is auto-stimulatory and operates in climacteric fruit and during petal senescence. Analysis of gene expression of members of the ACC synthase gene family in both the *rin* mutant and wild-type fruit has culminated in the model where System 1 ethylene is regulated by as yet unknown developmental pathway components through expression of *LeACS1A*

and *LeACS6* (Barry *et al*., 2000) (Figure 4). In fruit, transition from System 1 and System 2 is mediated by LeMADS-RIN which represents a key developmental signal indicating that the fruit has reached competency to ripen. During this transition period, combined induction of *LeACS1A* and *LeACS4* leads to the induction of *LeACS2* and autocatalytic ethylene production, defining System 2 (Figure 4). This model helps to explain why immature fruit do not ripen in response to exogenously applied ethylene even though the fruit induce a subset of ethylene inducible ACO genes, indicating that though they are able to perceive ethylene at that stage they are not competent to ripen (Lelievre *et al*., 1997).

In tomato, a number of ethylene signal transduction components homologous to those identified in Arabidopsis have been identified, some of which influence ripening. *Nr* (*Never-ripe*) is a naturally occurring semi-dominant mutant with fruits that do not fully ripen. In addition to delayed fruit ripening, *Nr* shows insensitivity to ethylene in the triple response and at the adult stage in leaves, flowers and abscission zones (Lanahan *et al*., 1994). *NR* was cloned and encodes a protein with homology to *ETR1* from Arabidopsis (Wilkinson *et al*., 1995; Yen *et al*., 1995). The *Nr* mutant contains a mutation in the ethylene binding site conferring ethylene insensitivity. The expression of *NR* mRNA is up-regulated during fruit ripening, flower senescence and abscission (Payton *et al*., 1996). Antisense repression of NR results in normal ripening fruit which suggests that while *NR* expression increases coincident with ripening, it does not appear to essential in the control of fruit ripening (Tieman *et al*., 2000). Since the cloning of *NR*, five additional ethylene receptors have been isolated from tomato (Klee

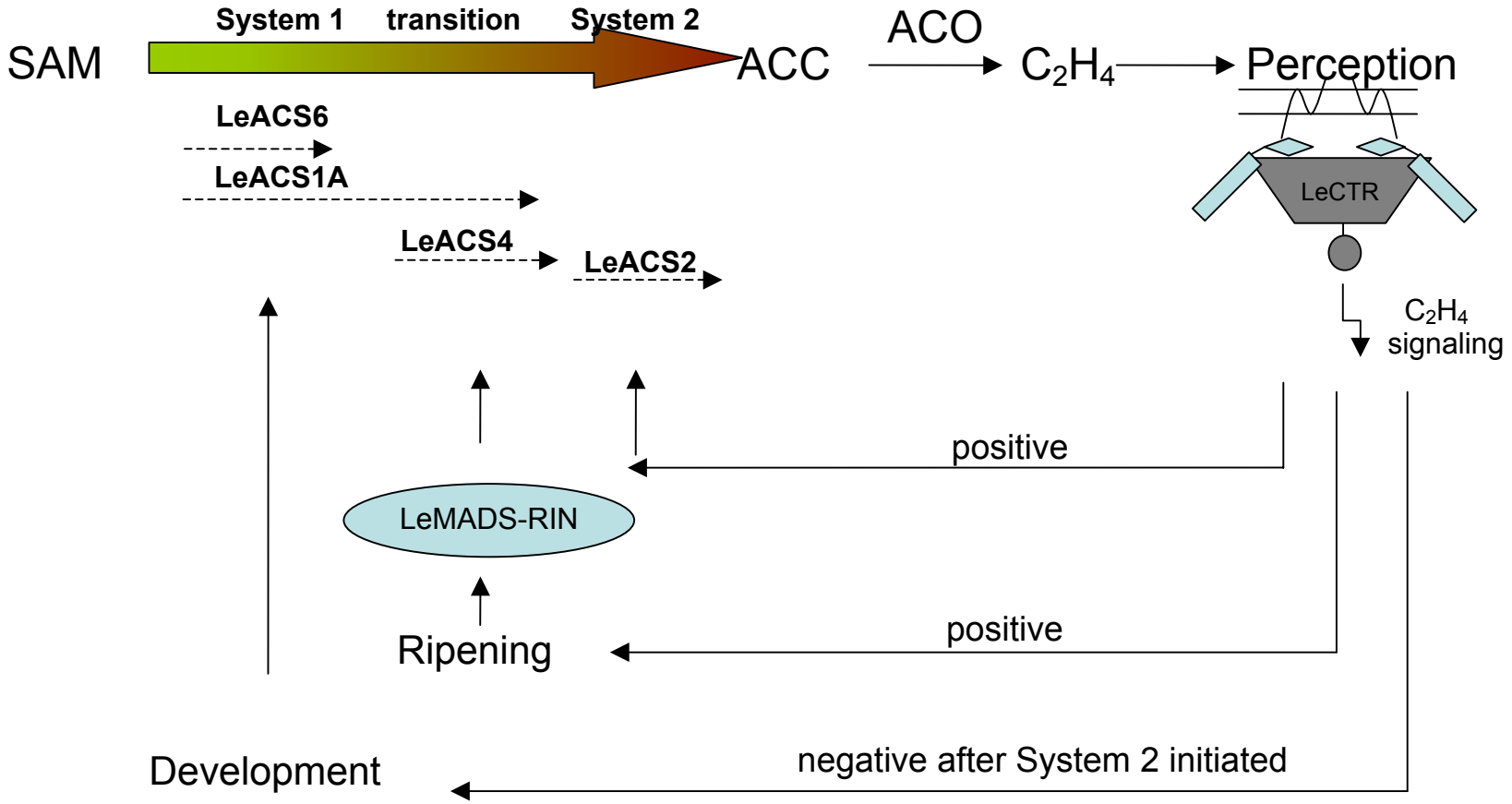


Figure 4. Model for ethylene biosynthesis regulation during fruit ripening.
System 1 ethylene is regulated through expression of *LeACS1A* and *LeACS6*. Transition from System 1 and System 2 is mediated by LeMADS-RIN. Combined induction of *LeACS1A* and *LeACS4* leads to the induction of *LeACS2* and autocatalytic ethylene production, defining System 2. Both positive and negative regulation of the ACS genes by ethylene is also indicated in this model. (based on the model presented in Barry *et al*., 2000)

2002; Lashbrook *et al*., 1998; Tieman and Klee, 1999; Zhou *et al*., 1996). Downstream of the receptors, three tomato *LeEIL* (*Ein3-like*) genes have been isolated (Tieman and Klee, 2001). These three tomato genes were shown to be functionally redundant and regulate multiple ethylene responses throughout plant development (Tieman and Klee, 2001). It appears most of the signaling components identified to date are global regulators of the ethylene response, thus the question of how specific responses occur in response to ethylene in tomato still remains unclear.

Modification of fruit quality and nutrition

Areas of research into fruit quality include modification of the fruit in terms of sugars and acids, flavor volatiles, cell wall softening, and color development, in addition to prevention of post-harvest degradation due to wounding and increased susceptibility to pathogens. Lately, there has been an increased interest in nutrient modification. While public regard of genetically modified crops has been lukewarm, it is possible that through promotion of the health benefits of modified fruits and vegetables, transgenic crops would gain greater acceptance by the public as the direct benefit is aimed more towards the consumer rather than the producer (Giovannoni, 2001; Grusak, 2002).

Lycopene content manipulation has received considerable attention, prompted in part by a study conducted by Giovannucci *et al*. in 1995 that uncovered a correlation between lycopene consumption and a decreased rate of prostate cancer. Since then, numerous studies have been undertaken that report the beneficial effects of lycopene (Minorsky, 2002). Tomatoes are a primary source of lycopene as it accumulates during ripening and account for >80% of the total lycopene intake of Americans (Minorsky,

2002). There appear to be many factors, including non-ethylene ones that mediate lycopene production. For example, ethylene induces phytoene synthase, an enzyme necessary for lycopene synthesis (Bird *et al*., 1991). However, fruit-localized phytochromes have been shown to regulate lycopene accumulation independently of ethylene biosynthesis (Alba *et al*., 2000). Unexpectedly, increased levels of polyamines resulted in the substantial increase in lycopene content exceeding that achieved through conventional methods thus far (Mehta *et al*., 2002). This represents a clear example of how the interplay of various factors can mediate one process and illustrates the complexity of fruit ripening.

The aim of this dissertation is to gain a better understanding of how climacteric fruit use ethylene to regulate ripening. Specifically, examination of the function and regulation of key regulatory components in ethylene signal transduction pathway will facilitate our understanding of the basic biological foundation by which climacteric fruit perceive and transduce the ethylene signal. As our understanding of the overall biology of fruit ripening improves, so will the ability to improve the quality and nutritional value of fruit through traditional or non-traditional means.

CHAPTER II

EVIDENCE THAT CTR1-MEDIATED ETHYLENE SIGNAL TRANSDUCTION IN TOMATO IS ENCODED BY A MULTIGENE FAMILY WHOSE MEMBERS DISPLAY DISTINCT REGULATORY FEATURES

Introduction

The model for ethylene signal transduction defined in Arabidopsis (Figure 2) and the associated gene and mutant resources have permitted comparative genomic and functional analyses in additional species, including important crops where the role of ethylene has important practical consequences. In some instances, the diversity of developmental and response programs may have been facilitated in evolution through modification of ethylene signaling components and/or their regulation. For example, in tomato a number of ethylene signal transduction components homologous to those identified in Arabidopsis have been identified and characterized. Six ethylene receptors have been isolated (Klee, 2002; Lashbrook *et al*., 1998; Tieman and Klee, 1999; Wilkinson *et al*., 1995; Zhou *et al*., 1996), five of which have been shown to bind ethylene (Klee 2002). Three of these are subfamily I receptors (LeETR1, LeETR2, and NR) while the remainder (LeETR4, LeETR5, and LeETR6) resemble subfamily 2 receptors (Bleecker, 1999). Each tomato receptor gene has a distinct pattern of expression throughout development (including a subset induced during ripening) and in

response to external ethylene and pathogens (reviewed by Klee and Tieman, 2002). For instance, *NR* and *LeETR4* gene expression is induced during fleshy fruit ripening (a developmental program non-existent in Arabidopsis) and further exhibit functional compensation indicating *in vivo* redundancy (Tieman *et al*., 2000). Three tomato *LeEIL* (*Ein3*-like) genes have also been isolated and were shown to be functionally redundant and to regulate multiple ethylene responses throughout plant development (Tieman *et al*., 2001).

A *CTR1-like* gene (*LeCTR1*) was isolated from tomato and shown through complementation of a *ctr1* Arabidopsis mutant to function in ethylene signaling (LeClerq *et al*., 2002). *LeCTR1* mRNA is upregulated by ethylene during fruit ripening (Giovannoni *et al*., 1998; LeClerq *et al*., 2002; Zegzouti *et al*., 1999), and as shown here, is part of a multigene family whose members possess CTR1 function and display differential gene expression. In contrast, in Arabidopsis only one *CTR1-like* gene has been implicated in ethylene signaling and its mRNA is constitutively expressed (Kieber *et al*. 1993). I present here the first experimental evidence of a multigene family of plant *CTR1-like* genes that are able to participate in ethylene signal transduction. The family is differentially regulated by ethylene and stages of development marked by increased ethylene biosynthesis, including fruit ripening. The presence of a multigene family of functional *CTR1* genes is not limited to tomato and the possibility of *CTR1-like* gene loss in Arabidopsis was examined. These results suggest that regulation of ethylene signal transduction machinery has been a target for selective pressure.

Results

Cloning of the tomato CTR1 gene family

To explore the complexity of *CTR1* sequences in tomato, the *LeCTR1* cDNA (Giovannoni *et al*. 1998) was used to screen an ordered tomato (cultivar Ailsa Craig) callus cDNA library (150,000 primary recombinants). This screen resulted in the recovery of two *LeCTR* cDNA sequences similar to yet distinct from the original *LeCTR1* cDNA and designated *LeCTR3* and *LeCTR4*, as well as additional clones corresponding to *LeCTR1*. Two apparent splice variants of *LeCTR4*, referred to hereafter as *LeCTR4sv1* and *LeCTR4sv2*, were also recovered from this screen. The predicted coding sequences of the *LeCTR4* isoforms vary as a result of differential processing of one exon (Figure 5). Specifically, a stop codon is introduced into the predicted coding sequence as a result of the splicing of the 6th intron in *LeCTR4sv1*. Both *LeCTR4sv1* and *LeCTR4sv2* have identical 3' UTR sequences and additionally share 67 bp of identical 3' UTR sequence with *LeCTR4* directly after the predicted stop codon of *LeCTR4*. The *LeCTR4sv1/2* 3' UTR sequence differs dramatically from *LeCTR4* downstream of this initial 67 bp (222 and 206 bp of 3'UTR for *LeCTR4* and the splice variants, respectively).

Predicted structural features of tomato CTR1 proteins

The *LeCTR3* cDNA contains 3,371 bp and translation of the largest open reading frame predicts a protein of 837 amino acids with a molecular mass of 92kD. There are 2,935 bp in the *LeCTR4* cDNA encoding a predicted protein of 793 amino acids with a molecular mass of 88.5 kD. LeCTR3 and LeCTR4 share 66 and 70% amino acid identity with the

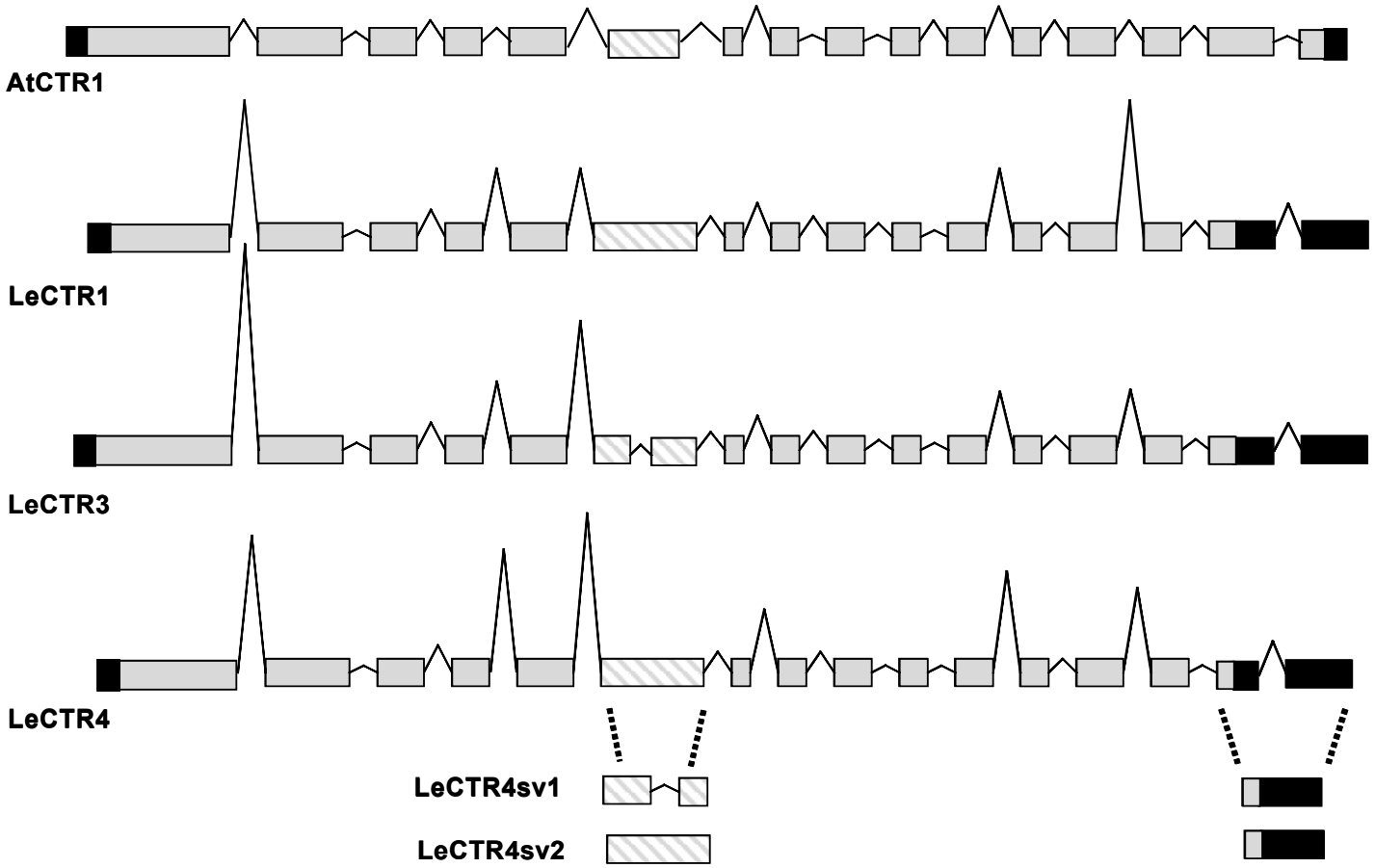


Figure 5. Comparison of the genomic structures of Arabidopsis *CTR1* (L08790) and *LeCTR1* (AY079028) to *LeCTR3* (AY382679), and *LeCTR4* (AY382677).

Exons are depicted as boxes and introns as variable sized wedges in proportion to the size of the intron. Regions upstream of the start codon and downstream of the stop codon are represented as black boxes. Exon 6 (with reference to Arabidopsis) is shown cross-hatched for each sequence. The dotted lines stemming from *LeCTR4* indicate portions of *LeCTR4* which are differentially spliced in transcripts designated *LeCTR4sv1* and *LeCTR4sv2*.

LeCTR1 protein sequence, respectively. Among all four *LeCTR-like* cDNAs identified to date (i.e. those described here and the *AtEDR1*-like *LeCTR2* reported by Lin *et al*., 1998), LeCTR3 shares the highest percent amino acid identity with AtCTR1 in both N-terminal (variable) and conserved C-terminal protein kinase domains (Table 1).

Table1. Percent amino acid identity for LeCTR sequences.

AtCTR1	LeCTR1	LeCTR2	LeCTR3	LeCTR4
N-terminal domain	**50.0**	**22.0**	**57.4**	**48.9**
Kinase domain	**84.0**	**59.5**	**87.7**	**83.4**
Full length cDNA	**60.7**	**32.4**	**67.2**	**59.6**

Percent amino acid identity that each of the four *LeCTR* cDNAs share with AtCTR1 in the N-terminal domain, kinase domain and across the full predicted amino acid sequence.

Within their respective kinase domains, LeCTR1, LeCTR2, LeCTR3 and LeCTR4 have a protein kinase ATP-binding site signature (IGAGSFGTVH) found in all protein kinases (Schenk and Snaar-Jagalska, 1999) as well as a serine/threonine protein kinase active site signature (IVHRDLKSPNLLV) found in serine/threonine kinases including Raf and AtCTR1 (Kieber *et al*., 1993). The 11 subdomains common to all known protein kinases (Hanks and Quinn, 1991; Hanks *et al*., 1988) are also perfectly conserved in LeCTR1, LeCTR2, LeCTR3 and LeCTR4. All of these aforementioned domains are conserved in the LeCTR4 splice variant, LeCTR4sv2. However, the stop

codon in the LeCTR4sv1 predicted peptide sequence occurs just before the kinase domain, thus the kinase domain would not exist in this isoform if it is successfully translated. The N-terminal domain of the predicted tomato and Arabidopsis CTR1 proteins, though more variable (Table 1), also possess a number of interesting structural features conserved to varying degrees among the various sequences. For example, LeCTR3 has an ATP/GTP binding site motif A (P-loop; [AG]-x(4)-G-K-[ST]) at amino acid residues 49-56 and proposed to be involved in binding ATP or GTP in Ras and other proteins (Saraste *et al*., 1990). This motif is also found in AtCTR1 but not in LeCTR1, LeCTR2 or LeCTR4. Additionally, LeCTR1, LeCTR2, LeCTR3, LeCTR4 and the LeCTR4 splice variants demonstrate conservation of the CN box, found in the N-terminal domain of AtCTR1 and other proteins with domains showing high homology to the CTR1 kinase domain (Huang *et al*., 2003).

AtCTR1 is one of six Arabidopsis *MAPKKK*s belonging to subclass B3 of group B *MAPKKK*s, which are related to the Raf kinases and have extended N-terminal domains (Ichimura *et al*., 2002). Surprisingly, phylogenetic analysis of the four LeCTR predicted peptide sequences, the six Arabidopsis sequences and several homologs from rice, barley and rose, indicated that *AtCTR1* is more similar to *LeCTR1*, *LeCTR3* and *LeCTR4* than to any of the other five members of the Arabidopsis MAPKKK subfamily (Figure 6). Based on amino acid identity and phylogenetic analysis, *LeCTR2* appeared to be more similar to *AtEDR1*, a *MAPKKK* involved in plant defense response, than the other *LeCTR* genes as was previously reported (Frye *et al*., 2001).

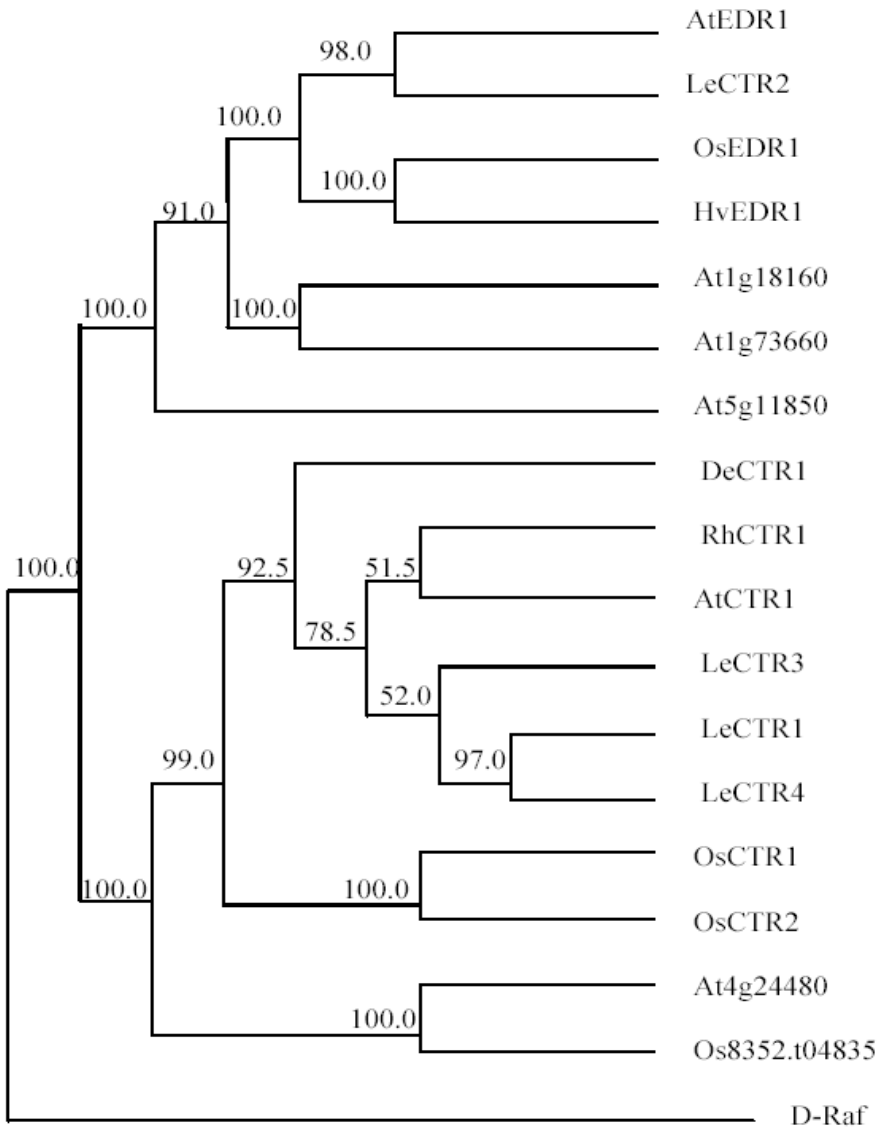


Figure 6. Phylogenetic analysis of tomato (Le), Arabidopsis (At), barley (Hv), rice (Os), *Delphinium* (De), and rose (Rh) reported and putative MAPKKKs.
Full-length amino acid sequences were aligned using ClustalX. The phylogenetic tree was constructed using programs from the Phylip package: the Seqboot program was used to generate a set of 100 bootstrapped sequence alignments, 100 bootstrapped trees were generated using ProtPars and then Consense was used to choose a consensus tree. D-Raf (*Drosophila* Raf) was used as an outgroup. The numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the trees, out of 100 trees.

Structure of the LeCTR gene family

The genomic structure of *LeCTR1* shares with *AtCTR1* conservation of the number, size and position of exons (LeClerq *et al*., 2002). To determine if this conservation in genomic structure was also preserved in *LeCTR3* and *LeCTR4*, genomic sequence information was obtained through screening an arrayed tomato genomic cosmid library with gel-purified gene-specific 3' UTR probes. Positive clones were subcloned and the inserts sequenced. Intron and exon positions were established through comparison of genomic to cDNA sequence. Structural analysis revealed that, similar to *LeCTR1* and *AtCTR1*, the *LeCTR4* coding sequence consisted of 15 exons interrupted by 14 introns while LeCTR3 coding sequence contained 16 exons and 15 introns (Figure 5). In most cases, the size of the introns remained conserved between the members of the *LeCTR* family with several notable exceptions. For example, intron #1 ranges from 2.18 Kb (*LeCTR4*) to 5.7Kb (*LeCTR3*). Intron size was not conserved between the tomato and Arabidopsis CTR1 genomic sequence, and was generally larger in tomato. In contrast, the size and position of exons was conserved between *AtCTR1* and all of the tomato *CTRs* with the exception of the number of amino acids in the first and last exons in addition to an intron in some versions of exon 6 (Figure 5). Exon 6 of *AtCTR1* is only 278 amino acids in length while in *LeCTR1* and *LeCTR4* it is 411 and 423 amino acids, respectively. Genomic sequences for both *LeCTR3* and *LeCTR4sv1* contain an intron that interrupts exon 6. The intron in both *LeCTR3* and *LeCTR4sv1* occurs in a region of the coding sequence after the CN domain and just before the start of the kinase domain where there is little conservation in amino acid sequence among all the *CTRs* (Figure 7),

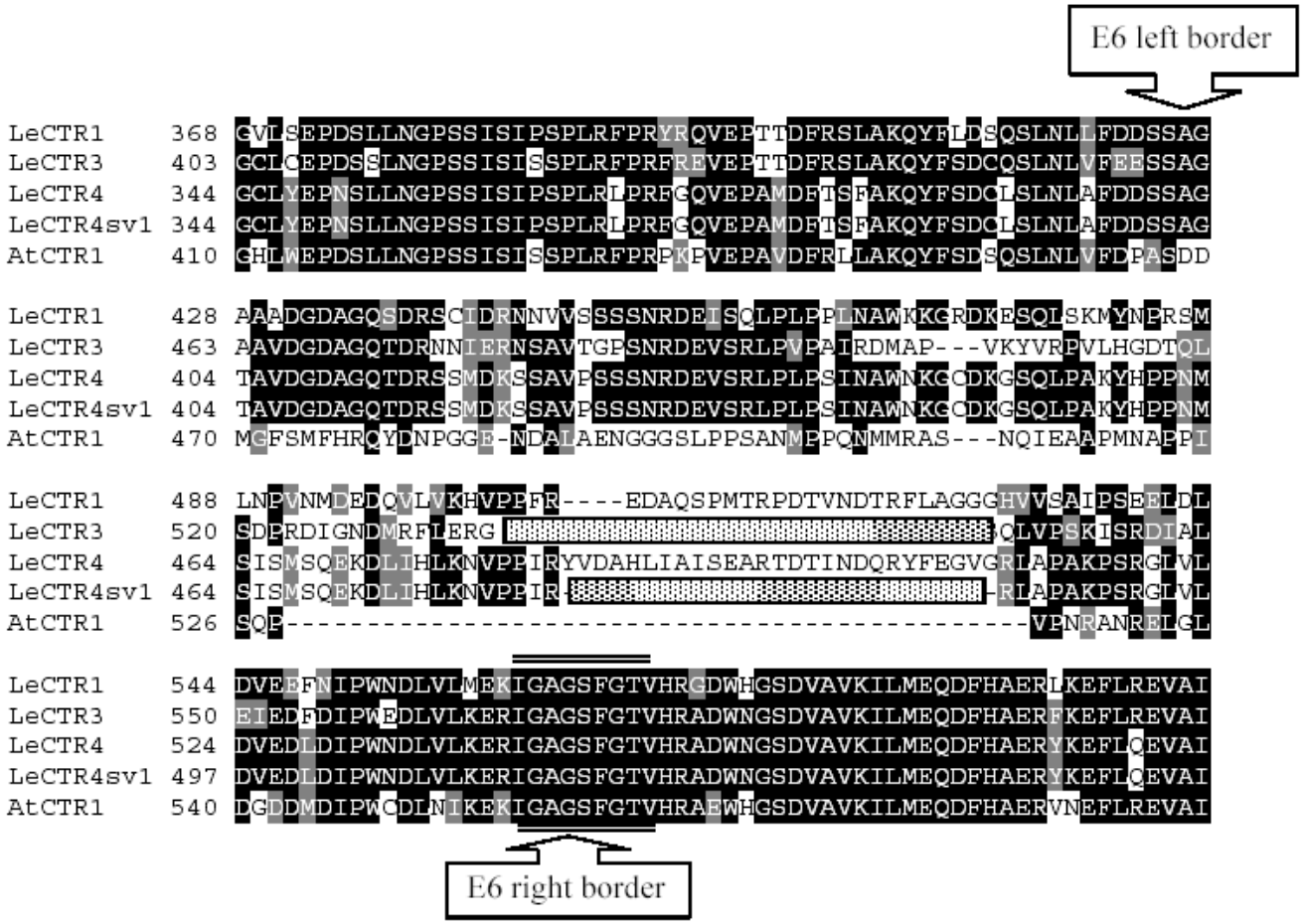


Figure 7. Amino acid alignments of AtCTR1, LeCTR1, LeCTR3, LeCTR4 and LeCTR4sv1 spanning exon 6. Identities between proteins are indicated by shaded squares. The left and right borders of exon 6 are indicated by arrows. The gray rectangles depict where introns exist in *LeCTR3* and *LeCTRsv1* genomic sequence that are spliced out in the coding sequence. The double line above and below highlights the first subdomain of the kinase domain.

suggesting a region whose function may be primarily to join adjacent domains. Amino acid sequences were examined for predicted secondary structure (see experimental procedures) and no obvious changes were predicted as a result of the lack or addition of the exon 6 intron sequence into the ORF.

LeCTR1, *LeCTR3* and *LeCTR4* genes have been placed on the tomato introgression line map developed by Eshed and Zamir (1994). The *LeCTR1* and *LeCTR4* loci both map to introgressions 10-2 and 10-3 on chromosome 10, while *LeCTR3* maps to introgression 9-1-3 on chromosome 9. None of these loci are linked to the tomato *Epi* locus (on chromosome 4) that when mutated results in seedling, leaf and root phenotypes consistent with those anticipated for a CTR1 mutation (Barry *et al*., 2001).

Complementation of Arabidopsis CTR1 mutants

To determine whether *LeCTR* genes indeed encoded MAPKKKs involved in ethylene signal transduction, constructs expressing each gene were transferred into Arabidopsis *ctr1* mutant genotypes to assay their respective abilities to complement loss of AtCTR1 function. LeCTR1 has been previously shown capable of complementing the constitutive triple response phenotype of the Arabidopsis *ctr1-1* mutant (Leclerq *et al*., 2002). *ctr1-1* harbors a mutation disrupting the kinase activity of CTR1 (Huang *et al*., 2003). To determine whether or not additional tomato *CTR1*-like genes also encode ethylene signaling CTR1 functions, we introduced constructs expressing LeCTR1, LeCTR3, LeCTR4 or LeCTR4 sv1 cDNA in the sense orientation behind the CaMV 35S promoter. The *ctr1-8* mutant was selected over *ctr1-1* in part because *ctr1-8* proved

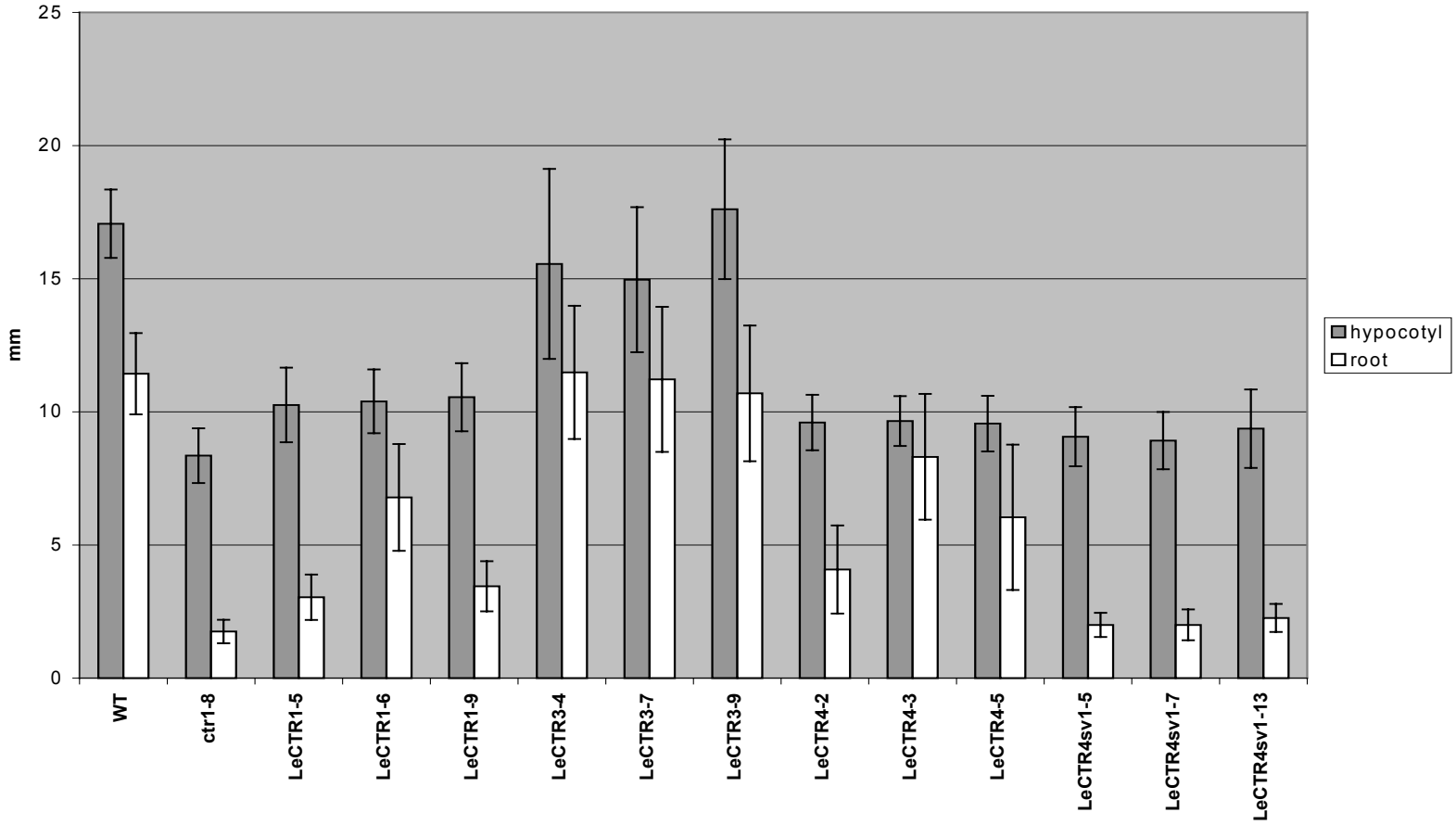


Figure 8. Complementation of *ctr1-8* constitutive triple response phenotype.
Hypocotyl and root length of 5-day-old etiolated Arabidopsis *ctr1-8* mutant seedlings in transgenic lines expressing the tomato LeCTR1 (1-5, 1-6, 1-9), LeCTR3 (3-4, 3-7, 3-9), LeCTR4 (4-2, 4-3, 4-5) or LeCTR4sv1 (4sv1-5, 4sv1-7, 4sv1-13) cDNA compared to the *ctr1-8* mutant and wild-type Arabidopsis.

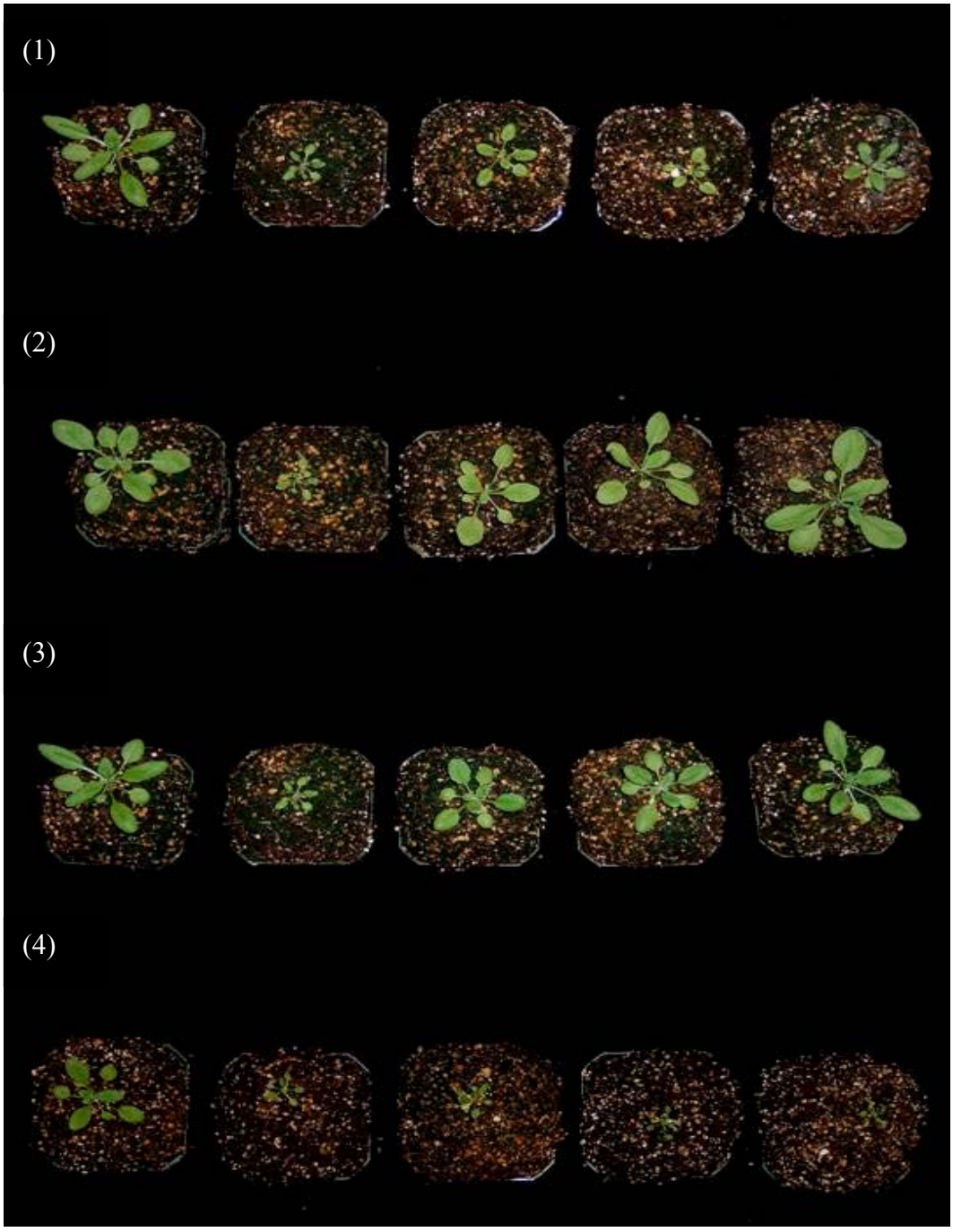


Figure 9. Complementation of the compact rosette phenotype of *ctr1-8*.
(1) WT, *ctr1-8*, LeCTR1-overexpressing lines, (1-5, 1-6, 1-9); (2) WT, *ctr1-8*, LeCTR3-overexpressing lines (3-4, 3-7, 3-9); (3) WT, *ctr1-8*, LeCTR4-overexpressing lines (4-2, 4-3, 4-5); (4) WT, *ctr1-8*, LeCTR4sv1-overexpressing lines (4sv1-5, 4sv1-7, 4sv1-1).

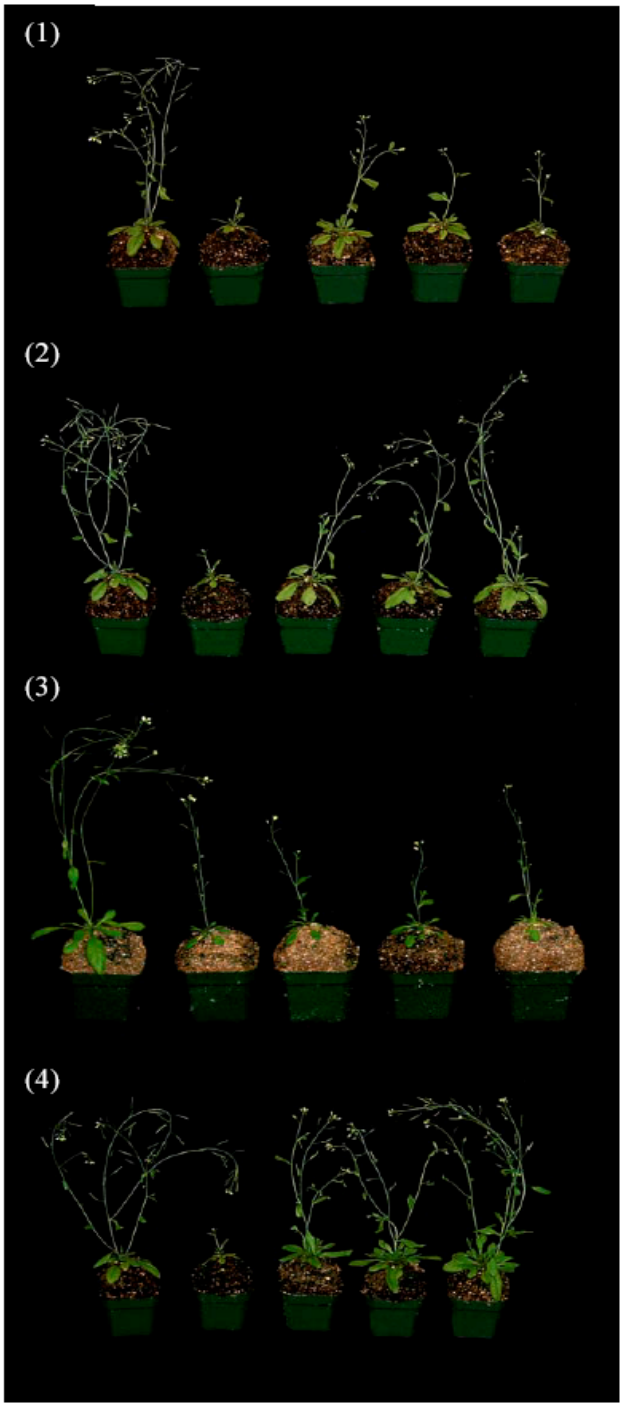


Figure 10. Complementation of *ctr1-8* at the flowering stage.
(1) WT, *ctr1-8*, LeCTR1-overexpressing lines, (1-5, 1-6, 1-9); (2) WT, *ctr1-8*, LeCTR3-overexpressing lines (3-4, 3-7, 3-9); (3) WT, *ctr1-8*, LeCTR4-overexpressing lines (4-2, 4-3, 4-5); (4) WT, *ctr1-8*, LeCTR4sv1-overexpressing lines (4sv1-5, 4sv1-7, 4sv1-1).
more amenable to transformation due to elevated sterility in *ctr1-1*. In *ctr1-8*, the highly

conserved Gly 364 residue is changed to a Glu in the CN box of the N-terminal domain rendering it constitutively responsive to ethylene (Huang *et al*., 2003). This mutant *ctr1* allele is the only one identified to date that does not effect the kinase activity, rather it disrupts the interaction with ETR1 (Huang *et al*., 2003) making it potentially more informative in functional analysis studies than a null mutation or other less well defined mutant alleles.

The ability of the constructs to complement the constitutive triple response and reduced adult plant size phenotypes of *ctr1-8* was assayed. When seedlings were grown in the dark for 6 days, LeCTR3 could fully restore the inhibited hypocotyl length and root length of the *ctr1-8* mutant to wild-type (Figure 8). LeCTR1 and LeCTR4 were not able to restore inhibited hypocotyl length but did partially restore root length. LeCTR4sv1 was unable to complement either hypocotyl or root length in *ctr1-8* (Figure 8). Adult rosette and inflorescence size could be fully restored to wild-type by LeCTR3 and LeCTR4 and was partially recovered by LeCTR1, but not by LeCTR4sv1 (Figure 9 and 10).

Expression Analysis of LeCTR genes

An expression profile for *LeCTR1* was reported previously (LeClerq *et al*., 2002) and was included here for comparison to *LeCTR3* and *LeCTR4* (Figure 11). RNAs were generated by extracting RNA from a range of tomato tissues at different stages of development and quantitating the message levels using real-time Quantitative RT-PCR for all three genes simultaneously. *LeCTR4* could be distinguished from the two

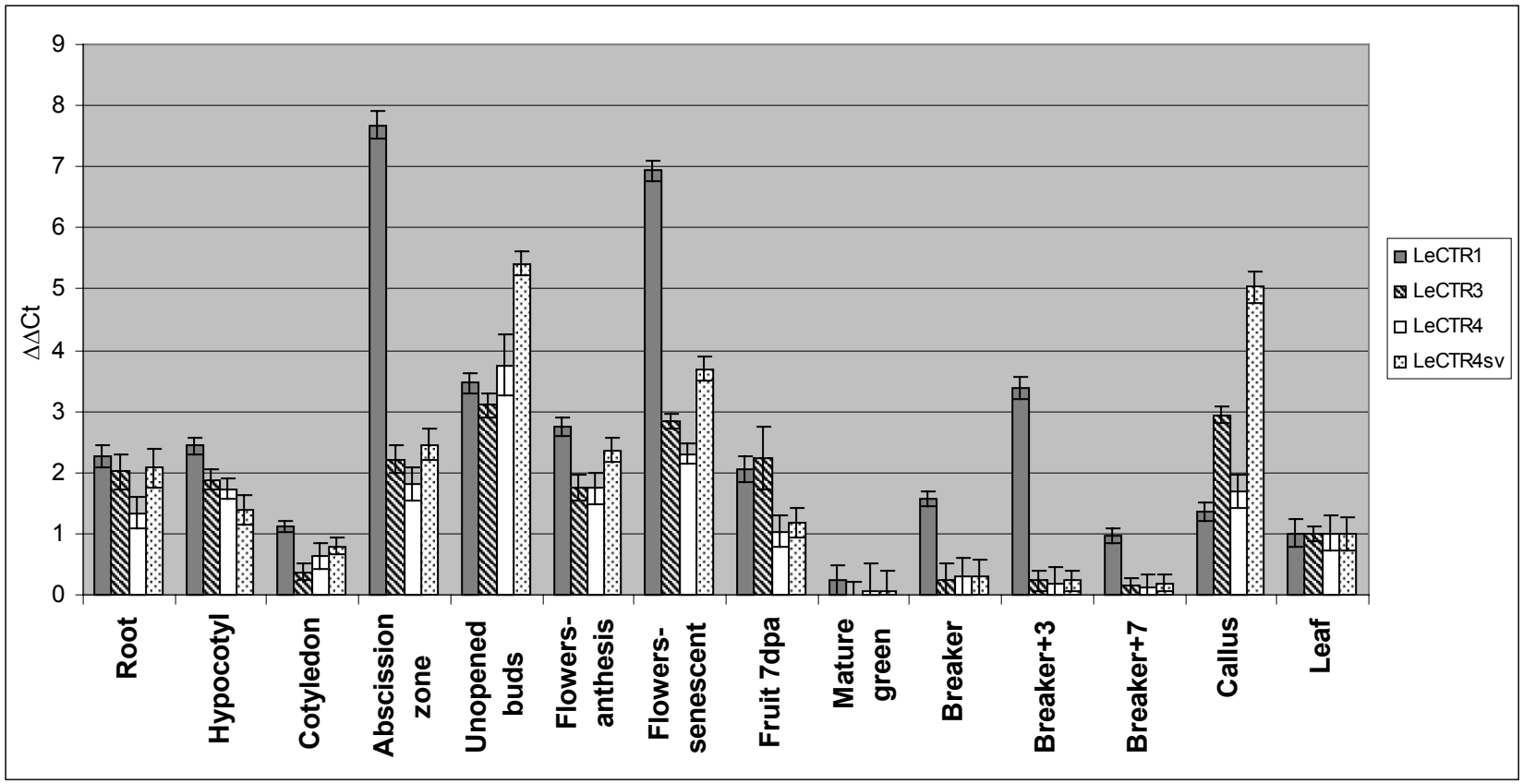


Figure 11. Differential expression of the LeCTR gene family.
RNA was extracted from different tissues at indicated stages of development and *LeCTR1*, *LeCTR3*, *LeCTR4* and *LeCTR4sv* transcript levels were assessed by real-time quantitative PCR. ΔΔCt on the y axis refers to the fold difference in a particular LeCTR message level relative to its level found in leaf.

LeCTR4 splice variants *LeCTR4sv1* and *LeCTR4sv2* (which were not distinguished from each other in this assay) due to the fact that both splice variants share a 3' UTR sequence distinct from *LeCTR4,* and thus employed as the target for expression monitoring. All messages were shown to be relatively low abundance based on difficulty of detection via RNA gel-blot analysis (data not shown), but could be detected at varying levels in all tissues examined by QRT-PCR.

LeCTR3, *LeCTR4* and *LeCTR4sv* accumulated to higher levels in leaves than fruit, which remained low for all three RNAs throughout fruit ripening. In contrast, *LeCTR1* transcript increased markedly coincident with the onset of ripening (Figure 11). During flower development, levels of all three *LeCTR* transcripts decreased 1-3 fold during anthesis compared to the levels observed in unopened buds. While there was a 1-2 fold increase in levels of *LeCTR3*, *LeCTR4* and *LeCTR4sv* in flowers undergoing senescence as compared to anthesis, clearly more pronounced is the 5 fold increase in *LeCTR1* transcript during that same developmental interval. In addition, *LeCTR1* transcripts were 5 fold higher in abscission zones harvested from pedicels of flowers at anthesis stage than in the corresponding flowers. No such abscission-related increase in transcript accumulation was observed for the *LeCTR3*, *LeCTR4* or *LeCTR4sv* transcripts (Figure 11). In summary, *LeCTR1* induction is associated with tissues at stages of development associated with increased ethylene (fruit ripening, pedicel abscission, petal senescence) as reported previously (LeClerq *et al*., 2002) while *LeCTR3* and *LeCTR4* transcripts are not.

It has been reported that *AtCTR1* is not inducible by ethylene in seedlings (Gao

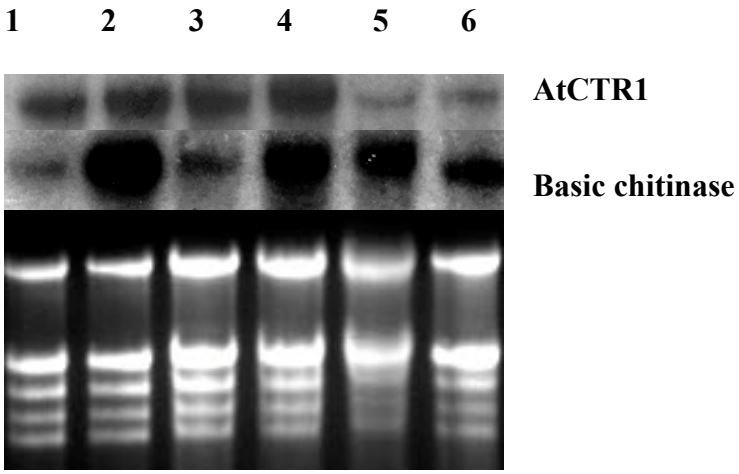


Figure 12. Ethylene inducibility of Arabidopsis *CTR1*.
RNA gel-blot of RNA isolated from leaves from 20 day plants (lanes 1 and 2) stems from 35 day old plants (lanes 3 and 4) and siliques from 35 day old plants (lanes 5 and 6) placed in sealed jars and treated with air (Lanes 1,3, and 5) or 50 ppm ethylene (lanes 2, 4 and 6) for 24 hours. Blots were probed with the 3' UTR region of *AtCTR1* and a fragment of basic chitinase (Samac *et al*., 1990). Different sized transcripts for chitinase found in siliques treated with and without ethylene could be attributed to preference in either tissue for transcripts harboring different polyadenylation sites located 85 bp and 214 bp from the termination codon (Samac *et al*., 1990) Equal loading of the RNA was checked by ethidium bromide staining (bottom).

et al., 2003; Kieber, *et al*., 1993), however, a more comprehensive analysis of the ethylene inducibility of *AtCTR1* that could address whether or not this is a tissue-specific phenomenon has not been published. Consequently we examined *AtCTR1* message levels in leaves, stems and siliques from adult plants treated with and without 50ppm ethylene for 24 hours and determined that *AtCTR1* is not induced by ethylene in these tissues under these experimental conditions (Figure 12).

We previously reported that *LeCTR1* is ethylene inducible in mature green fruit, leaves and roots of tomato. A timecourse of mature green fruit treated with ethylene was generated to more fully characterize the dynamics of ethylene responsiveness of all the *LeCTR* transcripts (Figure 13). While *LeCTR1* responded relatively rapidly to ethylene, maintaining elevated levels throughout the 24-hour time course, the other *LeCTR* messages failed to accumulate above levels observed in non-treated mature green fruit at any point throughout the experiment. Along the same lines, *LeCTR3*, *LeCTR4* and *LeCTR4sv* did not demonstrate significant accumulation in response to ethylene (as did *LeCTR1*) in either leaves or roots (Figure 14).

Evidence for a CTR1-like gene family in other species

There are extensive similarities in genome structure and sequence found among members of the corresponding families to which Arabidopsis and tomato belong (*Brassicaceae* and *Solanaceae*, respectively) facilitating a sequence based homology approach for determining the existence of multiple *CTR1-like* genes in the *Brassicaceae* and *Solanaceae*. *AtCTR1* cDNA nucleotide sequence was queried against the database of preliminary *B. oleracea* genomic sequence contigs (www.tigr.org/tdb/e2k1/bog1).

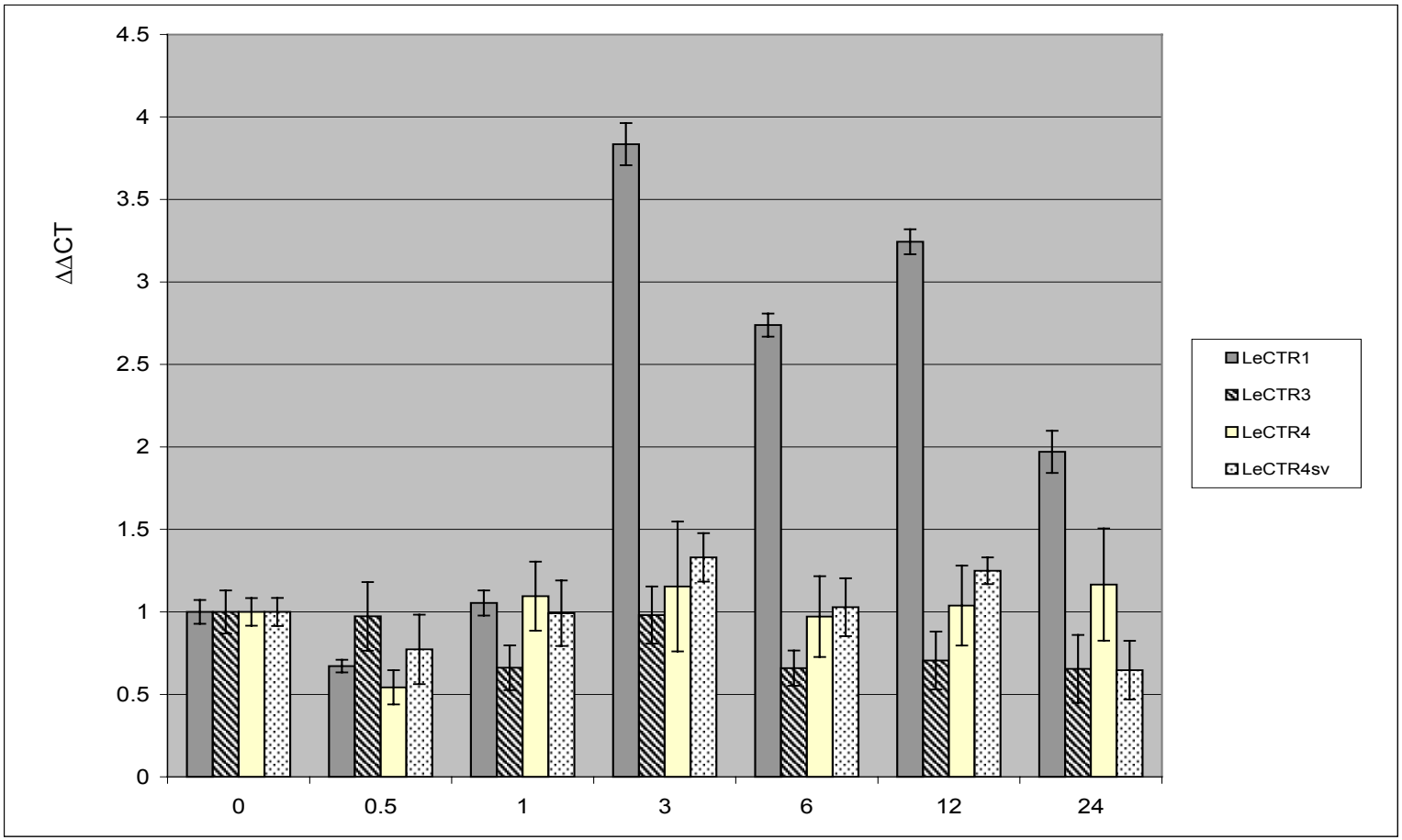


Figure 13. **Ethylene inducibility of tomato *CTR1*-like transcripts in mature green fruit.**
Mature green fruit were treated with 20 ppm ethylene for lengths of time ranging from 0.5 to 24 hours. RNA was extracted from the fruit and real-time quantitative RT-PCR was performed to determine relative fold differences in gene expression for *LeCTR1*, *LeCTR3*, *LeCTR4* and *LeCTRsv*. ΔΔCt on the y axis refers to the fold difference in a particular *LeCTR* message level relative to its level found in the non-treated control.

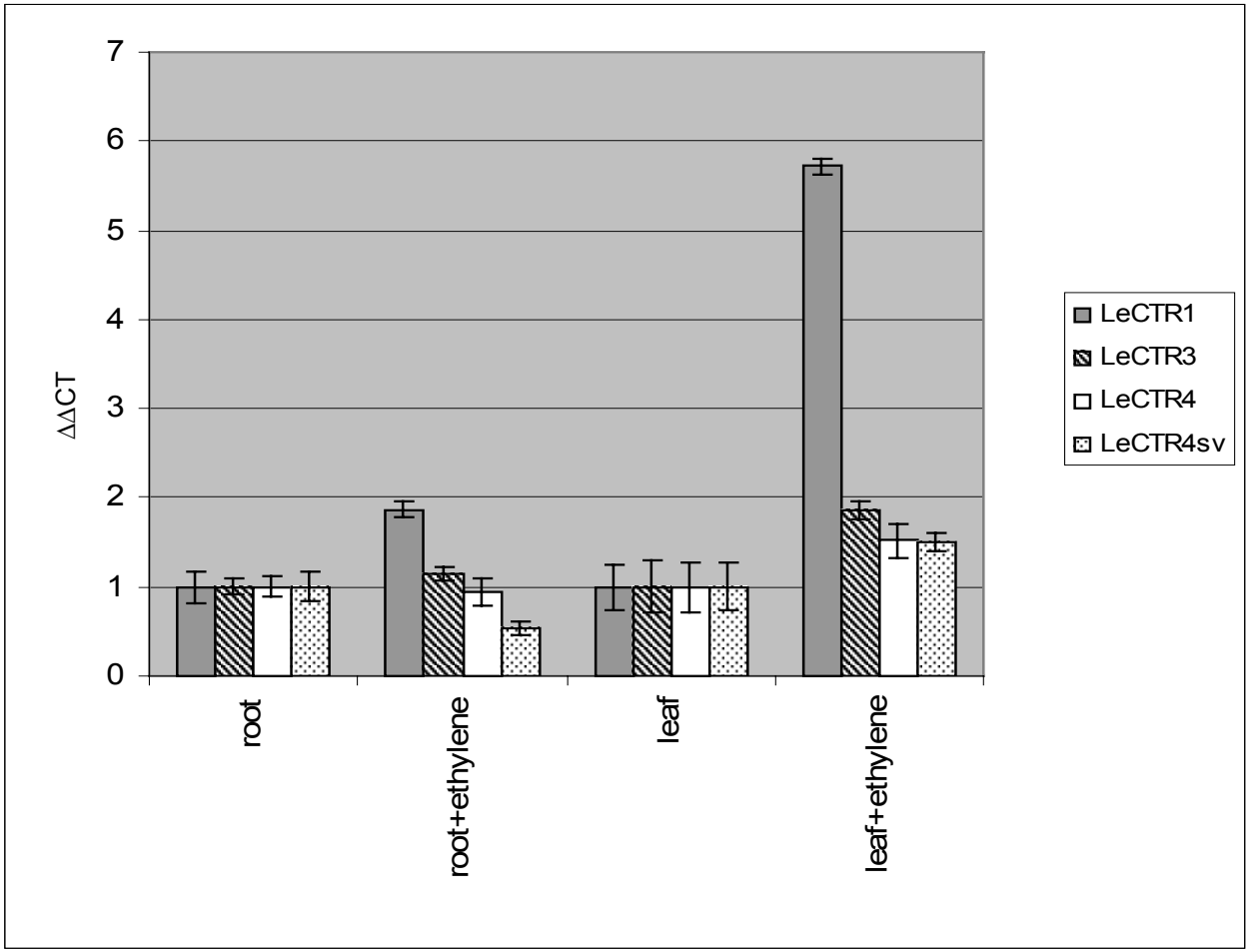


Figure 14. **Ethylene inducibility of tomato *CTR1*-like transcripts in leaves and roots.**
Six week old plants were placed in a sealed chamber and gassed with air or 20 ppm ethylene for 8 hours. RNA was extracted from the tissues and real-time quantitative RT-PCR was performed. ΔΔCt on the y axis refers to the fold difference in a particular LeCTR message level relative to its level found in air treated root and leaf, respectively.

Two sequences were retrieved which spanned the corresponding region of exon 2 in Arabidopsis sharing 92% nucleotide identity to each other and 91% nucleotide identity to *AtCTR1,* indicating the presence of multiple copies of *CTR1* in *B. oleracea* (Table 2). These two sequences share only 41-42% amino acid identity to *At4g24480* which is the next most similar sequence to *AtCTR1* in the Arabidopsis genome, providing further evidence that the two sequences retrieved were in fact both more similar to *AtCTR1* than any other sequence in the Arabidopsis genome.

In an effort to identify *CTR1*-like genes in the *Solanaceae*, each *LeCTR* cDNA was queried against the TIGR potato EST collection (www.tigr.org) and two single ESTs and one contig were identified (Table 2). One of the singletons (BE919922) does not overlap the other two sequences, thus it is possible that it does not represent a distinct gene. Nevertheless, each sequence corresponded to a different *LeCTR* with 94-98% nucleotide identity, indicating the existence of a *CTR1* multigene family in potato (Table 3).

To identify *CTR1* multi-gene families in other plant species, we submitted both the *AtCTR1* and *LeCTR1* N-terminal domain amino acid sequences into the TIGR database of EST collections for each of the plant gene indices available. We retrieved 13 putative *CTR* sequences from 9 different species (Table 2). All of these sequences contained conservation in the CN domain and those sequences that extended just downstream of the CN domain show additional conservation, which based on our analysis appears to be specific to *CTR-like* genes involved in ethylene signaling (i.e. not in *LeCTR2* or *AtEDR1*) (Figure 15). We have designated the region the EC (Ethylene

Table 2. Putative *CTR1*-like sequences obtained from TIGR genome and EST database searches.

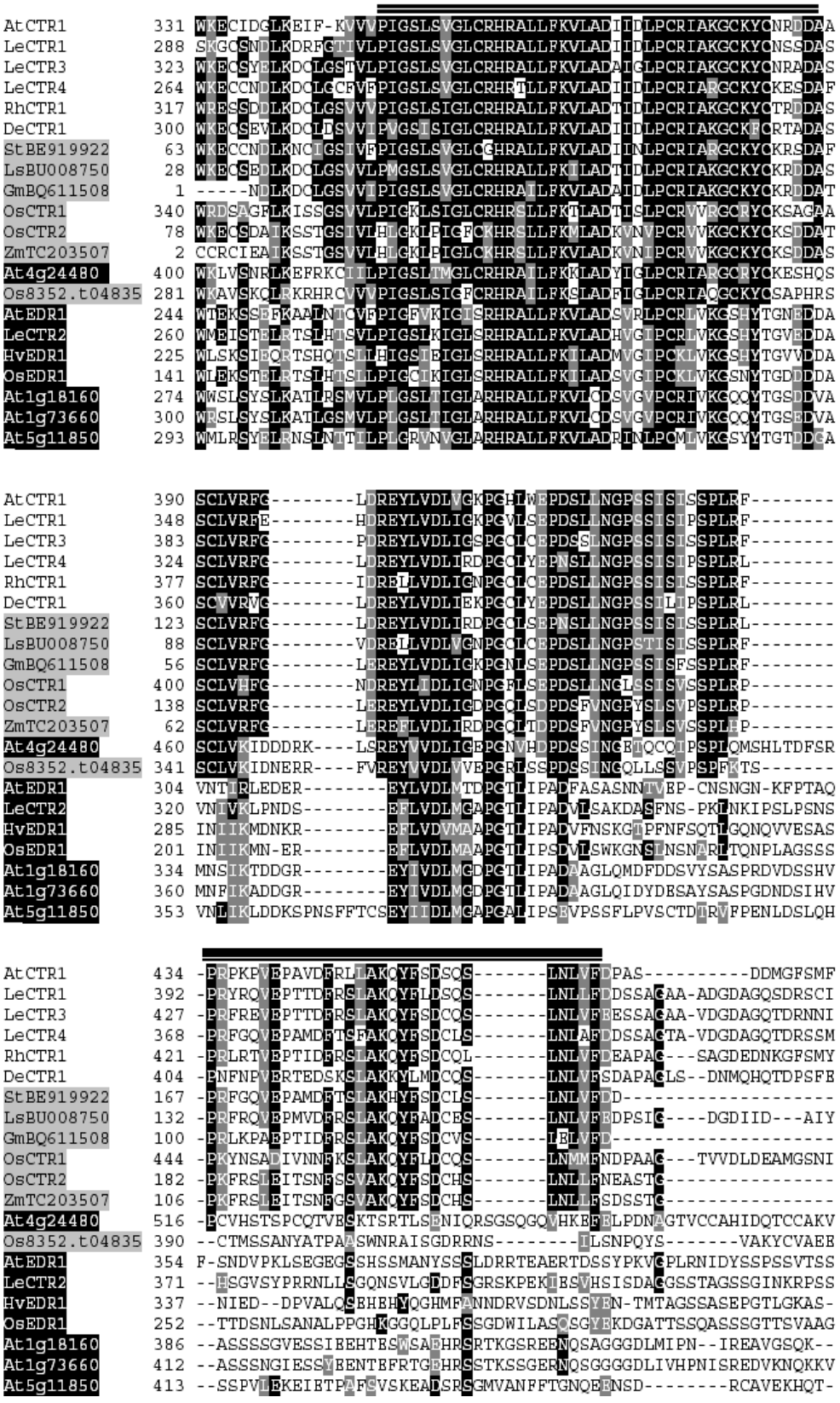
B. oleracea	BOGAC87TR, BOHCQ46TR
G. arboreum	BF274343
G. max	TC193259, BQ611508
H. annuus	BU026195
L. sativa	TC5349, BU008750
M. truncatula	TC93812, TC81131
O. sativa	OsCTR1 (TC136191) (8351.t030726) OsCTR2 (CB626810) (8352.t04853)
S. bicolor	CD229655
S. tuberosum	BE919922, BE342235, TC72396
T. aestivum	BJ315794
Z. mays	TC203507

Table 3. Percent nucleotide identity of LeCTR sequences with potato.

S. tuberosum	LeCTR1	LeCTR3	LeCTR4
*BE919922	81	81	**94**
BE342235	**98**	90	90
TC72396	84	**98**	85

Percent nucleotide identity of each *S. tuberosum* putative CTR1-like EST sequence to LeCTR1, LeCTR3 and LeCTR4 cDNA sequences.
* This sequence does not overlap BE342235 and TC72396, thus it is possible that it does not represent a distinct gene.

Figure 15. Conserved regions in the N-terminal domain are present in both AtCTR1 and putative CTR1-like amino acid sequences.
Amino acid alignments were preformed by ClustalX. Amino acid residues identical to the consensus sequence are shaded black while residues which are not identical but similar are shaded gray. Sequences shown highlighted in gray are novel EST sequences retrieved from TIGR EST database searches. These putative *CTR1*-like sequences are preceded by a two-letter prefix to indicate the species of origin: St, *Solanum turberosum* (potato), Ha (*Helianthus annuus* (sunflower), Ls, *Lactuca sativa* (lettuce), Mt, *Medicago truncatula*, Gm, *Glycine max* (soybean), Os, *Oryza sativa* (rice), Zm, *Zea mays* (corn). Sequences highlighted in black are both reported and putative MAPKKKs which belong to the same subfamily as AtCTR1 and are shown here to illustrate similarities and differences from *CTR1*-like sequences. The CN box (described by Huang et. al., 2003) is denoted with the double line. Downstream of the CN box, with a triple line above, is a region which appears to be conserved only in the CTR1-like sequences which we have designated the EC (Ethylene CTR specific) domain.


AtCTR1 331
LeCTR1 288
LeCTR3 323
LeCTR4 264
RhCTR1 317
DeCTR1 300
StBE919922 63
LsBU008750 28
GmBQ611508 1
OsCTR1 340
OsCTR2 78
ZmTC203507 2
At4g24480 400
Os8352.t04835 281
AtEDR1 244
LeCTR2 260
HvEDR1 225
OsEDR1 141
At1g18160 274
At1g73660 300
At5g11850 293
AtCTR1 390
LeCTR1 348
LeCTR3 383
LeCTR4 324
RhCTR1 377
DeCTR1 360
StBE919922 123
LsBU008750 88
GmBQ611508 56
OsCTR1 400
OsCTR2 138
ZmTC203507 62
At4g24480 460
Os8352.t04835 341
AtEDR1 304
LeCTR2 320
HvEDR1 285
OsEDR1 201
At1g18160 334
At1g73660 360
At5g11850 353
AtCTR1 434
LeCTR1 392
LeCTR3 427
LeCTR4 368
RhCTR1 421
DeCTR1 404
StBE919922 167
LsBU008750 132
GmBQ611508 100
OsCTR1 444
OsCTR2 182
ZmTC203507 106
At4g24480 516
Os8352.t04835 390
AtEDR1 354
LeCTR2 371
HvEDR1 337
OsEDR1 252
At1g18160 386
At1g73660 412
At5g11850 413

CTR) domain. Because of the approximate 3 kb transcript length of *CTR* genes, some were likely missed due to incomplete cDNA synthesis in EST library construction. The kinase domain could not be used for comparative analysis due to the overwhelming number of non-*CTR* kinases that were returned (data not shown).

Multiple CTR1-like sequences were obtained for lettuce, soybean, *Medicago*, and rice. Of most interest were one EST contig (TC136191) and one EST singleton (CB626819) retrieved from the rice EST collection that share 65.8-71% amino acid identity to *AtCTR1* in the CN domain while only 51.2 and 58.8% identity to *At4g24480*. The TC136191 and CB626819 sequences were queried against the rice genomic sequence database (http://www.tigr.org/tdb/e2k1/osa1/) in order to obtain putative full length protein sequences for both genes. The TC136191 and CB626819 EST sequences corresponded to 8351.t03037 and 8357.t03295 predicted protein sequences, respectively. A third putative CTR1-like rice gene (8352.t04835) was also identified during this search. A phylogenetic tree was constructed using the full length protein sequences of the putative *CTR1-like* clones from rice in order to determine if they were more similar to reported and putative CTR1-like genes or other subgroup B3 MAPKKK genes (Figure 6). Both 8351.t03037 and 8357.t03295 were more similar to CTR-like genes than an other MAPKKKs, while 8352.t04835 was more similar to At4g24480. We designated the rice gene represented by 8351.t03037, *OsCTR1*, and that represented by 8357.t03295, *OsCTR2*. Interestingly, OsCTR1 and OsCTR2 show conservation of both the CN domain and the EC domain while Os8352.t04835 only shares conservation in the

CN domain (Figure 15). All three sequences contain signatures described earlier that are important for serine/threonine kinase activity.

As *At4g24480* is the gene most similar in sequence to *AtCTR1* in the Arabidopsis genome, it might be a likely candidate to exhibit CTR1 function. However, two homozygous lines obtained from SALK containing verified T-DNA inserts in the *At4g24480* did not display constitutive ethylene response in etiolated seedlings or in the adult plants (data not shown). Furthermore, EDR1, which is also a member of this MAPKKK family, has been implicated in the negative regulation of defense responses in plants and does not exhibit any CTR1-like phenotypes indicating it probably functions in a pathway separate from the ethylene-response pathway (Frye *et al*., 2001). Together, these results provide supporting (though not conclusive) evidence that CTR function is most likely encoded by only one *CTR1* gene in Arabidopsis.

Discussion

Through isolation and functional characterization of three *LeCTR* cDNAs and corresponding genomic clones from tomato, we have provided here experimental evidence of a multigene family of *CTR1-like* genes which are functionally able to participate in ethylene signal transduction. Isolation and structural analysis of the genomic clones of the tomato *CTR1-like* genes revealed that intron sizes were considerably larger than those found in Arabidopsis *CTR1* while the organization of introns/exons remained conserved. This is consistent with the observation that while the position of the introns was probably established before the divergence of tomato and

Arabidopsis, differences exist between the two species in their rates of accumulation or loss of noncoding DNA (Ku *et al*., 2000). Exon size and position appeared to be quite conserved between the tomato and Arabidopsis sequences with the notable exception of exon #6. *AtCTR1* exhibits variability in transcript processing within this region. The longest intron in the Arabidopsis *CTR1* sequence precedes exon #6 and was found to be spliced at reduced efficiency in the mRNA population (Kieber *et al*., 1993). Structural comparison of the tomato *CTR* genomic sequences revealed that exon #6 was interrupted by an intron in different locations in both *LeCTR3* and *LeCTR4sv1* coding sequences. It has been well documented that a common form of alternative splicing in plants is intron retention and presumably reflects poor recognition of the intron (Brown and Simpson, 1998). This may be the case for the *LeCTR1* and *LeCTR4* and *LeCTR4sv2* transcripts. While no intron is spliced out, consensus acceptor sites and donor sites are present. Of note is the fact that if the *LeCTR3* intron were read through in frame, several stop codons would be encountered which would render the protein non-functional. In the case of *LeCTR4sv1*, when the intron is spliced, a stop codon is brought into frame rendering the predicted protein non-functional, which would explain the lack of complementation of the *ctr1-8* mutant for this construct. Further, the identification of two *LeCTR4* splice variants each differing only in the processing of this same intron permits speculation that splicing in the junction region which connects the N-terminal domain to the kinase domain could serve in autoregulation or pathway control as a trans-dominant inhibitor. In such a scenario, it would be possible that each *LeCTR* transcript could have splice variants that differ in the processing of this intron. This phenomenon has also been

shown to occur in broccoli, rice and wheat mRNA transcripts. (reviewed by Brown and Simpson, 1998).

Attempts to complement the Arabidopsis *ctr1-8* mutation with three different tomato CTR1 genes suggest all encode functional CTR1 proteins *in vivo*. Specifically, I have shown that all three genes have similar percent predicted amino acid identity to AtCTR1 (Table 1), all are more similar to *AtCTR1* than any other genes in the Arabidopsis genome (Figure 6) and when expressed in the *ctr1-8* mutant under the direction of the CaMV35s promoter each resulted in partial to full complementation of mutant seedling (Figure 8) and mature plant phenotypes (Figures 9 and 10). While RNAi of each *LeCTR1* gene is in progress in our lab, it is noteworthy that viral induced gene silencing (VIGS) of the *LeCTR1* gene resulted in constitutive ethylene response phenotypes in tomato (Liu *et al*., 2002).

The *LeCTR* gene family is differentially regulated by ethylene and during stages of development marked by increased ethylene biosynthesis. Similarly, ethylene receptors are encoded by a multi-gene family, differentially regulated by ethylene, and function to negatively regulate ethylene responses in both Arabidopsis and tomato (Hua and Meyerowitz, 1998; Lashbrook *et al*., 1998; Tieman and Klee, 1999). Somewhat paradoxical is the notion that expression of a negative regulator of ethylene response would increase in response to ethylene. This phenomenon may serve as a mechanism to modulate the sensitivity of a tissue to ethylene to provide the range of responses under various conditions/tissues observed for ethylene (Hua and Meyerowitz, 1998; Klee, 2002; Tieman *et al*., 2000). When ethylene is present it binds to the receptors to inhibit

their biochemical activity, causing CTR1 to become inactive and unable to repress downstream responses leading to ethylene associated phenotypes (Huang *et al*., 2003). The ratio of receptors encoded by different family members in a particular cell type might influence the dose-response relationships which can vary for different tissues and responses (Bleecker, 1999). In apparent contrast to Arabidopsis, modulation of said ratio in tomato occurs at the levels of both receptors and CTRs, while only receptors respond transcriptionally to ethylene in Arabidopsis. The combination of a larger repertoire of inducible *CTR* genes, in concert with an apparently greater range of inducibility of ethylene receptors in tomato as compared to Arabidopsis, may represent an adaptation to promote important biological functions dependent upon ethylene in the *Solanaceae*. It will be interesting to determine whether or not specific tomato CTRs will interact with specific tomato receptors. One might predict that LeCTR1, which is inducible in ripening fruit, might interact specifically with the predominant fruit ethylene receptors Nr and LeETR4. A combination of differential expression of receptors and *CTR* genes in conjunction with differential interaction kinetics could represent a mechanism for optimizing fidelity of ethylene responses.

While *AtCTR1* is a part of the large *MAPKKK* gene family in Arabidopsis, it is curious as to why there is only one gene encoding CTR1 function in Arabidopsis while there are two *CTR1-like* sequences in its close relative *B. oleracea*. Additionally, there seemingly exists a small family in tomato, potato, lettuce and soybean. It will be interesting to ascertain whether or not multiple *CTR1*-like genes is a reflection of multiple MAP kinase cascades capable of participation in ripening.

To gain clearer insight into the evolution of the *CTR* family in plants it is advantageous to examine rice as it is a monocot and the second plant for which comprehensive genome sequence is available. Monocots and dicots appear to have diverged from a common ancestor between 130 and 240 million years ago (Patterson *et al*., 2000). Rice has at least two transcripts (OsCTR1 and OsCTR2) that based on amino acid identity and phylogenetic analysis appear to be more related to AtCTR1 indicating the possible existence of a multigene family of CTR1-like genes in rice. Furthermore, rice appears to have at least one transcript more related to At4g24480, the next most similar gene to AtCTR1 in the Arabidopsis genome, providing further support that OsCTR1 and OsCTR2 represent two CTR1-like sequences. This raises the possibility that there were multiple copies of *CTR1-like* genes in plants before monocots and dicots diverged, and while this family apparently persisted in the *Brassicaceae*, Arabidopsis appears to have lost one or more of these members. The most common fate of duplicated genes is non-functionalization (gene silencing), while in order to be retained, the duplicated gene must either acquire a novel, beneficial function or both copies must undergo subfunctionalization whereby the total capacity of both genes is reduced to the level of the single-copy ancestral gene (Lynch and Conery, 2000). The retention of multiple *CTR1-like* genes in rice might reflect the impact that ethylene has on a process crucial to the survival of the plant. For example, an adaptive feature of rice is the capacity to elongate rapidly when submerged. Ethylene has been shown to enhance the internodes of rice to gibberellic acid, thereby inducing the rapid elongation of submerged deepwater rice (Kende *et al*., 1992).

It is widely accepted that at least one or more large-scale gene or entire genome duplications have likely occurred during the evolutionary history of Arabidopsis (Ku *et al*., 2000; Lynch and Conery, 2000; Vision *et al*., 2000). It has also been proposed that a second genome wide duplication event occurred in Arabidopsis after divergence from tomato, which was followed by accelerated selective gene loss in Arabidopsis (Vision *et al*., 2000; Ku *et al*., 2000; Van der Hoeven *et al*., 2002). As a result, the estimated percentage of gene families in tomato does not appear to be significantly higher than that in Arabidopsis (Van der Hoeven *et al*., 2002). As an exception, the *E8* gene family, whose functions are associated with fruit development and ripening, is also larger in tomato than in Arabidopsis (Van der Hoeven *et al*., 2002). It was suggested that this might reflect a more complex fruit development/ripening process in tomato compared with Arabidopsis. This might also prove to be the case for some of the components of the ethylene signal transduction pathway given the impact that ethylene has on fruit ripening.

Experimental procedures

Plant material

Arabidopsis thaliana ecotype Columbia plants were grown in a growth chamber under 16h days at 22°C. Tomato (*Lycopersicon esculentum* cv. Ailsa Craig) plants were grown in a naturally illuminated greenhouse under standard conditions. Where indicated, fruits were harvested at the following stages: (MG) “mature green” stage occurs prior to ripening when seeds are mature but fruit remain green, approximately 5 days before

breaker; (B) “breaker” stage fruit take on a hint of yellow color and exhibit increased autocatalytic ethylene production; (B+3) fruit are harvested three days post breaker and are typically orange-red; (B+7) “red- ripe” fruit turn a bright red color and start to exhibit obvious softening at this stage.

Isolation of full-length cDNA and genomic clones

An arrayed tomato (cultivar Ailsa Craig) callus cDNA library (150,000 primary recombinants) was screened at low stringency with the full-length sequence of *LeCTR1*. Two positive clones with the largest inserts, cLEC056D21 (*LeCTR3*) and cLEC071P14 (*LeCTR4*), were sequenced using an ABI3700 Capillary DNA sequencer and Applied Biosystems BigDye dideoxy terminator reagents (Perkin-Elmer). Two splice variants of *LeCTR4* were recovered and designated *LeCTR4sv1* (cLEC071F7) and *LeCTR4sv2* (pGEMT LeCTR4sv2#5).

5’ RACE-PCR (Marathon Kit, Clontech) was employed to obtain cDNA spanning the missing 5' coding sequences of both genes. For *LeCTR3*, the clone obtained through RACE-PCR designated LeCTR3 5’(2B-1) did not contain the complete coding sequence so an arrayed *Lycopersicon cheesmannii* BAC library (J. Vrebalov and J. Giovannoni, unpublished) was screened with a probe designed from the first 150 bases of LeCTR3 5’(2B-1). The resulting BAC (LA483 O17 H23) was digested with HindIII and shotgun cloned into pBluescript (Stratagene). The 5’ end was retrieved via colony lift hybridization to the same probe used to screen the BAC library resulting in identification of LeCTR3 BAC (H1-4). The insert of LeCTR3 BAC (H1-4) was sequenced first with the following primer toward the putative *LeCTR3* start of

transcription: TCTR3RevRACE6 5'-CAAATGACGCCTCCGCATTAGACAAC-3'. Additional primers were designed as new sequence became available until the complete putative coding sequence was obtained. *Pfu* polymerase (Stratagene) was used to PCR the corresponding region from Ailsa Craig genomic DNA using the following primers: TCTR3 BAC H1-4For1 5'-TCCGATGTGCTTTTTAAGTCAAG-3' and TCTR3 5' Rev 5'-TACTCCCCGGAGATCGAACTTTCACC-3'. The resulting PCR product was cloned into pGEMT (Promega) to yield a plasmid designated LeCTR3 (Ac+/+Pfu#6) and 3 independent plasmids were sequenced to identify any PCR-induced mutations. LeCTR3 (Ac+/+Pfu#6) extended 513 bases upstream of the predicted start of transcription. Due to difficulties in cloning the full length *LeCTR3* RT-PCR product a full length cDNA sequence was constructed by ligating LeCTR3(Ac+/+Pfu#6) to LeCTR3 5'(2B-1) using the EcoRV internal restriction site found in the overlapping regions [bases 222 to 228 of LeCTR3 5' (2B-1)] to create plasmid LeCTR3 (PCR2.1#1)

The full length cDNA for *LeCTR4* was obtained by performing PCR on callus cDNA using the following primers designed to the predicted sequence ends: TCTR4 5' For1 5'-GAAGTTGGGGAACTGAATTTGT-3' and LeCTR4 3'UTR Rev 5'-CTTATTTAGCCGCCGAAGAGAAT-3. The resulting PCR product was cloned into PCR2.1 (Invitrogen) to yield plasmid LeCTR4 (pCR2.1 #8). Three clones were sequenced to identify any PCR-induced mutations. The full length cDNA for *LeCTR4sv1* was obtained by cloning the 5' end obtained from RACE PCR into the 3' end clone (cLEC071) using the NsiI internal restriction site found in the overlapping regions (bases 130 to 136 of cLEC071) to yield plasmid LeCTR4sv1 (pBS 2B-2).

To obtain genomic sequence for both *LeCTR3* and *LeCTR4*, an arrayed Ailsa Craig cosmid library (S. Tracy and J. Giovannoni, unpublished) was screened with gel purified gene specific 3' UTR probes for *LeCTR3* and *LeCTR4* (described below). Two cosmid clones for *LeCTR3* (Ac+/+ cos 91J17, 153O18) and 4 cosmid clones for *LeCTR4* (28P4, 60O6, 232E16, and 232I8) were subcloned into pBluescript and 19 of the resulting subclones were sequenced using gene specific primers. Junction regions of the cosmid subclones were sequenced directly from the cosmid to ensure proper assembly of the contigs. Intron/exon boundaries were determined by utilizing the large gap alignment function of the SEQUENCHER program (Gene Codes, Ann Arbor, MI), which allows alignment of cDNA to genomic sequence. Sequences of the cDNA and genomic sequences have been deposited into GenBank (*LeCTR3* cDNA, AY382575; *LeCTR3* genomic, AY382679; *LeCTR4* cDNA, AY382678; *LeCTR4* genomic, AY382677).

Generation of LeCTR gene specific probes

3' UTR probes were generated by PCR from the corresponding full length *LeCTR* cDNA sequence using the following primers: LeCTR1 3'UTR For 5'-GCACATATTCTGCC GGTCAT-3'; LeCTR1 3' UTR Rev 5'-CAAGAAATCCTGGGCAGA-3'; LeCTR3 3' UTR For 5'-TTTCTGCACATATTTGGCATTC-3'; LeCTR3 3'UTR Rev 5'-GAACTG TGCATTCCCATTATAAA-3'; LeCTR4 3' UTR For 5'-CATTTGCACTTGGTATTT GGCTTA- 3'; LeCTR4 3' UTR Rev 5'- CTTATTTAGCCGCCGAAGAGAAT- 3'; LeCTR4sv 3'UTRFor 5'-TGTATGATTCCTGCACATCTTTGG-3', LeCTRsv 3'UTR Rev 5'-TGGACGAATTATTGTTGACATACC-3'.

Sequence Analysis

Amino acid sequence identities were calculated using the ALIGN program (GeneStream Server, http://www.genestream.org). Amino acid sequence alignments were performed using the CLUSTALX program (Thompson *et al*., 1997). The amino acid sequences for LeCTR3 and LeCTR4 were scanned against the PROSITE database of protein families and domains for predicted patterns and motifs through the ExPASy server (Appel *et al*., 1994). Amino acid sequences were submitted to the PSIpred (McGuffin *et al*., 2000) program through the ExPASy server in order to predict secondary structure. Phylogenetic trees were constructed using programs from the PHYLIP package (Felsenstein, 1989). Preliminary genomic sequence data for *B. oleracea* and *O. sativa* as well as EST sequences retrieved from the plant gene indices were obtained from The Institute for Genomic Research (TIGR) website at http://www.tigr.org. All sequences obtained from any of the TIGR databases were reported using the sequence identifier number annotated by TIGR. *AtCTR1* cDNA nucleotide sequence was queried against the database of preliminary *B. oleracea* contigs utilizing the BLASTn function. The AtCTR1 and LeCTR1 N-terminal domain amino acid sequences were queried against the TIGR database of EST collections for each of the plant gene indices available utilizing the tBLASTn function. Sequences that shared at least 50% amino acid identity to either AtCTR1 or LeCTR1 were retained. Sequence IDs were reported as the EST ID if only one EST was identified or as the TC number if more than one EST was identified.

Mapping

Probes for *LeCTR1* (generated by PCR from 800 bp of the promoter region), *LeCTR3* (generated by PCR from the last 1200bp of *LeCTR3* cDNA) and *LeCTR4* (3'UTR probe described above) were surveyed against *L. pennelleii* and *L. esculentum* genomic DNA digested with 5 different restriction enzymes (DraI, EcoRI, EcoRV, BstNI, HaeIII) via DNA gel-blot analysis. After determining which enzyme would provide a useful polymorphism for mapping each gene in a previously developed *L. esculentum/L. pennelleii* introgression population (Eshed and Zamir, 1994), DNA gel blots with 50-76 *L. esculentum/L. pennelleii* introgression lines digested with the appropriate enzyme were hybridized with the same *LeCTR* probe used in the initial survey filter to determine to which introgression each locus mapped. BstN1, EcoRV, and DraI provided RFLPs for *LeCTR1*, *LeCTR3* and *LeCTR4*, respectively.

Plant transformation

Full length cDNA sequences for *LeCTR1*, *LeCTR3*, *LeCTR4*, and *LeCTR4sv1* designated LeCTR1 (pGEMT#8), LeCTR3 (PCR2.1#1), LeCTR4 (pCR2.1 #8), and LeCTR4sv1 (pBS 2B-2) respectively were cloned into the binary plant transformation vector pBI121 (Invitrogen) in the sense orientation and under the control of the CaMV 35S promoter and employing the nopaline synthase (nos) 3' terminator. The resulting LeCTR1/S, LeCTR3/S, LeCTR4/S and LeCTR4sv1/S constructs were transformed into *A. tumefaciens* strain GV3101 carrying the helper plasmid pMP90. Arabidopsis *ctr1-8* seeds were grown under 12h day length for 2 weeks, transferred to 16h day length for 4 weeks and then transformed using the floral dip method (Clough and Bent, 1998).

Putative transformants were screened on MS medium containing 50μg/μl Kanamycin, 1X Gamborg's vitamins (Sigma), 1% sucrose and 0.7% Phytagar (Gibco) under 16 hours of light.

Confirmation of transgenic lines

Genomic DNA was extracted from each putative transformant and Southern analysis was performed (described below) using NPTII as a probe in order to confirm transgene integration and estimate copy number. The NPTII probe was generated by PCR using the following primers: NPTII For 5'-TGAAGCGGGAAGGGACTG-3' and NPTII Rev 5'-AAGGCGATAGAAGGCGATG-3'. PCR on the genomic DNA was also used for confirmation. In each case the forward primer was CaMV 35S For 5'-GGAAAAAGAAGACGTTCCAACCAC-3'. The reverse primer used for LeCTR1 plants: TCTR1intR1 5'-AAAGCAAAGCACGATGCC-3'; LeCTR3 plants: TCTR3 5' rev 5'-TCAGGCACATGATCCAAAA-3'; LeCTR4 plants: TCTR5R1 5'-GGATCA CTTTGCCGATCAAT-3'.

Southern Analysis

10μg of Arabidopsis DNA was digested with EcoRI and then electrophoresed through a 0.8% agarose gel. The gel was blotted to a Hybond-N+ membrane (Amersham) following the manufacture's instructions. Probes were radiolabeled with [32P] dCTP (Perkin Elmer) using random hexamers and Klenow fragment of DNA polymerase I (Promega). Blots were pre-hybridized and hybridized at 65°C in the following buffer: 5X SSC,0.025M KPO_4 buffer pH=6.5, 0.005% SDS, 5X Denhardt's solution. Blots were washed at 65°C and were carried out to 1XSSC and 0.05% (w/v) SDS.

Seedling triple response assay

Arabidopsis seeds were sterilized with 95% ethanol for 1 minute followed by 5 minutes with 50% bleach (2.625% sodium hypochlorite final volume) and resuspended in 0.1% agarose. Sterilized seeds were plated on sterile cellulose membranes (Bio-Rad) placed on medium containing MS salts, 1X Gamborg's vitamins, 1% sucrose, and 1.2% Phytagar. The plates were incubated at 4ºC in the dark for 4 days and then moved to room temperature and incubated in the vertical position for another 6 days in the dark. Measurements of the hypocotyls and roots were taken for each numbered seedling. The plates were then placed under low light for 2 days and then in 16 hour days of high light to allow greening of the cotyledons and true leaf formation. Genomic DNA was extracted from each numbered seedling according to Edwards *et al*., 1991. The pellet was allowed to air dry and was resuspended in 10 µl of H20. PCR was performed on 1 µl DNA isolated from each seedling using the 35S forward primer and a *LeCTR* gene specific reverse primer in order to determine which seedlings were azygous.

RNA Isolation

2-3 grams of tissue was ground to a powder with liquid nitrogen using a mortar and pestle and extracted with phenol as previously described (Leclerq *et al*., 2002). The pellet was allowed to air dry and was resuspended in DEPC water. The RNA was treated with DNAseI (Promega) followed by a phenol-chloroform extraction.

Real-time Quantitative PCR

Real-time quantitative PCR was performed using 250 ng total RNA for *LeCTR1*, *LeCTR3*, and *LeCTR4sv*, 350 ng for *LeCTR4*, and 2.5 pg for 18S in a 20 µL reaction

volume using Taq-Man One-Step RT-PCR Master Mix reagents (PE Biosystems) on an ABI PRISM 7900HT sequence-detection system. PRIMER EXPRESS software (Applied Biosystems) was used to design gene-specific primers and Taq-Man probes: LeCTR1 forward primer 5'-CATCCTCTTTCTTACTGTGAGAAAATTTAGA-3'; LeCTR1 reverse primer 5'-CATTTCCCTGTATAAAAACGTTCAGTT-3'; LeCTR1 Taq-Man probe 5'-VIC-CCAACTGCCATTAGCAATTTTCAGCTCAA-TAMRA-3'; LeCTR3 forward primer 5'-ACTTCAGGCTTTTGTTCCGTACA-3'; LeCTR3 reverse primer 5'-CCACGAGGAAACGTACAAGTCA-3'; LeCTR3 Taq-Manprobe5'-VIC-CAGCCATTTCTCCCAGAAGAGCATTTGC-TAMRA-3'; LeCTR4 forward primer 5'-CATTTGCAC TTGGTATTTGGCTTA-3'; LeCTR4 reverse primer 5'-CTTATTTAGCCGCCGAAGA GAAT-3'; LeCTR4 Taq-Man probe 5'-VIC-CAAAATCAATCCTGGACAGATGCAGAAACTCAT-TAMRA-3'; LeCTR4sv forward primer 5'-CTTGGACCATGTCTGTTTGTGTATC-3', LeCTR4sv reverse primer, TGGACGAATTATTGTTGACATACCA; LeCTR4sv Taq-Man probe 5'-VIC-CTGTCTCTTGAATCTAATGAATTTAAGAGCTGTTGCCC-TAMRA-3'; 18S forward primer 5'-CGGAGAGGGAGCCTGAGAA-3'; 18S reverse primer 5'-CCCGTGTTAGGATTGGGTAATTT -3'; 18S Taq-Man probe 5'-6FAM-CGGCTACCACATCCAAGGAAGGCA-TAMRA-3'. For LeCTR1, LeCTR3, LeCTR4 and LeCTR4sv, the optimal primer concentration was 900 nM and optimal probe concentration was 250 nM. Optimal primer and probe concentrations for 18S were 300 nM and 125 nM, respectively. RT-PCR conditions were as follows: 48°C for 30 min., 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Samples

were run in triplicate on each 384 well plate and were repeated on at least two plates for each experiment. For each sample, a Ct (threshold cycle) value was calculated from the amplification curves by selecting the optimal ΔRn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method using 18S as a reference. To demonstrate that the efficiencies of the LeCTR (target) and 18S (reference) were approximately equal, the absolute value of the slope of the log input amount (ng of total RNA) vs. delta Ct was calculated and determined to be <0.1 for each LeCTR and 18S set. To determine relative fold differences, the average Ct value for each target was normalized to the average Ct value for 18S and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$.

RNA Gel-Blot Analysis

10 μg of total RNA was fractionated on a 1.2% agarose gel containing formaldehyde in Phosphate buffer and transferred to a Hybond-N membrane (Amersham) following the manufacturer's instructions. Probes were radiolabeled as described previously. Blots were hybridized and washed at 65°C. Washes were carried out to 1XSSC and 0.05% (w/v) SDS.

CHAPTER III

DEVELOPMENT OF TRANSGENIC TOOLS FOR FUNCTIONAL ANALYSIS OF THREE CTR1-LIKE GENES IN TOMATO

Introduction

Mechanisms by which plants perceive and respond to ethylene during the process of fruit ripening remain an interesting question difficult to address in the otherwise excellent model for analyzing ethylene signal transduction, *Arabidopsis thaliana*. While the Arabidopsis carpal (silique) matures and senesces, it does not undergo the type of changes in expansion and maturation associated with ripening of fleshy fruits (reflecting differences in seed dispersal mechanisms). The physiological importance of ethylene for fruit ripening in tomato has been demonstrated as plants altered in their expression of genes involved in ethylene biosynthesis and perception exhibit inhibition or delay of ripening (Klee, 1993; Lanahan *et al*., 1994; Oeller *et al*., 1991). In contrast, ethylene may not play a very important role in silique dehiscence in Arabidopsis, as ethylene insensitive receptor mutants exhibit a normal time-course and manifestation of dehiscence (Ferrandiz, 2002). In this regard an important question is: has the critical dependence on ethylene for normal maturation of climacteric fruits in particular, resulted in any alteration or modification of the general ethylene signaling model defined in Arabidopsis? Indeed, in apparent contrast to Arabidopsis, there are at least three functional CTR1-like genes in tomato while only one *CTR1* gene in Arabidopsis. It is

possible that these multiple CTRs in tomato participate in ethylene signal transduction as it is defined in Arabidopsis, though offering the opportunity for further levels of regulatory complexity that may facilitate biological differences influenced by ethylene, such as ripening.

In support of this hypothesis, several notable differences exist between Arabidopsis and tomato ethylene signal transduction at the receptor/CTR1 complex level. Reduced expression of just one receptor in tomato causes a dramatic constitutive ethylene phenotype (Tieman *et al*., 2000), in sharp contrast to what has been reported for Arabidopsis (Hua and Meyerowitz, 1998). Additionally, reduced expression of subfamily II receptor, *NR*, results in increased expression of subfamily I receptor *LeETR4* resulting in normal progression and completion of ripening (i.e. functional compensation) (Tieman *et al*., 2000). This phenomenon has not been reported for Arabidopsis ethylene receptors, in fact, loss of function of both subfamily 1 receptors could not be rescued by subfamily II receptors (Wang *et al*., 2003). However, it is possible that this difference reflects the fact that tomato has three subfamily I receptor isoforms and only one receptor was suppressed in the tomato study. Further differences exist at the expression level of the receptor/CTR1 complex as modulation of mRNA expression in tomato occurs at both the levels of receptors and CTRs, while only receptors respond to ethylene in Arabidopsis (see chapter 2). The significance of the apparent ethylene-inducibility of *LeCTR1* in root, leaf, and fruit tissues remains unknown. Rather than regulating irreversible processes such as abscission or fruit ripening, ethylene inducibility of *LeCTR1* might serve to mediate responses stresses such

as flooding or wounding that disappear over time and which elicit the production of ethylene, as has been predicted for the receptors (Klee, 2000).

Ectopic over-expression of a gene is a powerful method for examining its function, particularly if it is a member of a gene family. Briefly, the gene of interest is placed in front of a strong constitutive promoter (eg. CaMV 35S or any of a number of enhanced versions of this or other promoters for plants) followed by transcription terminator sequences (eg. Nos terminator from *Agrobacterium* T-DNA). The construct including selection for genome integration (eg. NPTII) is transformed into plants via *Agrobacterium tumefaciens*. The resulting transgenic lines will generate various levels of expression of the transgene depending on insertion site and number of inserts including instances of reduced expression (co-suppression) of both the transgene and endogenous gene (Fray and Grierson, 1993).

RNAi (RNA interference) is a relatively new technology to plants and is an effective method of reducing the level of a specifically targeted endogenous gene (Wang and Waterhouse, 2001). The principle behind RNAi is that double stranded RNA can trigger post-transcriptional gene silencing (PTGS) in plants, which results in sequence specific degradation of the corresponding RNA sequence. RNAi constructs are designed so that a small (300-500bp) inverted repeat is formed resulting in hairpin RNA, which has been shown to highly effective in inducing the silencing machinery in 90-100% of independently transformed lines (Wesley *et al*., 2001).

In order to determine the individual functions for each member of the tomato *CTR1-like* gene family (if any), and to shed light on questions regarding the evolutionary

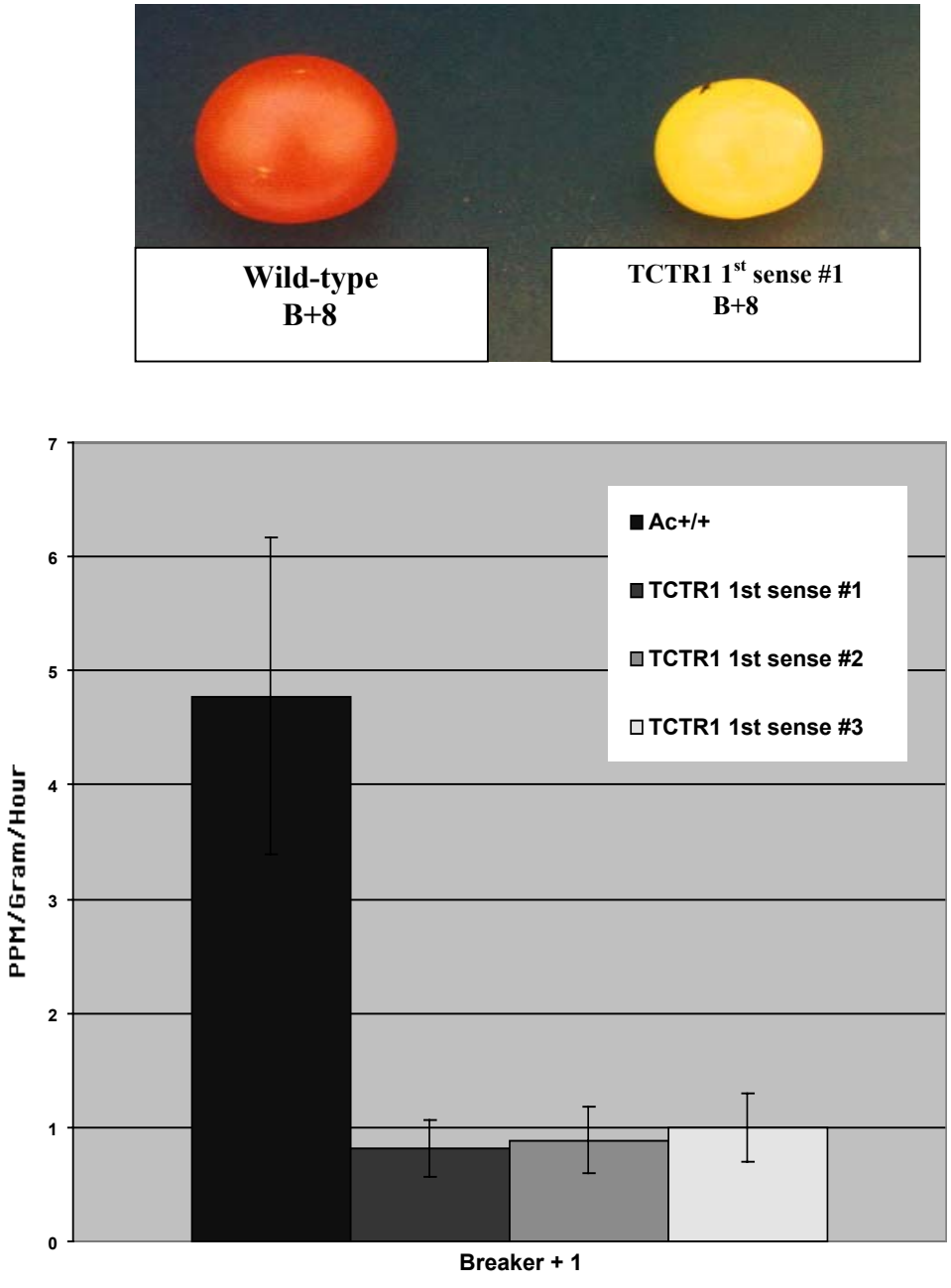


Figure 16. TCTR1 1st sense construct transgenic lines exhibit a delay in fruit ripening.
Fruit from the T1 generation were harvested at breaker and allowed to ripen detached from the vine. At 8 days post-breaker, fruit from transgenic lines harboring the 1st sense construct remained yellow (top). Gas chromatograph readings indicated these fruit were producing less ethylene than wild-type fruit (bottom).

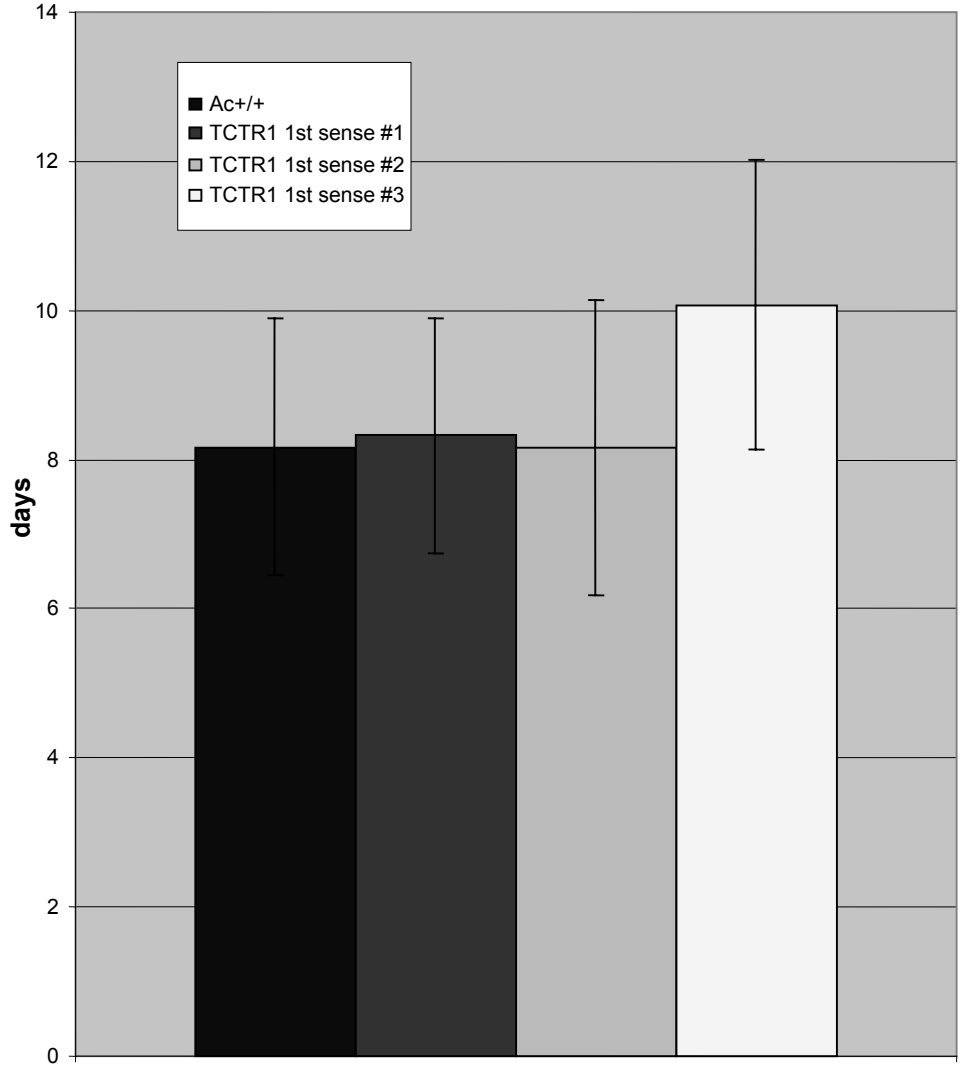


Figure 17. T2 generation TCTR1 1st sense construct fruit do not exhibit a delay in ripening.
T2 generation fruit were examined for ripening inhibition by recording the number of days it took for fruit to go from breaker stage to red ripe while on the vine. There was no significant delay in ripening recorded for any of the lines relative to wild-type.

basis for the ethylene inducibility and retention of multiple CTRs in tomato, constructs were designed to over-express as well as repress the expression of each individual gene in transgenic tomatoes. Transgenic plants over-expressing LeCTR1 generated by a previous graduate student were analyzed and a plan to functionally characterize new transgenics is outlined.

Results

Two constructs, designated TCTR1 1st sense and TCTR1 2nd sense, which overexpressed LeCTR1 behind the CaMV35S promoter, were transformed into tomato. The progeny from the T1 generation of plants harboring the TCTR1 1st sense construct were characterized (Kannan, unpublished results). As LeCTR1 is presumed to act as a negative regulator of ethylene responses, it was expected that overexpression might result in ethylene insensitivity. Indeed, these plants displayed a remarkable delay in ripening, although seedlings, leaves and flowers did not exhibit ethylene insensitivity (Kannan, unpublished results). In an effort to confirm the delayed fruit ripening and lower ethylene production phenotype observed by Kannan (Figure 16), plants for each of the 3 lines which exhibited this delay were allowed to self pollinate and seed was harvested for study in the next generation. The progeny were tested for presence of the transgene through both Southern analysis and PCR confirmation as described in Experimental Procedures (data not shown). Flowers were tagged at anthesis and marked at breaker and red ripe stages. Fruits were harvested at MG, B, B+3 and B+7 stages of fruit development and ethylene measurements were taken with a gas chromatograph

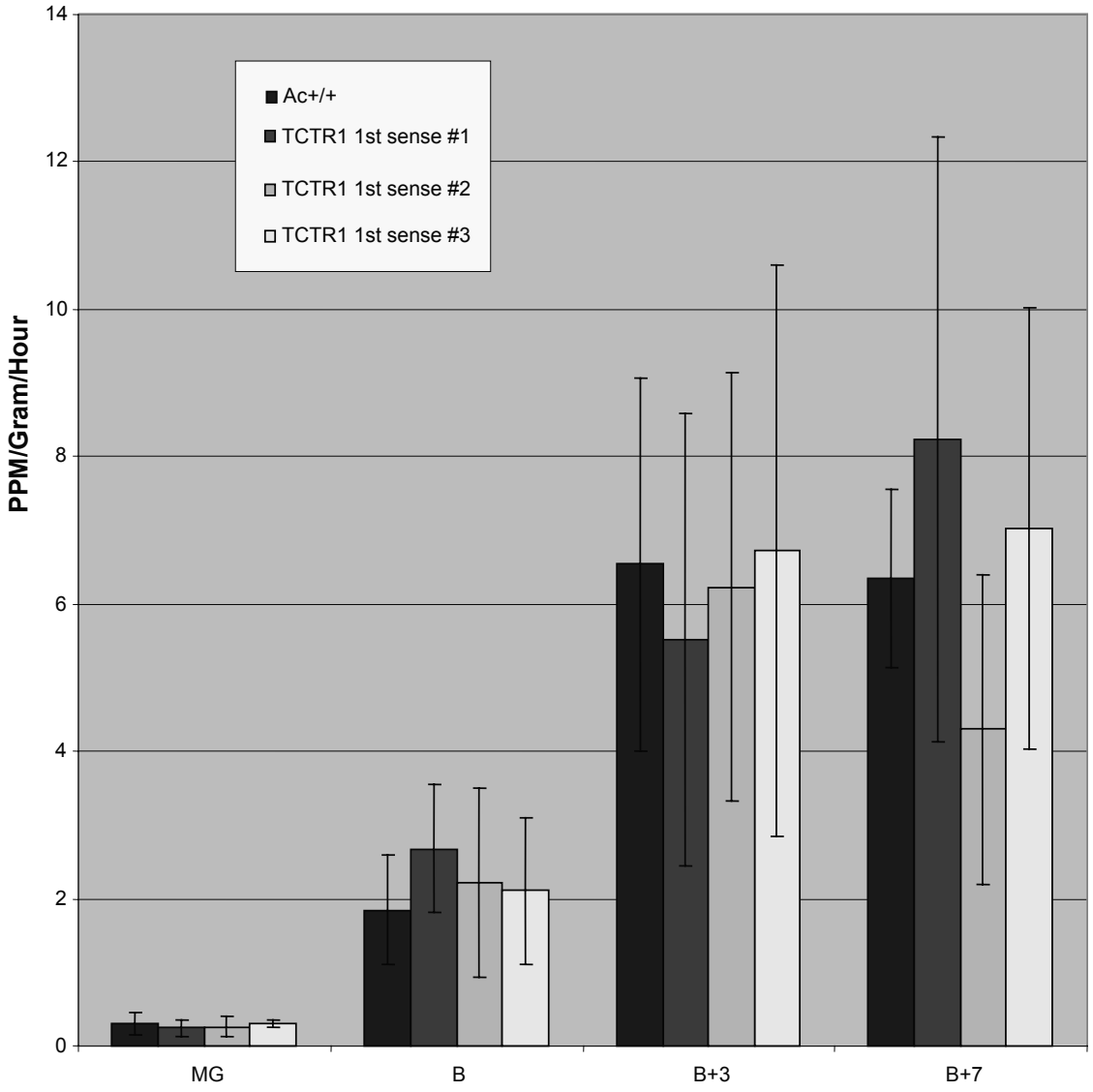


Figure 18. T2 generation TCTR1 1st sense construct fruit do not produce less ethylene than wild-type fruit.
T2 generation fruit were harvested at the indicated developmental stage and measurements of ethylene evolution were recorded using a gas chromatograph. No significant difference from wild-type was observed during any stage of fruit development tested.

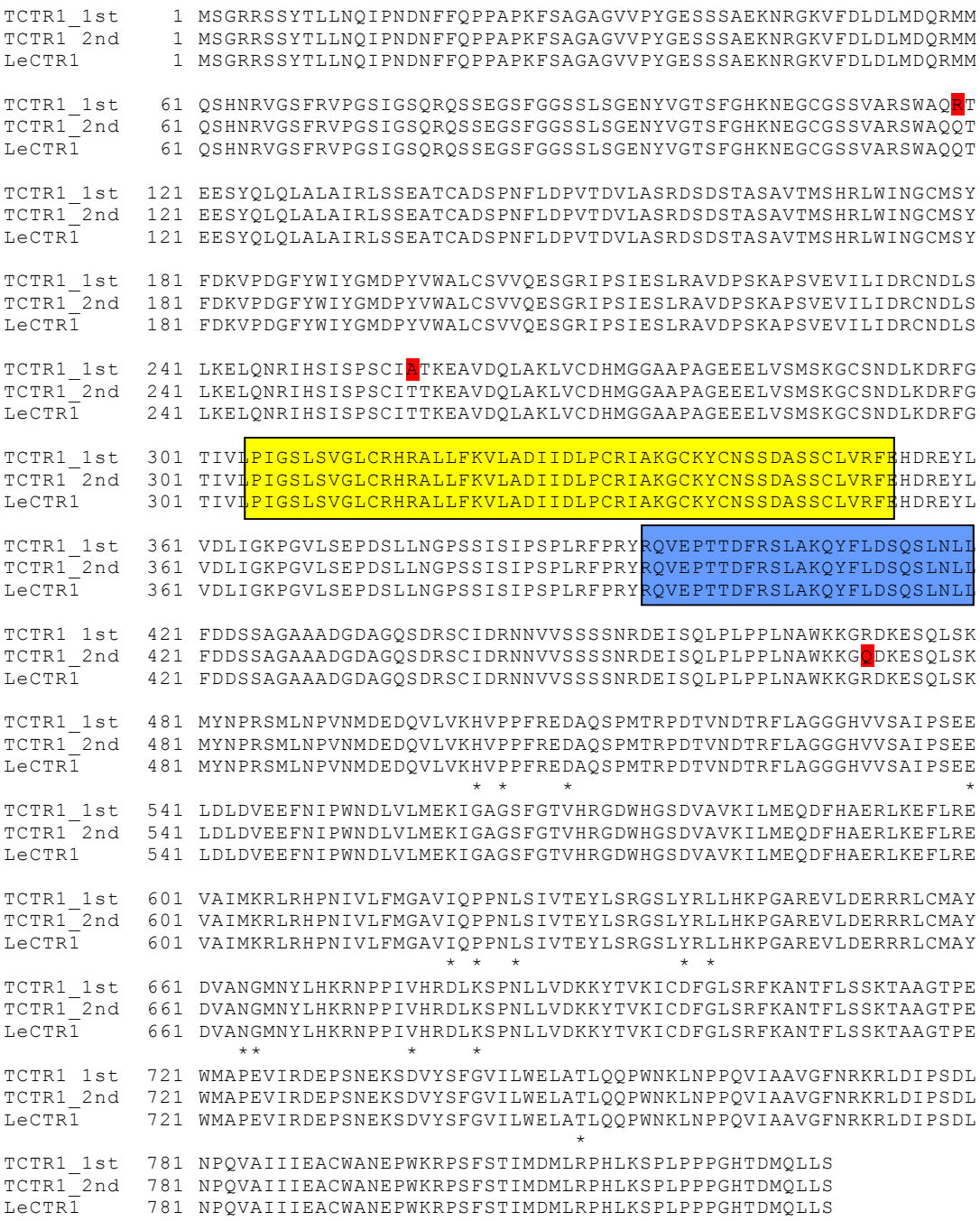


Figure 19. Amino acid sequence alignments for TCTR1 1st and 2nd sense constructs compared to *LeCTR1* sequence.

Mutations in both TCTR1 constructs inherited at the beginning of this project were discovered and are shown highlighted in red. However, none of the mutations occurred in conserved N-terminal domains (CN in yellow and CS in blue) or conserved residues in the kinase domain (denoted with asterisks).

(Photovac Markham, Ontario, Canada) and recorded for each stage of fruit development. Careful and repeated examination revealed that there were no significant differences from the wild-type control plants in ripening time neither on the vine (Figure 17) nor off the vine (data not shown). Likewise, there were no significant differences during any stage of fruit ripening examined (Figure 18). I also determined through DNA sequencing that the construct used in this study had 2 nucleotide substitutions which resulted in the alteration of the predicted peptide sequences, although none of the altered residues occurred in conserved regions (Figure 19).

Five independent lines harboring another sense construct designed by Kannan, designated TCTR1 2nd sense, were generated. Plants at the T0 generation were self-pollinated to allow for further analysis on the T1 generation. In line #3, leaves from plants which were positive for the transgene senesced earlier and showed severe epinasty compared to wild-type (Figure 20), and flowers on the transgenic lines senesced before they reached maturity (Figure 20). The phenotype was delayed in manifesting itself, so that early flowers and fruit developed normally. Additionally, in two separate plantings, approximately 4 out 30 plants were dwarfs (Figure 21) and did not produce fruit.

In line #2, the leaves were not epinastic, but the fruit ripened abnormally in "sectors" (Figure 22). However, the fruit would eventually turn completely red. An RNAse protection assay determined that the transgene was indeed being expressed indicating that the phenotypes could not be due to co-suppression (Figure 23). No observable differences from wild-type in any of these phenotypes were recorded for the other three transgenic lines (data not shown). It was also determined that this construct

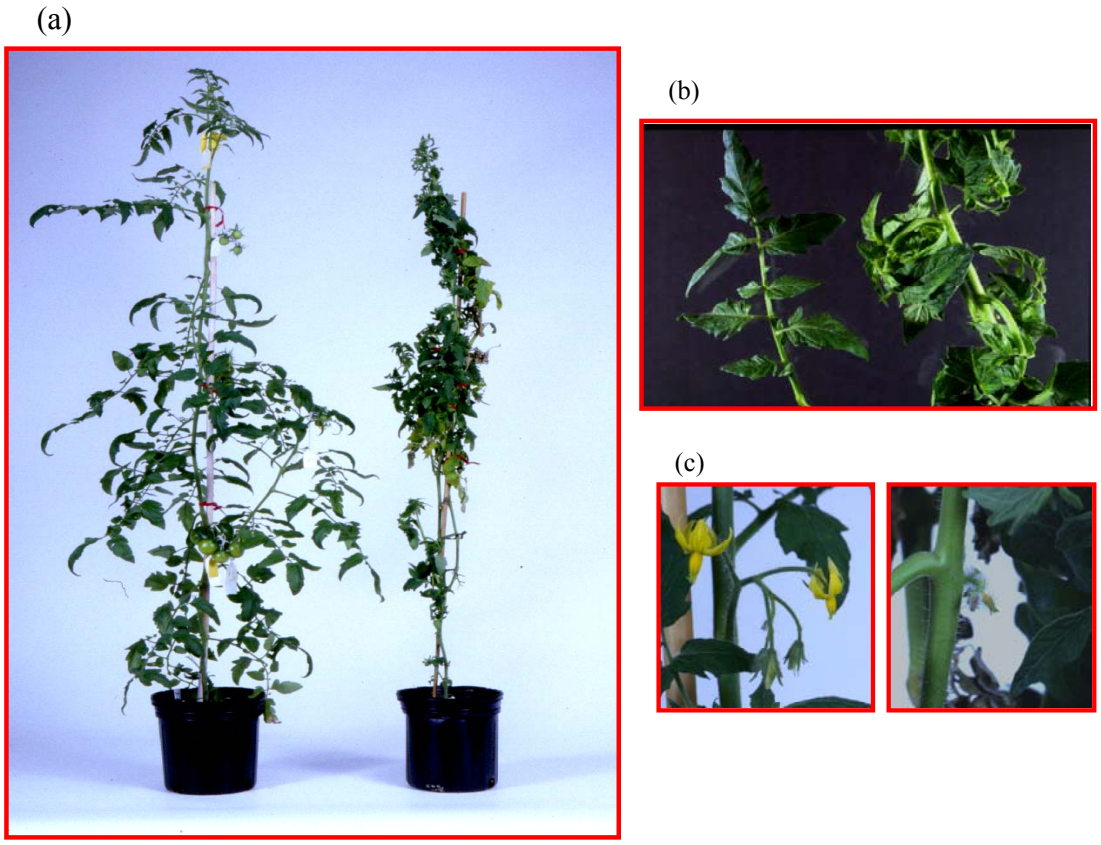


Figure 20. Tomato plants over-expressing TCTR1 2nd sense construct show constitutive activation of ethylene phenotypes in line #3.
(a) Wild-type tomato plant on the left compared to line#3 on the right at the same age reveals accelerated senescence of leaves in line #3. (b) Wild-type tomato leaf branch is shown on the left, compared to line #3 transgenic leaf branch, which exhibits severe curling of the petioles. (c) Flowers in line #3 (on the right) senesce before becoming mature compared to wild-type (on the left).

Figure 21. Wild-type tomato compared to a "dwarf" plant from TCTR1 2nd sense line #3.
Both genotypes were planted at the same time and are approximately 8 weeks post germination. Wild-type is shown on the left, a "dwarf" segregant is shown on the right.

Figure 22. Blotchy fruit phenotype displayed by TCTR1 2nd sense line #2.
Fruit from line#2 harboring the TCTR1 2nd sense construct ripened in a blotchy manner (top and bottom right) as compared to fruit taken from wild-type plants grown side-by-side in the greenhouse (bottom left).

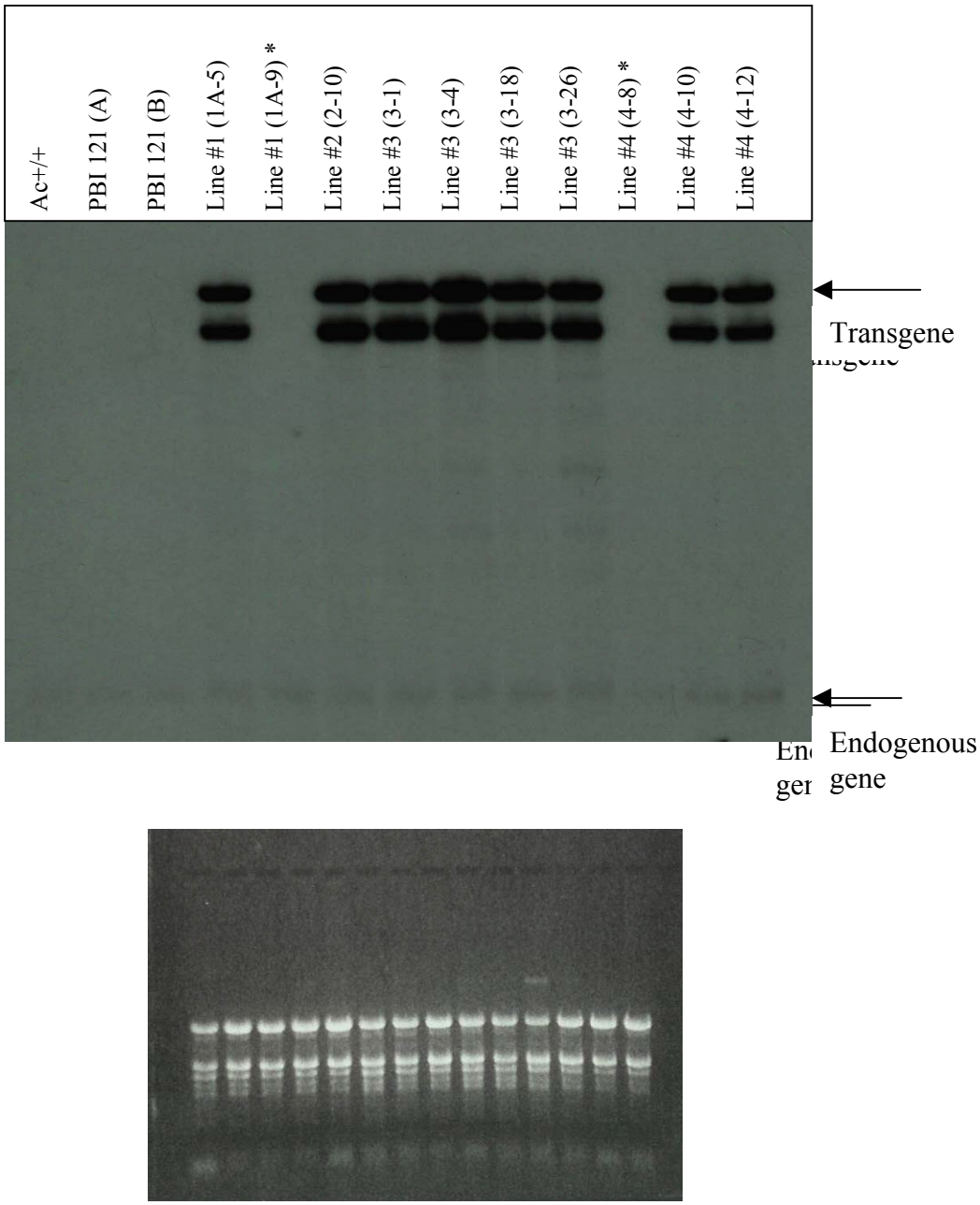


Figure 23. RNAse protection assay indicates that the transgene is being expressed in TCTR1 2nd sense construct transgenic lines.
The probe I designed for this assay contained a portion of the endogenous gene and the Nos terminator from the construct. This assay was performed by C.S. Barry from RNA that I isolated from control plants and transgenic plants. Line #1 contains the TCTR1 1st sense construct. Below is the ethidium bromide stained RNA used in the assay. The asterisk (*) denotes confirmed non-transformed segregants from that line.

had one nucleotide substitution which resulted in the alteration of one amino acid in the predicted peptide sequence and which was also not in a conserved domain (Figure 19).

A new construct for LeCTR1 and constructs containing LeCTR3, LeCTR4, and LeCTR4sv1 in the sense orientation behind the CaMV35S promoter were engineered when I took over this project (Figure 24). In addition, I designed and created RNAi constructs for LeCTR1, LeCTR3, and LeCTR4 (Figure 24). Transformation of tomato is underway and several lines have already been generated for each construct (Table 4). Selection for homozygous plants is in progress. In order to expedite the process, a method to determine heterozygous and homozygous plants at the T1 generation has been developed. Briefly, the membrane containing digested genomic DNA from T1 plants is hybridized to P32 labeled NPTII and "control" probes simultaneously. Presence of the NPTII band indicates that the plant is transformed, while the "control" is simply intron sequence, which should hybridize equally to any plant (essentially a DNA loading control). The intensity of the NPTII band is compared to the "control". A homozygous plant (i.e. with double the copies of NPTII) should be twice the relative intensity as compared to the "control" hybridization signal. (Figure 25). After selecting putative homozygous plants, PCR on the next generation of plants will be performed, to ensure homozygosity.

Discussion

Analysis of two separate generations of plants harboring the TCTR1 1st sense construct gave contrasting results. It is important to note that the study on the T1 generation was

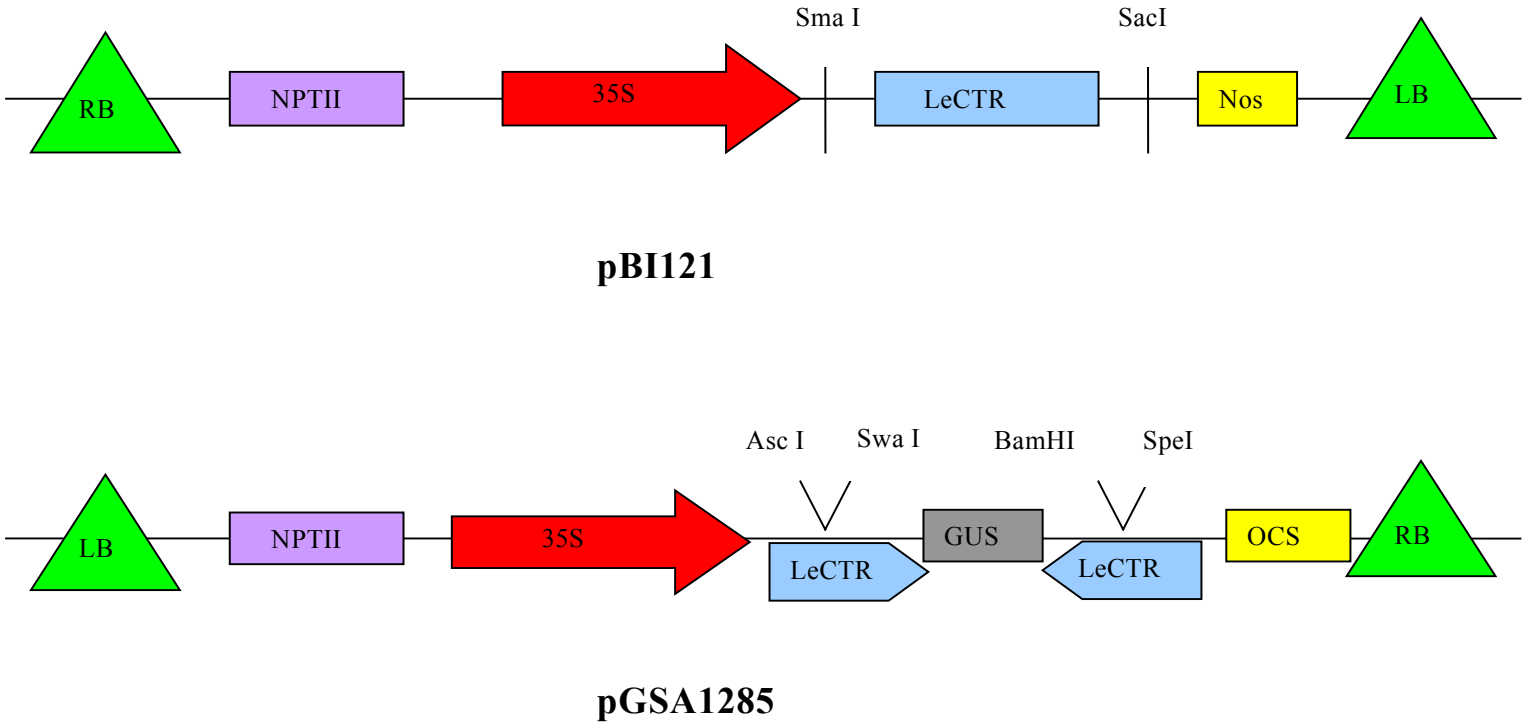


Figure 24. Plasmid vector maps for sense and RNAi constructs.
The sense construct is shown at the top and the RNAi construct at the bottom. For the sense construct, the full-length LeCTR sequence is placed between the SmaI and SacI restriction sites so that it is in front of the 35S promoter in the sense orientation. For the RNAi construct, a portion of the LeCTR gene is placed between AscI/SwaI and BamHI/SpeI restriction sites on either side of the GUS gene so that double stranded RNA will form from a hairpin loop when transcribed from the 35S promoter. LB and RB refer to left border and right border sequences of the T-DNA, respectively, NPTII refers to the kanamycin resistance, and Nos and OCS are termination sites.

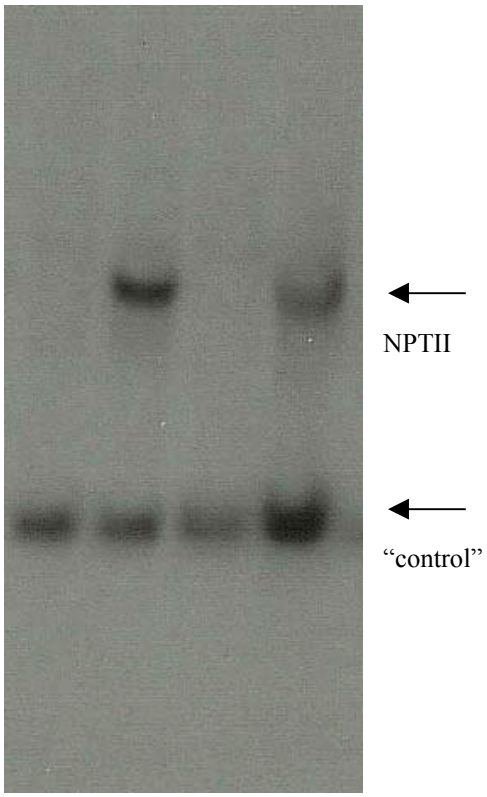


Figure 25. Screen for homozygous transgenic tomato plants.
DNA digested with EcoRI, is blotted to a membrane and probed simultaneously with P32 labeled NPTII and "control" probes. 1st lane, wild-type; 2nd lane, homozygous plant; 3rd lane, non-transformed plant; 4th lane, heterozygous plant.

Table 4. Number of independent tomato transgenic lines generated for each construct.

Construct	# of lines
LeCTR1 sense	18
LeCTR3 sense	13
LeCTR4 sense	18
LeCTR4sv1 sense	20
LeCTR1 RNAi	4
LeCTR3 RNAi	1
LeCTR4 RNAi	5

performed during the summer while the study on the T2 generation was performed in the late fall. Additionally, in the first study, fruit that were detached from the vine ripened more slowly than on the vine and delayed ripening was more dramatic the older the plants grew, or as the summer progressed (data not shown). These differences in analysis may explain the opposing results. High temperatures for an extended period of time can cause inhibited carotenoid biosynthesis and lower production of ethylene in tomato by inhibiting the accumulation of ripening-related mRNAs (Lurie *et al*., 1996). Transgenic fruit with lowered ethylene production also are known to ripen more slowly when detached from the stem than on the vine (Klee, 1993). This is presumably due to escape of internal ethylene by diffusion through the stem scar lowering the concentration of ethylene below the threshold that is necessary for normal ripening (Klee, 1993). While no differences in ripening time or ethylene evolution were evident in the T2 generation, it is possible that the delay of ripening in plants harboring the TCTR1 1st construct was enhanced due to lower production of ethylene as a result of high temperatures during the summer and when detached from the vine, thus enabling a measurable difference from wild-type to be recorded for those lines. There could be a threshold between ethylene produced and levels of the TCTR1 negative regulator present in the fruit. With less ethylene being produced, high levels of TCTR1 could have more of an impact.

The constitutive ethylene response exhibited by line #3, which contained theTCTR1 2nd construct, was unexpected as it is the opposite phenotype that would be predicted by over-expressing a negative regulator of ethylene response. It was the only line out of 5 that showed this severe ethylene phenotype. Even though the fruit in line#2

ripened in sectors, the fruit eventually ripened normally. This "blotchy fruit" phenomenon has been seen in transgenic tomato fruit engineered to over-express isopentenyl transferase behind a fruit specific promoter though a definitive reason for the phenotype could not be given (Martineau *et al*., 1994). It is possible that the constitutive ethylene phenotype displayed by line #3 is due to the insertion of the T-DNA itself and is not related to the transgene. While line #3 had more insertions of the transgene (data not shown), RNAse protection analysis did not indicate any increased expression over the other lines that contained fewer insertions (Figure 23). In a population of 30 plants, there was significant segregation of the transgenes; however no correlation could be made with phenotype to a single locus. Further studies could be initiated to determine if the phenotypes observed are due to insertion into an important ethylene-regulated gene.

Analysis of the transgenic plants over-expressing LeCTR1, LeCTR3, LeCTR4, and LeCTR4sv1 and with reduced expression of LeCTR1, LeCTR3 and LeCTR4 are underway. Homozygous plants are currently being selected. The fruit and leaves will be frozen for RNA extraction for real time Q-RT PCR analysis. The levels of transgene and endogenous genes will be monitored as well as expression of the other *CTR1-like* genes for each line to see if functional compensation exists at the CTR level in tomato. Seedlings will be measured in their response to treatment with the ethylene precursor, ACC (essentially an ethylene treatment). Flowers will be tagged at anthesis to determine if there is any delay in the time from anthesis to breaker and breaker to red ripe. Ethylene measurements on the fruit and leaves will be taken with a gas chromatograph to determine if any change in ethylene production occurs. Further analysis could include

examination for altered response to salt stress or pathogens and microarray analysis to gain a broader view of global gene expression in response to the various transgenes.

Experimental Procedures

Plant material

Tomato (*Lycopersicon esculentum* cv. Ailsa Craig) plants were grown in a naturally illuminated greenhouse under standard conditions and tissue was collected as described in Chapter 2.

Generation of sense and RNAi constructs

Sense constructs were engineered by cloning the full length cDNAs for each LeCTR gene (described in Chapter 2) into the SacI and SmaI sites of the pBI121 vector. Each sense construct was sequenced to check for errors. LeCTR1, LeCTR3 and LeCTR4 sequences were engineered into the pGSA1285 dsRNA vector (www.chromdb.org) to form an inverted repeat when transcribed in the plant. For each construct, 2 primers were designed which included approximately 300 bp of the 5' UTR and 300 bp of coding sequence in order to target each specific gene. The forward primer contained an adapter with Spe1 and Asc1 restriction sites while the reverse primer contained an adapter with BamH1 and SwaI restriction sites. The sequences for each primer are as follows: TCTR1 RNAi For1 5'-GGACTAGTGGCGCGCCAGCCATGGGTTAAGTTC AGC-3';TCTR1 RNAi Rev1 5'-CGGGATCCATTTAAATCGTTGATCCATCAAGTC AA3-3';TCTR3 RNAi For1 5'-GGACTAGTGGCGCGCCAGGCCAAATGGGTTGTA AGA-3'; TCTR3 RNAi Rev1 5'-CGGGATCCATTTAAATCACCTGTTTTGCCTTT ACCC-3'; TCTR4 RNAi For1 5'- GGACTAGTGGCGCGCCTTGGGGAACTGAATT

TGTCC-3'; TCTR4 RNAi Rev1 5'- CGGGATCCATTTAAATGTTGAGCCCAACTT TTCGAC-3'. For each primer pair, PCR was performed with *Pfu* Polymerase (Stratagene). The gel purified PCR product was digested with Asc1 and SwaI and ligated into AscI/SwaI cleaved pGSA1285 vector (http://www.chromdb.org). Once the construct was confirmed through sequencing, PCR was performed again with the same set of primers, and this time was digested with BamHI and SpeI and inserted into the BamH1/SpeI cleaved vector containing the first insert. Each construct was confirmed by digesting with appropriate restriction enzymes to indicate both inserts were in the proper orientation.

Once each construct was confirmed, it was transformed into *Agrobacterium tumefaciens* strain LBA4404. Transformation into wild-type tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) followed the protocol as outlined in Fillatti *et al*., 1987.

Confirmation of transgene and determination of homozygous plants

DNA was isolated following the protocol described in Fulton *et al*., 1995. PCR was performed as described in Chapter 2 to confirm the presence of the transgene. Southern analysis was performed as described in Chapter 2 to determine transgene copy number. In addition to NPTII, the membrane was simultaneously hybridized to a "control" probe generated from an intron sequence from LeCTR3 using the following primers: TCTR3 2nd gap For 5'-CGTCCTGATAGCTGCTCCTC-3' and TCTR3 2nd gap Rev 5'-TGGTTAGTGAGGTTCCAGCTT-3'.

RNA isolation and RNAse Protection Assay

RNA was isolated as described in Chapter 2. The primers used to generate the probe for the RNAse protection assay were as follows: TCTR (RNAP) FOR 5'-ACCAAGTTTTCTTCGCAAGCTC-3' and NOS 163BP REV 5'-AAAACCCATCTCA TAAATAACGTCA-3'. The RNAse protection assay was performed by C.S. Barry.

CHAPTER IV

CHARACTERIZATION OF AT4G24480: THE NEAREST CTR1 HOMOLOGUE IN THE ARABIDOPSIS GENOME IMPACTS ABA BUT NOT ETHYLENE RESPONSE

Introduction

It is intriguing that while three *CTR1* gene family members possessing CTR1 function exist in tomato, there only appears to be only one *CTR1* gene possessing ethylene signaling capability in Arabidopsis. *B. oleraceae*, a close relative to Arabidopsis, has two putative gene sequences more similar to *AtCTR1* than any other gene in the Arabidopsis genome, suggesting that Arabidopsis either lost or *B. oleraceae* gained one *CTR1-like* gene since their evolutionary divergence. One possibility is that *CTR1-like* sequences in Arabidopsis may have rapidly diverged through subfunctionalization if there was no selective pressure to retain multiple *CTR1* genes in Arabidopsis.

Mounting evidence implicates the involvement of an additional factor functioning similarly to CTR1 as a negative regulator of ethylene signaling in Arabidopsis. This hypothesis is based on the observations that 1) *ctr1* loss-of-function mutants retain ethylene responsiveness (Larsen and Chang, 2001), 2) quadruple mutants in the ethylene receptor gene family display a constitutive ethylene response phenotype more severe than *ctr1* loss-of-function mutants (Hua and Meyerowitz, 1998), 3) a mutation in *RAN1* which disrupts the assembly of the copper cofactor into the ethylene

receptor has a stronger constitutive ethylene response phenotype than *ctr1* mutants (Woeste and Kieber, 2000), and 4) *etr1-7;ctr1-1* double mutants have shorter hypocotyls and roots than the *ctr1-1* mutant alone (Hua and Meyerowitz, 1998). These observations suggest that either multiple *CTR1* function encoding homologues exist in Arabidopsis or an alternate protein functions in parallel to CTR1 in Arabidopsis.

An obvious candidate for a second *CTR1* functioning gene in Arabidopsis would be one with similar peptide structure as predicted by amino acid homology. Based on amino acid sequence and phylogenetic analysis, CTR1 belongs to subgroup B3 of group B MAPKKKs (Ichimura *et al*., 2002). *At4g24480* is a member of the subgroup B3 MAPKKKs and is predicted to encode a serine/threonine protein kinase. It is the next most similar gene to *AtCTR1* with respect to amino acid identity. At4g24480 shares only 38.8% amino acid identity for the full length protein sequence with CTR1. Broken down into N-terminal and C-terminal domains, At4g24480 shares 64% identity in the kinase domain and 28.5% in the N-terminal domain as compared to CTR1. Interestingly, the CN domain found within the N-terminal portion of the protein is highly conserved between At4g24480 and CTR1 (sharing 60% identity), while the EC domain (see Chapter 2) is not found in At4g24480.

The CN domain appears to be important for association of CTR1 with the receptors (Clark *et al*., 1998; Gao *et al*., 2003; Huang *et al*., 2003). In the *ctr1-8* mutant, the highly conserved glycine at residue number 364 is predicted to change to a glutamine in the CN box of the N-terminal domain rendering it constitutively responsive to ethylene (Huang *et al*., 2003). This mutation is the only mutant *ctr1* allele identified to

date that does not affect the kinase domain, rather it disrupts the interaction with ETR1 (Gao *et al*., 2003; Huang *et al*. 2003). A mutant allele typically used in the study of the CTR1 step in Arabidopsis ethylene signal transduction is *ctr1-1*. *ctr1-1* harbors an amino acid substitution in residue 694 changing this highly conserved kinase domain aspartate to glutamate (Kieber *et al*., 1993). While *ctr1-1* can associate with the receptor, it has been shown to be deficient in kinase signaling (Gao et al., 2003; Huang *et al*. 2003). Interestingly, all mutant alleles for *ctr1* recovered in screens for mutants displaying sensitivity to other hormones and signaling molecules (see Chapter 1) harbor mutations in the kinase domain. The nature of these two mutations makes the two corresponding alleles useful in understanding the biochemical mechanism of ethylene signaling and possibly cross-talk with other signal transduction pathways.

In order to gain insight into the potential function of At4g24480, characterization of two T-DNA insertion lines disrupting this gene was performed by measuring responses to ethylene, sugar, osmotic stress and abscisic acid. Due to the unique nature of the *ctr1-8* mutation residing in the N-terminal domain, *ctr1-8* was also examined for response to these same treatments.

Results

Characterization of ethylene related phenotypes in At4g24480

To determine whether At4g24480 plays a role in ethylene signaling, two putative mutant lines were obtained from SALK that contained T-DNA inserts in (S025685), or just upstream of (S123763), the *At4g24480* gene (Figure 26). Southern analysis indicated

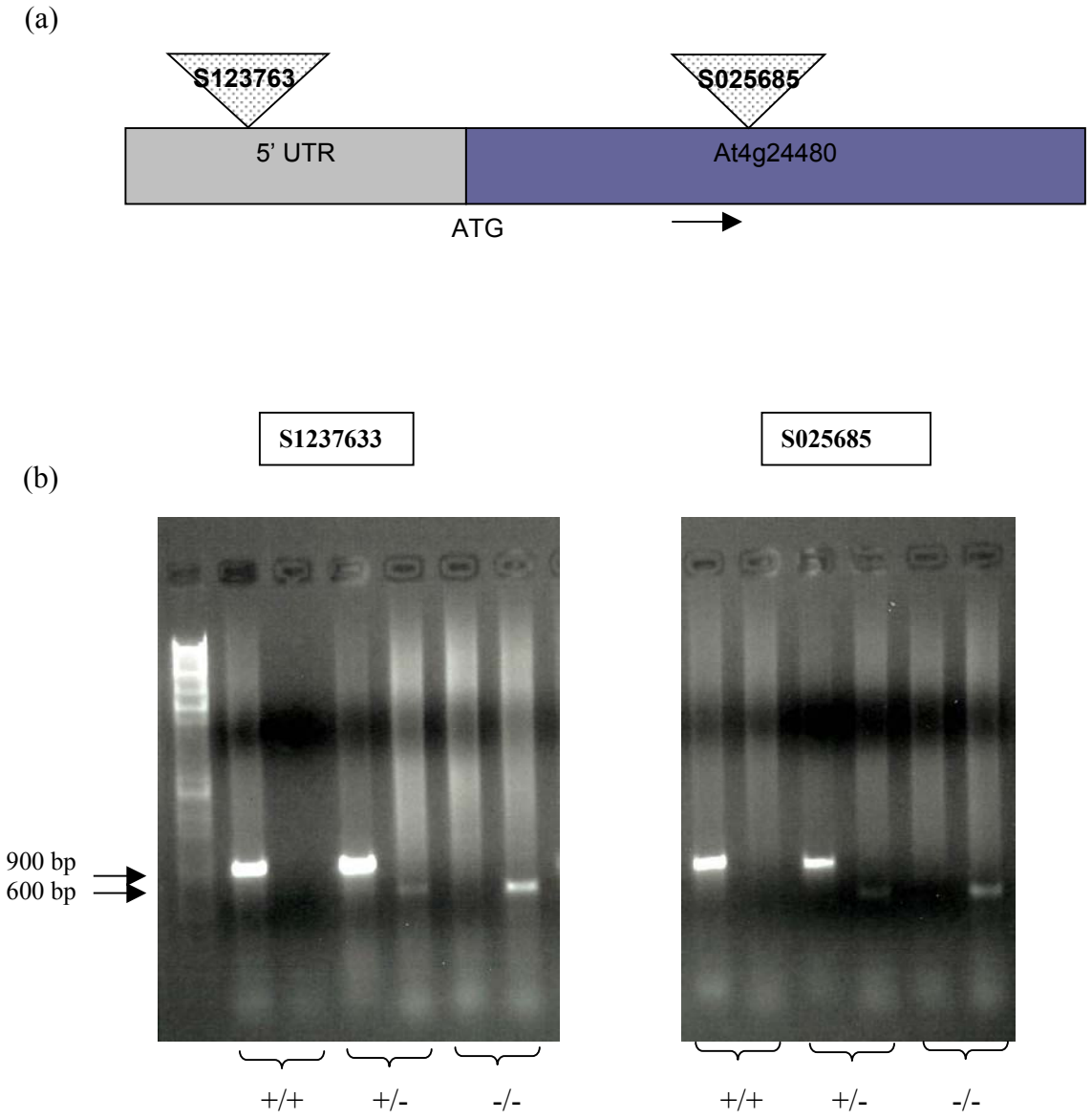


Figure 26. Confirmation of *At4g24480* T-DNA inserts.
(a) The T-DNA in line S123763 is inserted 319 bp upstream of the start codon within the 5'UTR. One of the T-DNAs in line S025685 is inserted 109 bp into exon5 upstream of the kinase domain and downstream of the CN domain. Arrows indicate location of primers used for homozygous screening for each T-DNA as described in Experimental Procedures. (b) T-DNA lines were tested for homozygosity at their respective insertion sites shown in A. Left gel: PCR was performed on DNA using S123763 specific primers from set A in lanes 1, 3, and 5 and from set B in lanes 2, 4, and 6. +/+ denotes wild-type, +/- denotes heterozygous insertion, and -/- denotes homozygous insertion. Right gel: PCR was performed on DNA using S025685 specific primers from set A in lanes 1, 3, and 5 and from set B in lanes 2, 4, and 6.

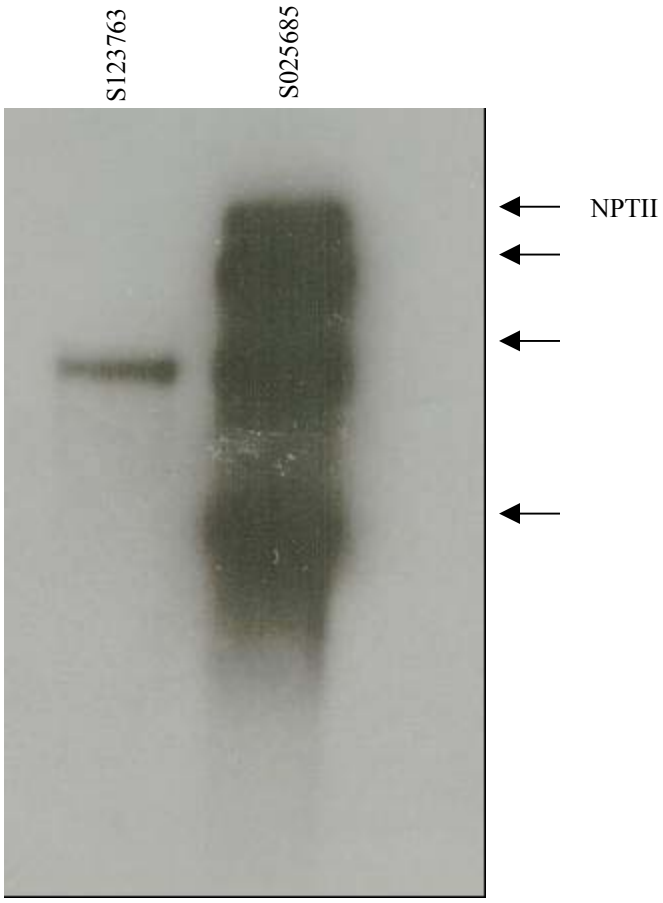


Figure 27. Southern analysis of *At4g24480* T-DNA lines.
DNA was extracted from S123763 and S025685 T-DNA lines and hybridized to a probe for the NPTII gene contained in the T-DNA. Arrows indicate the presence of multiple bands in the S025685 T-DNA line.

that S123763 contained 1 insert while S025685 contained 4 inserts (Figure 27). PCR was performed as described in experimental procedures to screen for plants that were homozygous at the respective integration sites shown in Figure 26. Homozygous *At4g24480* T-DNA lines were examined for seedling triple response phenotypes by growing seedlings in the dark for 5 days with and without ACC (Figure 28). No significant difference from wild-type was observed for either treatment. Loss-of-function mutations in *CTR1* result in global constitutive activation of ethylene response phenotypes including smaller adult rosette leaves and inflorescences, delayed bolting, and infertile flowers. S123763 and S025695 do not exhibit any of these phenotypes at the rosette stage or the adult inflorescence, if fact S123763 is slightly larger than WT (Figure 29).

The homozygous T-DNA lines were also examined for insensitivity to glucose and sucrose. The reason for these treatments is based on the observations that application of exogenous ethylene can phenocopy the glucose-insensitive mutant *gin1* (Zhou *et al*., 1998) and screens for sucrose sensitivity resulted in identification of a sugar-insensitive mutant (*sis1)*, found to be allelic to *ctr1* (Gibson *et al*., 2000). In tests for glucose and sucrose sensitivity, the T-DNA mutants were arrested in the same developmental stage as wild-type (Figures 30 and 31). However, while wild-type periodically exhibited 1 green cotyledon and one bleached cotyledon (Figures 30 and 31), the T-DNA mutants always exhibited two bleached cotyledons. This was not quantified by the criteria used to score the developmental stages (see experimental procedures) but could potentially represent a hypersensitive response. While both *ctr1-1*

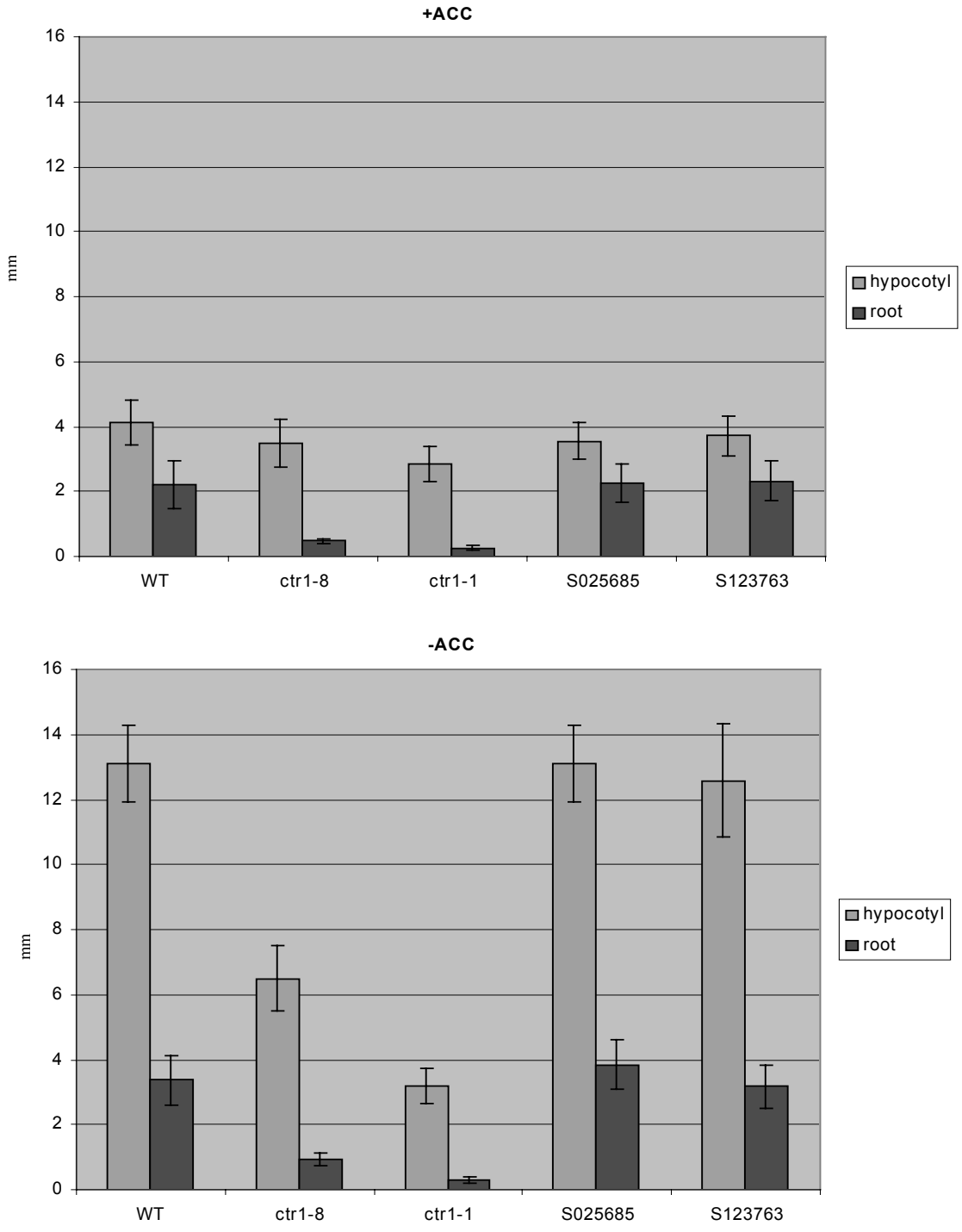


Figure 28. Hypocotyl and root length of etiolated seedlings in response to ACC (top) or without ACC (bottom).

Figure 29. Rosette phenotypes of *At4g24480* mutants.
The Arabidopsis plants shown here were grown for 3-4 weeks under 12 hours of light. S123763 and S025695 rosettes are more similar to wild-type rosette size compared to *ctr1* mutants.

and *ctr1-8* showed similar resistance to high levels of glucose (Figure 30), *ctr1-8* showed somewhat less resistance to high levels of sucrose, particularly with respect to true leaf formation (Figure 31).

At4g24480 exhibits ABA insensitivity

Mutant alleles of *CTR1* and *EIN2* have been discovered in screens for ABA sensitivity/insensitivity indicating cross-talk between the abscisic acid and ethylene pathways (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000), thus *At4g24480* T-DNA mutant lines were examined for atypical response to ABA. *At4g24480* T-DNA insertion line seeds plated on 1um ABA, were slower to germinate (data not shown) and after 10 days, line S025685 seedlings were still slower to emerge from the seed coat and form expanded cotyledons than wild-type (Figure 32). This response was similar to that exhibited by *ein2*, though not quite as dramatic. In contrast, *ctr1-1* germinated before wild-type and showed 100% cotyledon expansion and development of true leaves in agreement with the observations made by Beaudoin *et al*., 2000. Interestingly, *ctr1-8* did not show the same degree of insensitivity as *ctr1-1* (Figure 32).

Seeds were also tested for their ability to germinate under osmotic stress (Figure 33). Both *At4g24480* T-DNA lines were significantly inhibited in response to a high concentration of sorbitol compared to wildtype with respect to seedling emergence and cotyledon expansion. While *ctr1-1* was completely insensitive to sorbitol as described by Gibson et al., *ctr1-8* was slightly more sensitive than wild-type (Figure 33). Seedlings were grown on media without ABA, and then transferred after 5 days relative to control plates without ABA in wild-type seedlings and in *ctr1-1* mutants (Figure 34). However,

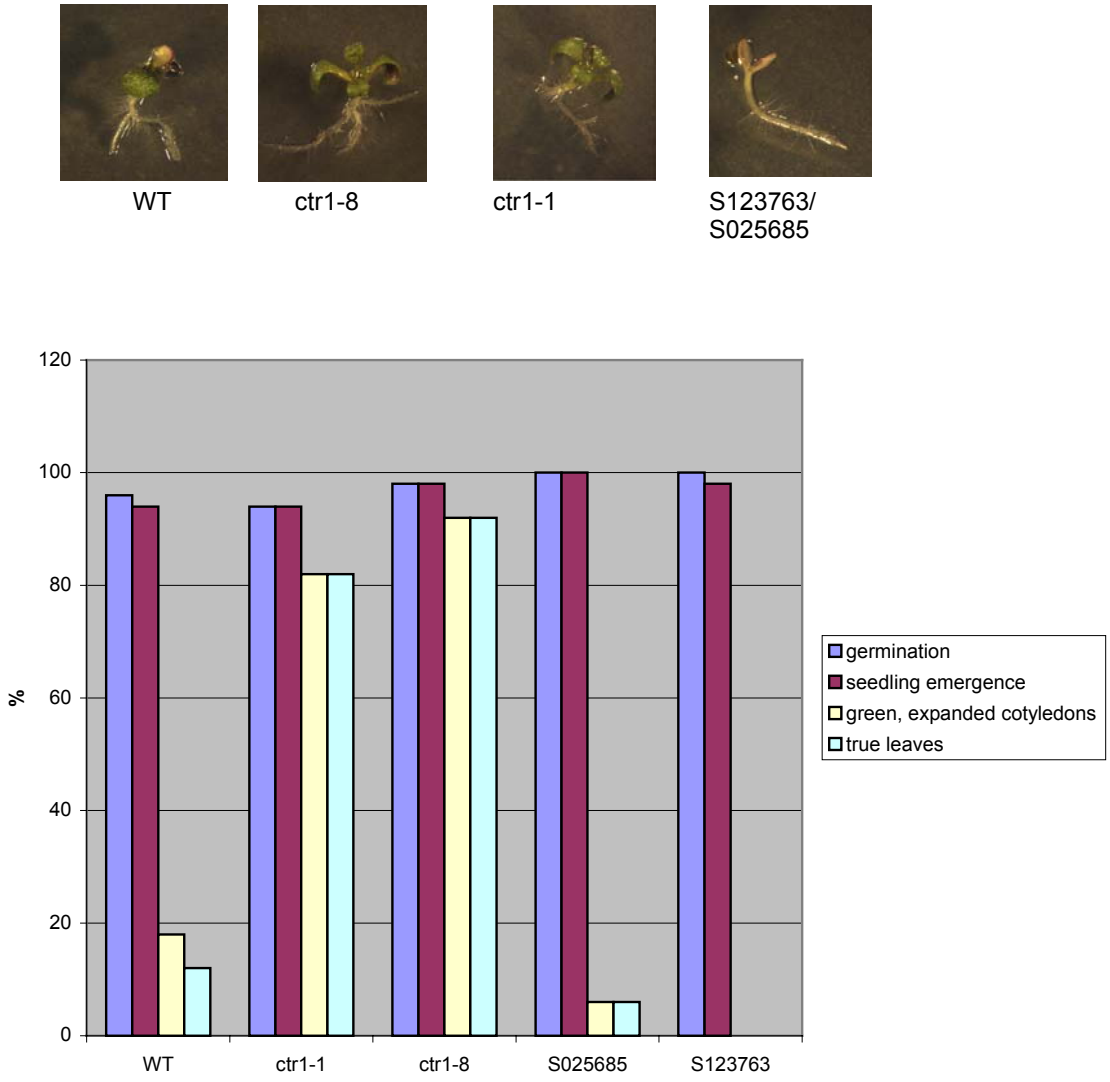


Figure 30. Sensitivity of *ctr1* and *At4g24480* mutants to germination on glucose. seeds were germinated on 6% glucose and the percentage of seedlings in the indicated developmental stage was recorded after 10 days. Pictures above the graph were taken after 10 days and are representative of the stage of development of the majority of the seedlings for that genotype.

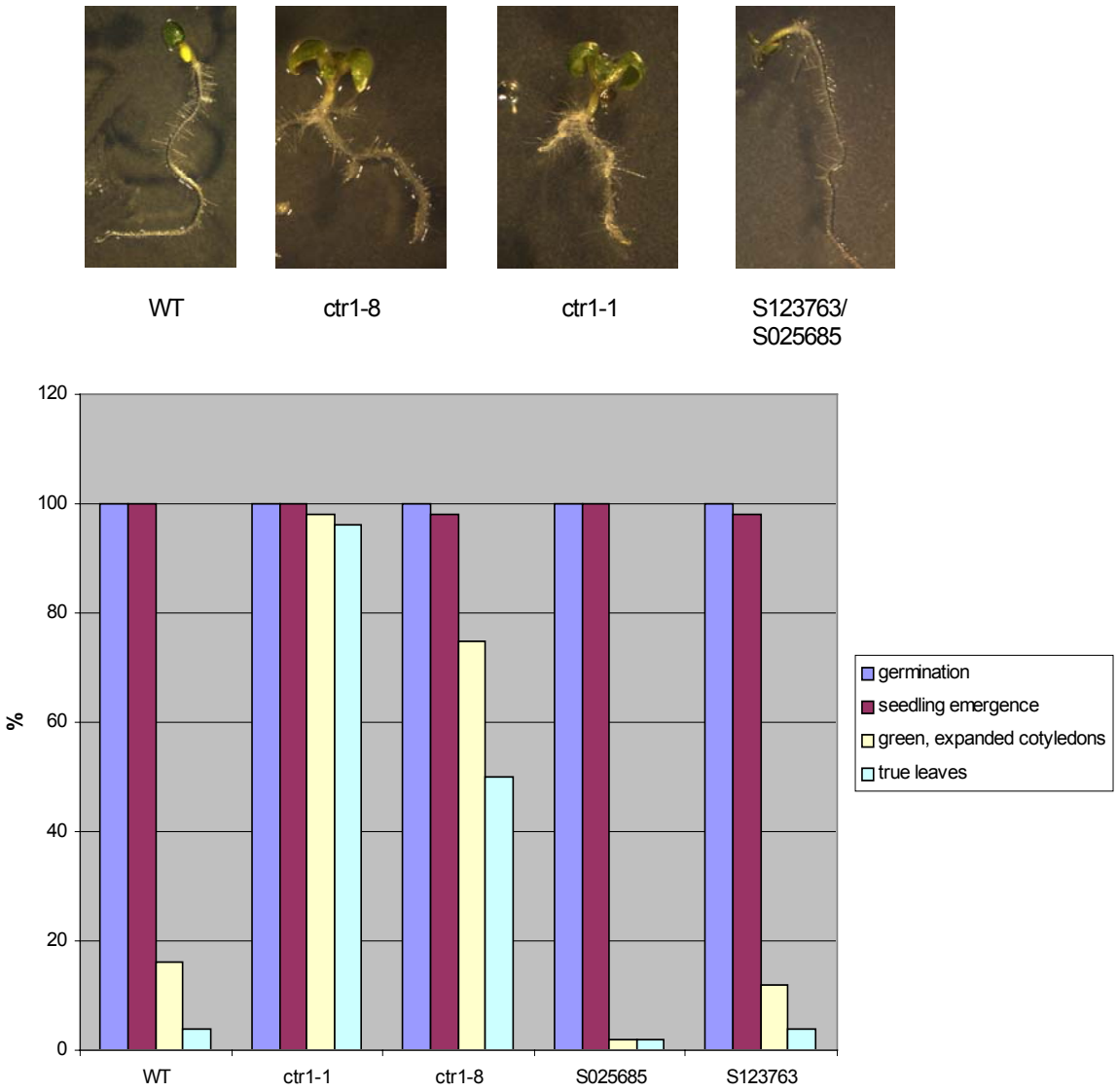


Figure 31. Sensitivity of *ctr1* and *At4g24480* mutants to germination on sucrose. Seeds were germinated on 10% sucrose and the percentage of seedlings in the indicated developmental stage was recorded after 10 days. Pictures above the graph were taken after 10 days and are representative of the stage of development of the majority of the seedlings for that genotype.

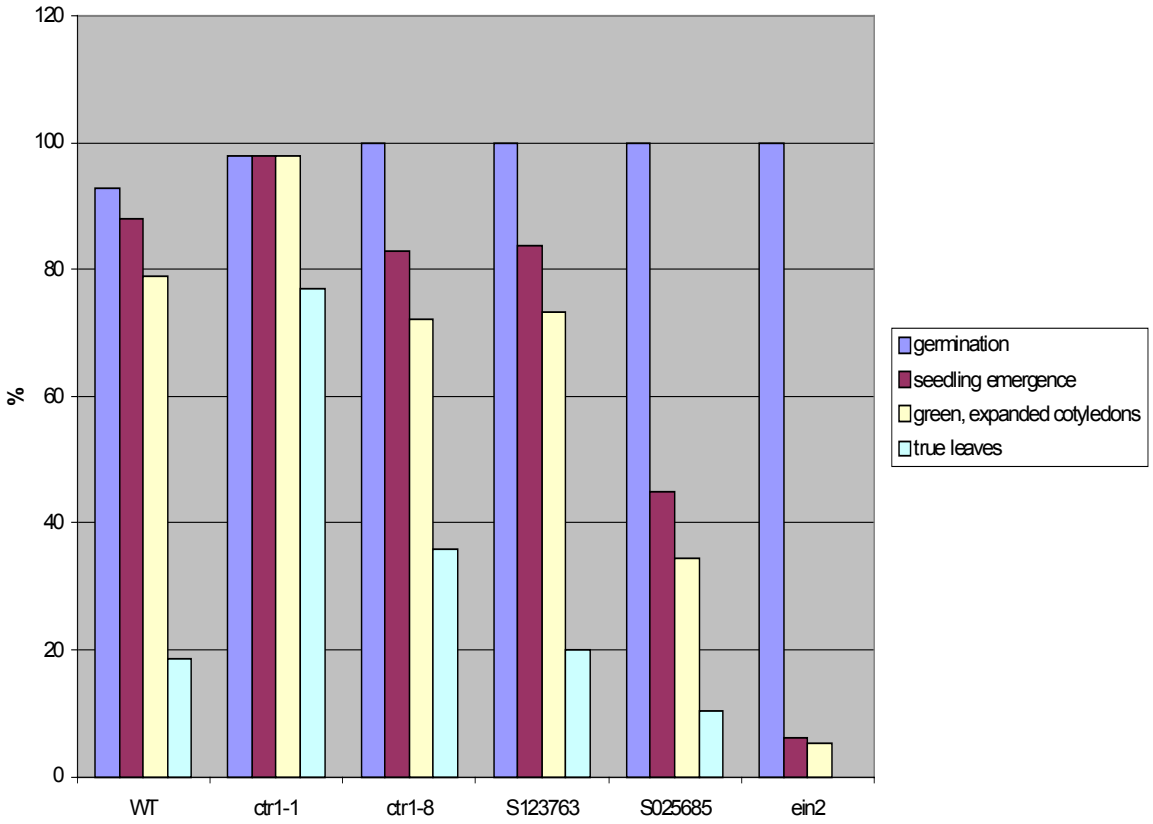
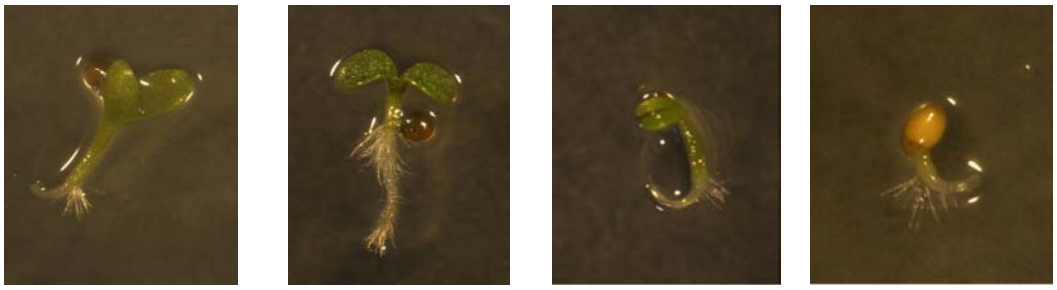


Figure 32. Sensitivity of *ctr1* and *At4g24480* mutants to germination on ABA.
Seeds were germinated on 1uM ABA and the percentage of seedlings in the indicated developmental stage was recorded after 10 days.



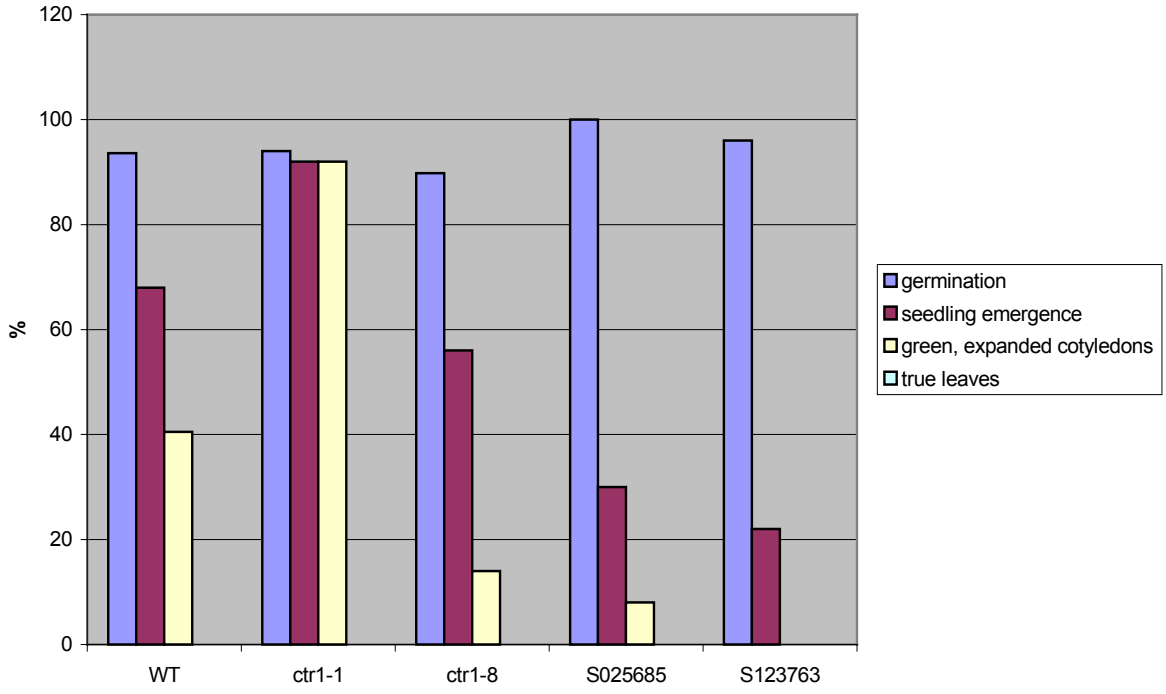


Figure 33. Sensitivity of *ctr1* and *At4g24480* mutants to germination on sorbitol. Seeds were germinated on 0.5 M sorbitol and the percentage of seedlings in the indicated developmental stage was recorded after 10 days. Pictures above the graph were taken after 10 days and are representative of the stage of development of the majority of the seedlings for that genotype.

ABA did not significantly inhibit roots of *At4g24480* T-DNA lines. In fact, ABA seemed to promote root growth, although the difference was not significant as it was for *ein2* (Figure 34). Tests for NaCl sensitivity were also performed in the same manner and while root growth was inhibited in all genotypes tested, wildtype and *ctr1-1* roots were the most severely inhibited by salt, between 72-85% (Figure 35). The *At424480* lines, *ein2* and *ctr1-8* lines were only inhibited between 38-55%.

Based on the atypical ABA and osmotic stress responses, the promoter region of *At4g24480* was scanned for cis-acting elements related to either of these signals (see experimental procedures). ABA has been shown to induce transcriptional activators that recognize cis-acting elements including ABRE, DRE, and Sph elements found in promoters of genes involved in stress response (see Zhu, 2002 and references therein) as well as genes involved in signaling, transport, and cell rescue (Hoth *et al*., 2002). Indeed, *At4g24480* has several of these elements in its promoter region that could indicate induction by ABA or osmotic stress (Figure 36). Furthermore, a genome-wide expression study identified *At4g24480* as one of 660 genes induced by ABA in Arabidopsis (Hoth *et al*., 2002 supp. data).

ctr1-8 allele displays exaggerated root phenotype in the light

Through the course of this analysis, it was discovered that the roots of the *ctr1-8* mutant display a much more pronounced phenotype than *ctr1-1* roots (Figure 37). The roots are shorter and the root hairs are more dense compared to *ctr1-1*. The difference in root length from *ctr1-1* becomes more exaggerated the longer the seedlings stay in the light and is even more dramatic when sucrose is added to the media (Figure 38). This is in

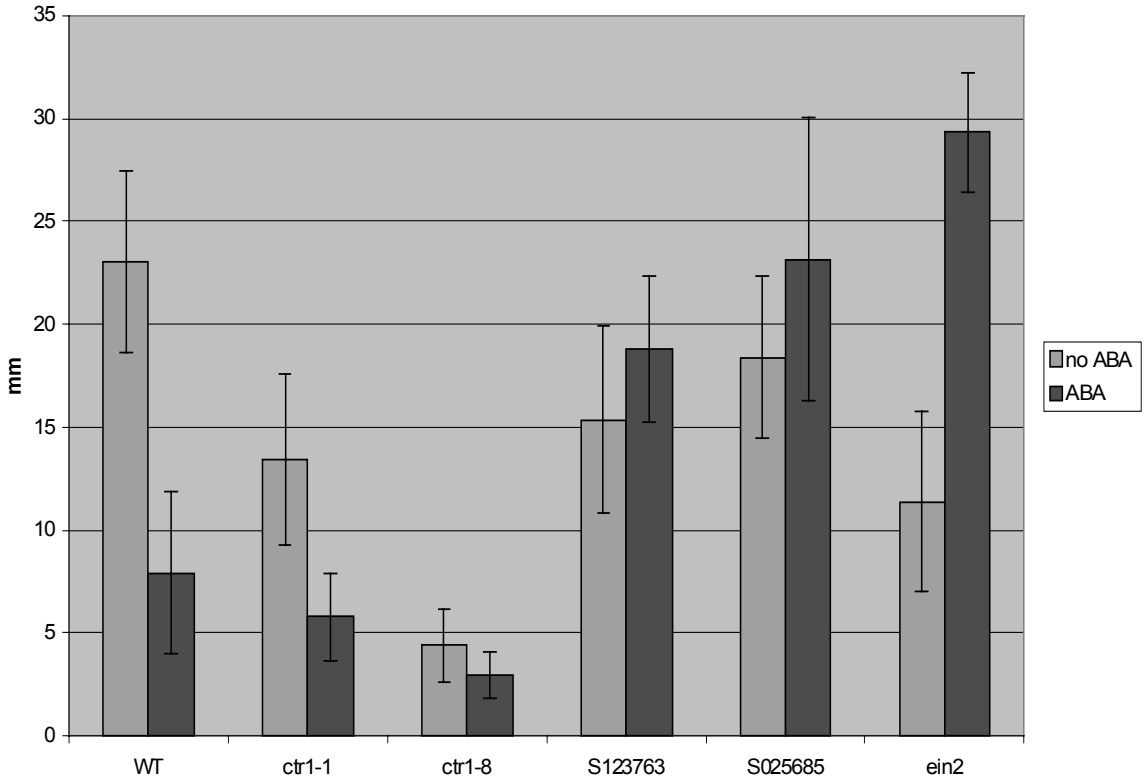


Figure 34. Root length inhibition in response to ABA.
Sterilized seeds were plated on sterile cellulose membranes placed on medium without 1uM ABA for 5 days, and were transferred to 1uM ABA. Root length was marked on the both control plates and treatment plates at the time of transfer and was measured after 5 days.

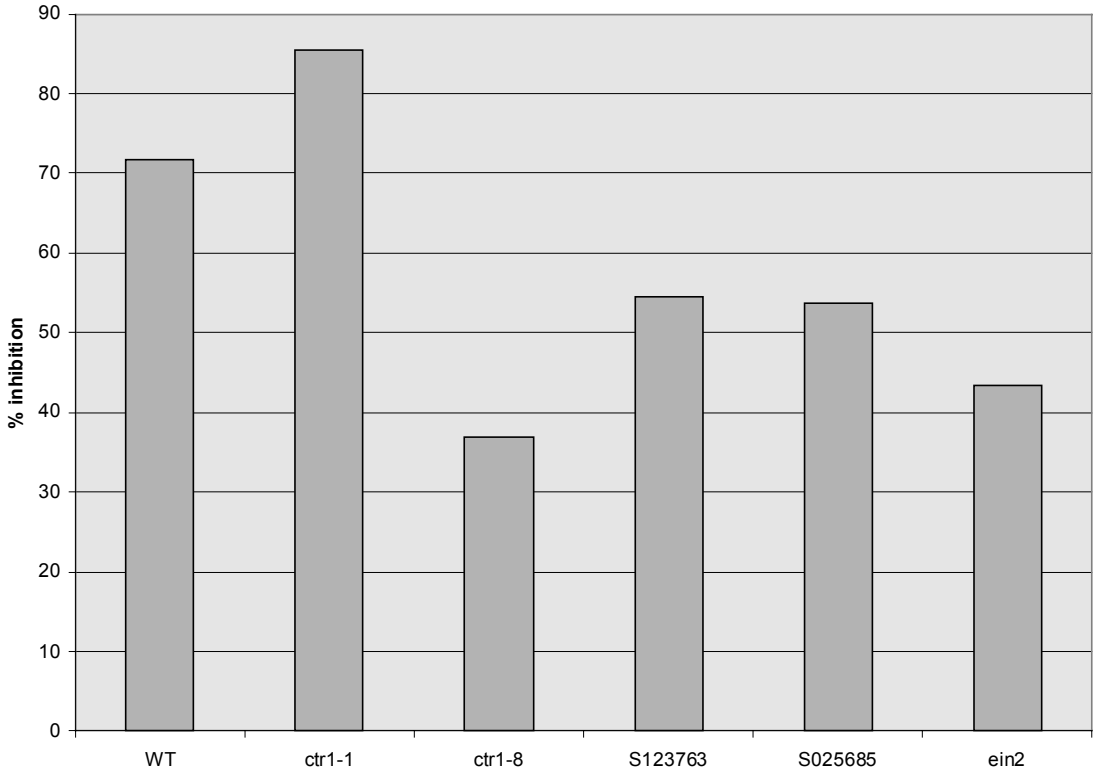
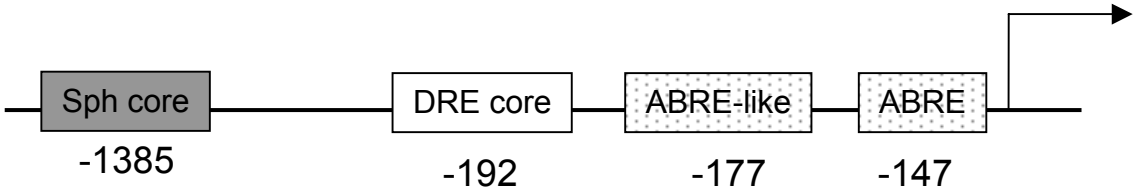


Figure 35. Percentage of root length inhibition in response to NaCl.
Sterilized seeds were plated on sterile cellulose membranes placed on medium without 160mM NaCl for 5 days, and were transferred to 160mM NaCl. Root length was marked on the plate at the time of transfer and was compared to root length from non-treated seedlings after 5 days to determine relative root growth to the control and is expressed here as percentage of inhibition.



ABRE: ACGTGT

ABRE-like: ACGTG

DRE1core: ACCGAGA

Sph core: TCCATGCAT

Figure 36. ABA response elements found in the *At4g24480* promoter.
The promoter sequence of *At4g24480* was scanned against the Plant cis-acting regulatory DNA elements (PLACE) database (Higo *et al.*, 1999). Positions relative to the putative start of transcription are shown underneath.

contrast to seedlings grown in the dark where the *ctr1-8* mutant has longer roots and hypocotyl than *ctr1-1* (Figure 39). When grown in soil, the *ctr1-8* mutant has a larger rosette, bolts earlier than *ctr1-1*, and does not produce the extra leaves that *ctr1-1* does (Figure 40).

Complementation of ctr1-8 phenotypes with tomato CTRs

Lines containing each of the tomato *CTR1-like* genes (Chapter 2) were checked for their ability to complement this *ctr1-8* specific phenotype (Figure 41 and Figure 42). Consistent with their ability to complement the triple response phenotype (Chapter 2), *LeCTR1* lines showed the least rescue while *LeCTR3* completely rescued the root phenotype. As further confirmation of their ability to complement the *ctr1-8* allele, rescue of glucose was examined in each of the *LeCTR* lines. (Figure 43).

Discussion

Preliminary data suggests that while *At4g24480* mutants do not appear to show abnormal ethylene mediated triple response, they do exhibit abnormal sensitivity to abscisic acid and osmotic stress. Abscisic acid mutants involved in ABA signaling such as *abi1-1* show the same sensitivity or insensitivity to abscisic acid during germination as during root growth, but do not show abnormal sensitivity to ethylene (Beaudoin *et al*, 2000; Ghassemein *et al*., 2000). However, the *At4g24480* mutants respond to ABA and osmotic stress in the same manner as the ethylene insensitive *ein2* mutants (though lacking ethylene insensitivity in etiolated seedlings) in that they are sensitive to ABA

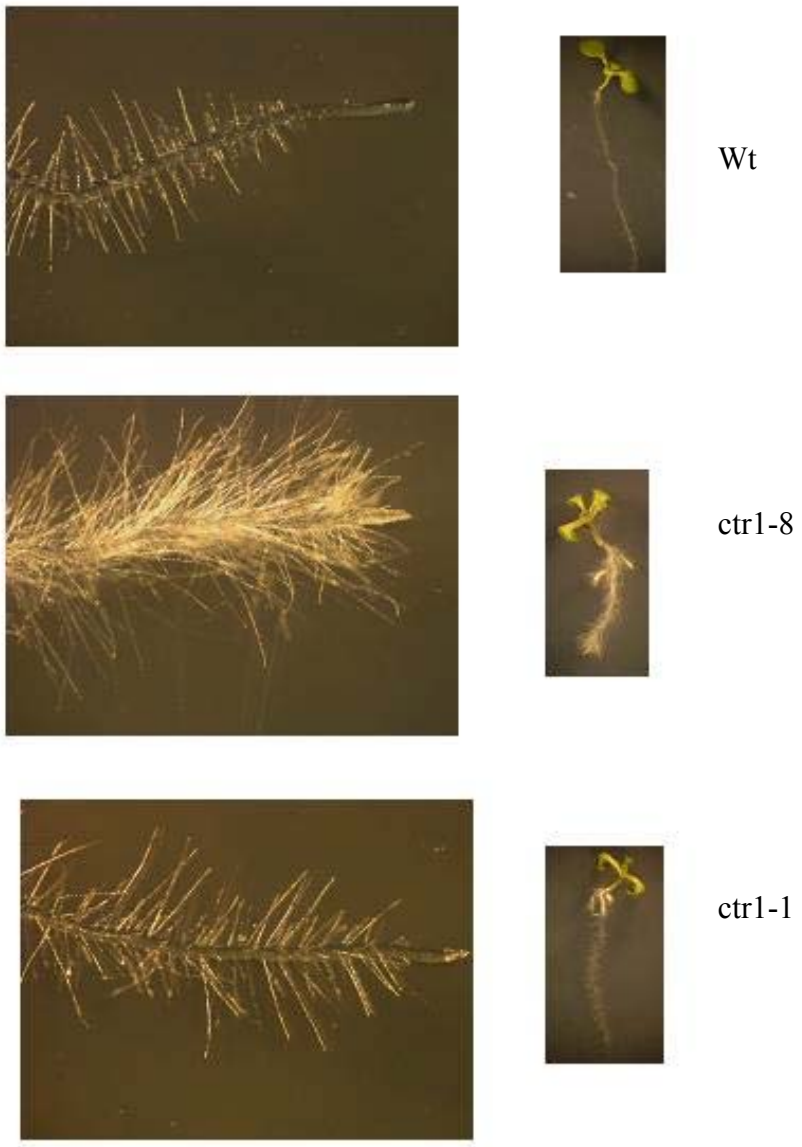


Figure 37. *ctr1-8* displays a more pronounced root phenotype than *ctr1-1* when grown in the light.
Seedlings shown in the pictures were grown for approximately 10 days in the light in the vertical orientation. All pictures are at the same magnification.

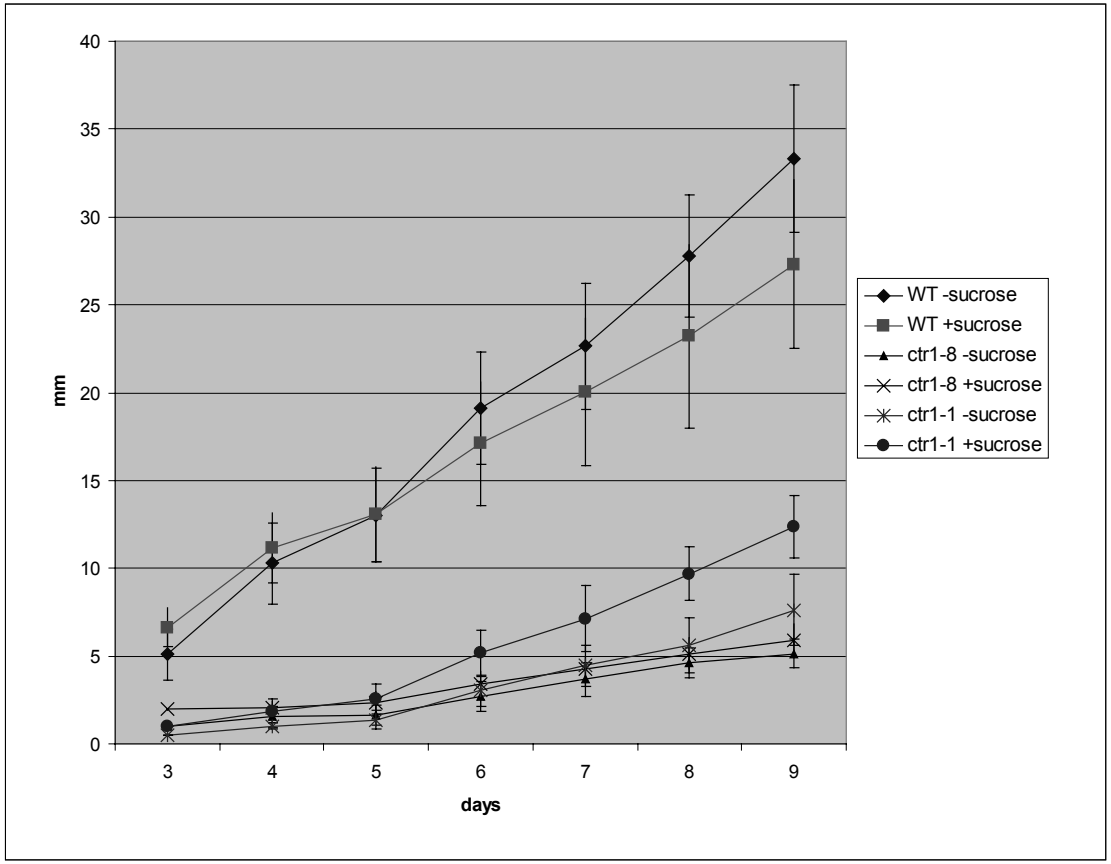
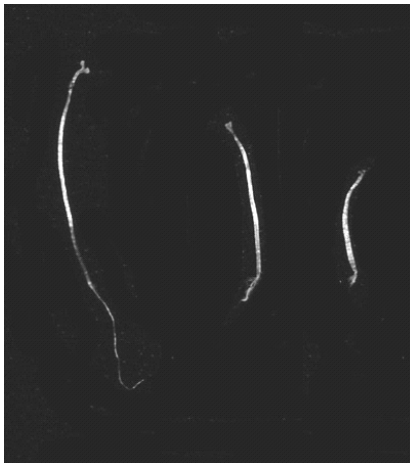


Figure 38. *ctr1-8* roots are shorter than *ctr1-1* when grown in the light. Seeds were plated on medium with and without 1% sucrose and were grown in the light in the vertical orientation for 9 days. Root length was measured daily starting on day 3.



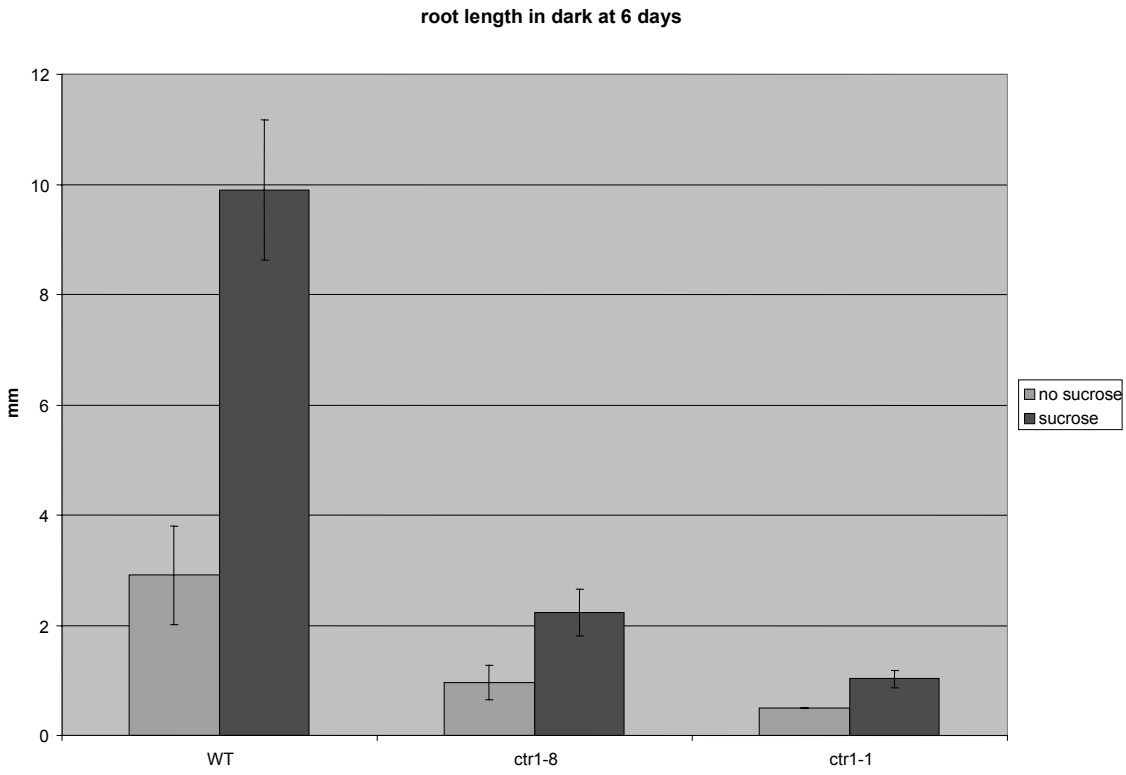


Figure 39. *ctr1-8* roots are longer than *ctr1-1* when seedlings are grown in the dark. Seeds were plated on medium with and without sucrose and grown in the dark in the vertical orientation for 6 days. Root length was recorded and a picture was taken at that time.

WT ctr1-8 ctr1-1

Figure 40. *ctr1-8* displays an intermediate ethylene response phenotype when grown in the soil.
Picture of adult Arabidopsis grown under 16 hour daylength for approximately 4 weeks. Both WT and *ctr1-8* have produced an inflorescence (indicated by the arrow) while *ctr1-1* remains in the rosette stage and produces more leaves.

during germination, but are insensitive in their roots. This could indicate that *At4g24480* and *EIN2* are involved in the same ABA pathway, mediating response to ABA in a subset of developmental stages and/or tissues.

One possible mechanism to modulate developmental or tissue-specific response to ABA could be through regulation of ABA biosynthesis and/or response by ethylene and osmotic status. Osmotic stress triggers the biosynthesis of ABA (reviewed in Zhu, 2002) though little is known about the signals involved in this process. Interestingly, *eto1* mutants exhibit decreased sensitivity to ABA in the roots (Ghassemian *et al*., 2000) suggesting cross-talk or overlap between the ethylene and the osmotic status/ABA signaling pathways. One possible explanation for this second phenomenon is that the ethylene signaling pathway in Arabidopsis is also involved in transmission of non-ethylene signal(s) (possibly including osmotic status or ABA related signals) and that ethylene competes with said signal(s) resulting in modified ABA synthesis and/or responses (Gazzarrini and McCourt, 2001). In support of this hypothesis, ethylene mutants spanning the signaling cascade from receptors to *ein2* show abnormal responses to ABA (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000). *ein2* is especially interesting in that this mutant displays an in increase in transcript accumulation of zeaxanthin epoxidase, the first committed enzyme in ABA biosynthesis (Ghassemian *et al*., 2000) and suggesting altered ABA response phenotypes may represent perturbation of endogenous ABA levels. To fully understand the relationships between ethylene signaling, osmotic status and ABA synthesis and signaling more experimentation is clearly needed. For example, whether or not ethylene signal transduction mutant

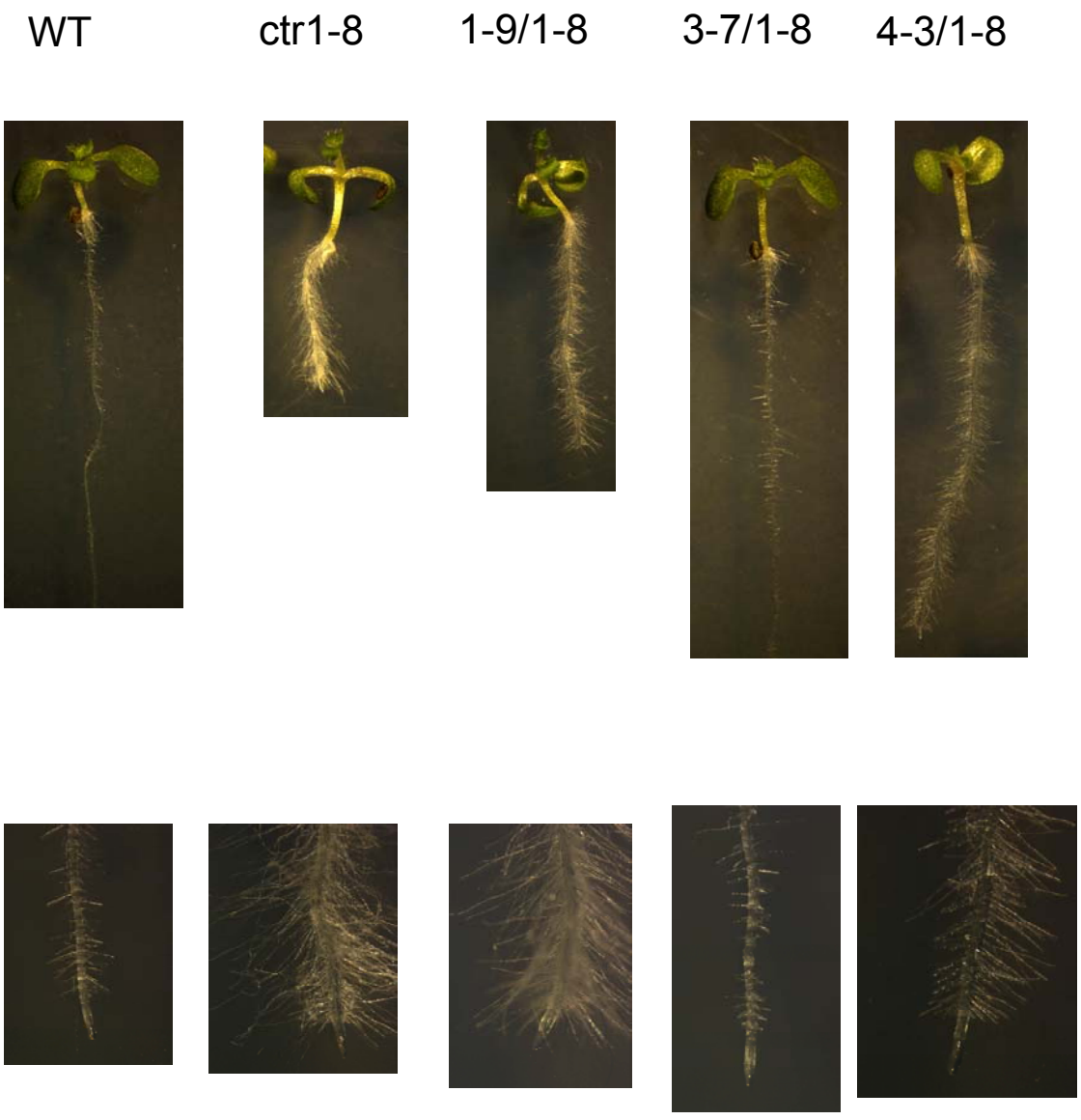


Figure 41. Complementation of *ctr1-8* light grown root phenotype.
Seedlings were grown for 8 days in the light. A seedling from a line representative for each construct overexpressing a tomato *LeCTR* gene is shown along with wild-type and *ctr1-8* seedlings for reference.

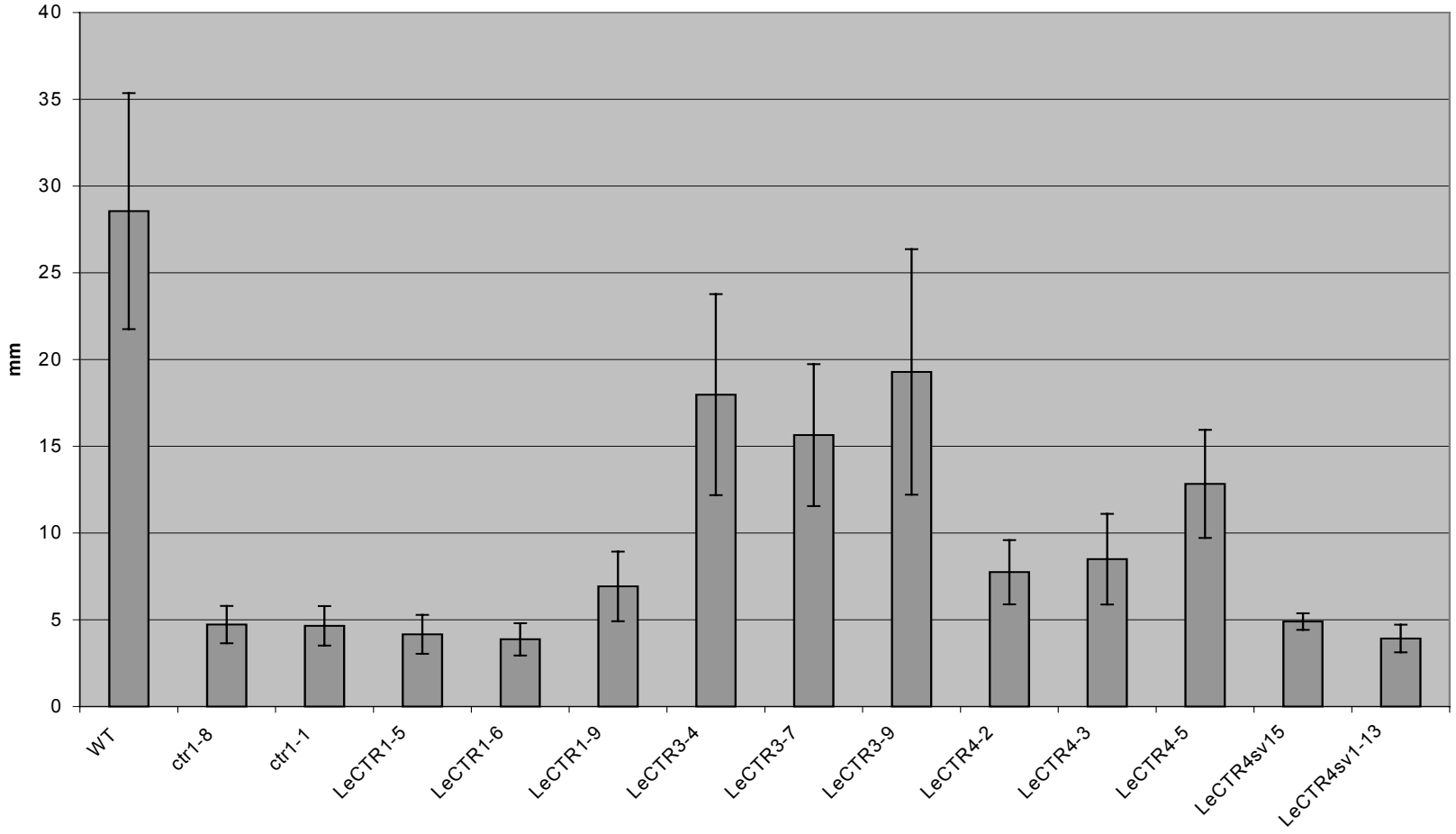


Figure 42. Complementation of *ctr1-8* root length in seedlings grown in the light.
Constructs overexpressing each *LeCTR* gene [LeCTR1-overexpressing lines, (1-5, 1-6, 1-9); LeCTR3-overexpressing lines (3-4, 3-7, 3-9); LeCTR4-overexpressing lines (4-2, 4-3, 4-5); LeCTR4sv1-overexpressing lines (4sv1-5, 4sv1-7, 4sv1-1)] were assayed for their ability to complement the light grown root phenotype of *ctr1-8* compared to wild-type. Seedlings were grown for 8 days in the light and root length was measured at that time.

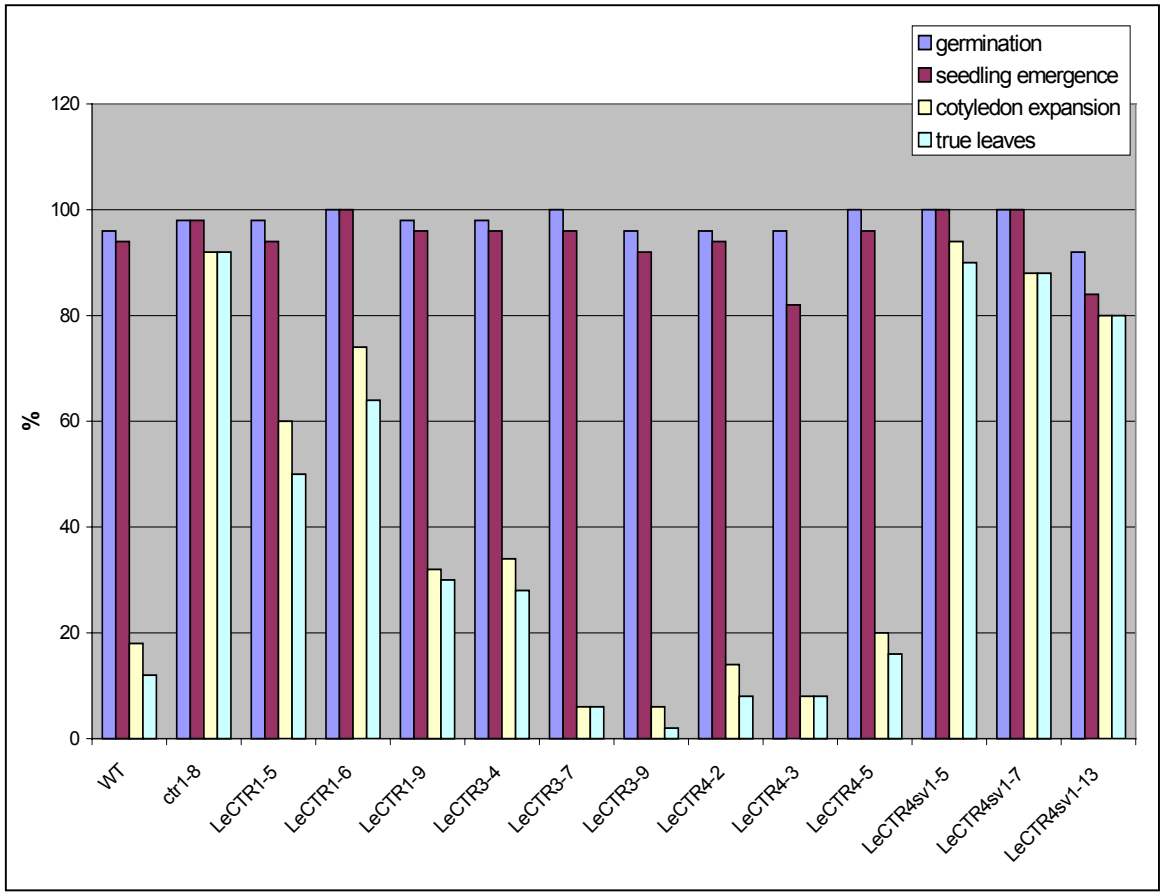


Figure 43. Complementation of *ctr1-8* glucose insensititivity.
Constructs over-expressing each *LeCTR* gene were assayed for their ability to complement the glucose insensitive phenotype of *ctr1-8*. Seeds were germinated on 6% glucose and the percentage of seedlings in the indicated developmental stage was recorded after 10 days.

displaying altered ABA response increase or decrease endogenous ABA levels should be clarified.

Lacking additional evidence, a simple working model could be envisioned where one or more members of the ethylene receptor family also act as an osmosensor, in addition to an ethylene receptor, mediating osmotic responses through regulation of a MAPKKK (At4g24480) cascade that signals to EIN2 (Figure 44). Depending on the developmental stage of the plant, and the signal involved, this could result in positive or negative regulation of ABA biosynthesis/response.

Providing support for an association between osmotic signaling and ethylene signaling, it has been shown that alfalfa SIMK, the same kinase shown to be induced by ACC and proposed to mediate ethylene signaling in plants (Ouaked *et al*., 2003), is activated in response to hyperosmotic stress (Munnik *et al*., 1999). Additionally, it is known that the osmolarity-response pathway in yeast involves a two-component regulator and signaling through a MAPKKK cascade (Maeda *et al*., 1994 and Posas *et al*., 1996). ETR1 possesses the histidine kinase activity shown to be required for activation of the osmosensor in yeast (Gamble *et al*. 1998) even though it is not necessary for ethylene signal transmission (Wang *et al*., 2003). It has been proposed that this activity might allow a subset of the ethylene receptors (ETR1 and ERS1) to participate in additional pathways and phosphorylation of histidine residues could serve to recruit specific proteins into the formation of signaling complexes (Gamble *et al*., 1998). Thus, a connection could be made between osmotic signaling and ethylene signaling. Furthermore, it has been shown that excess Na+ and osmotic stress triggers increases in

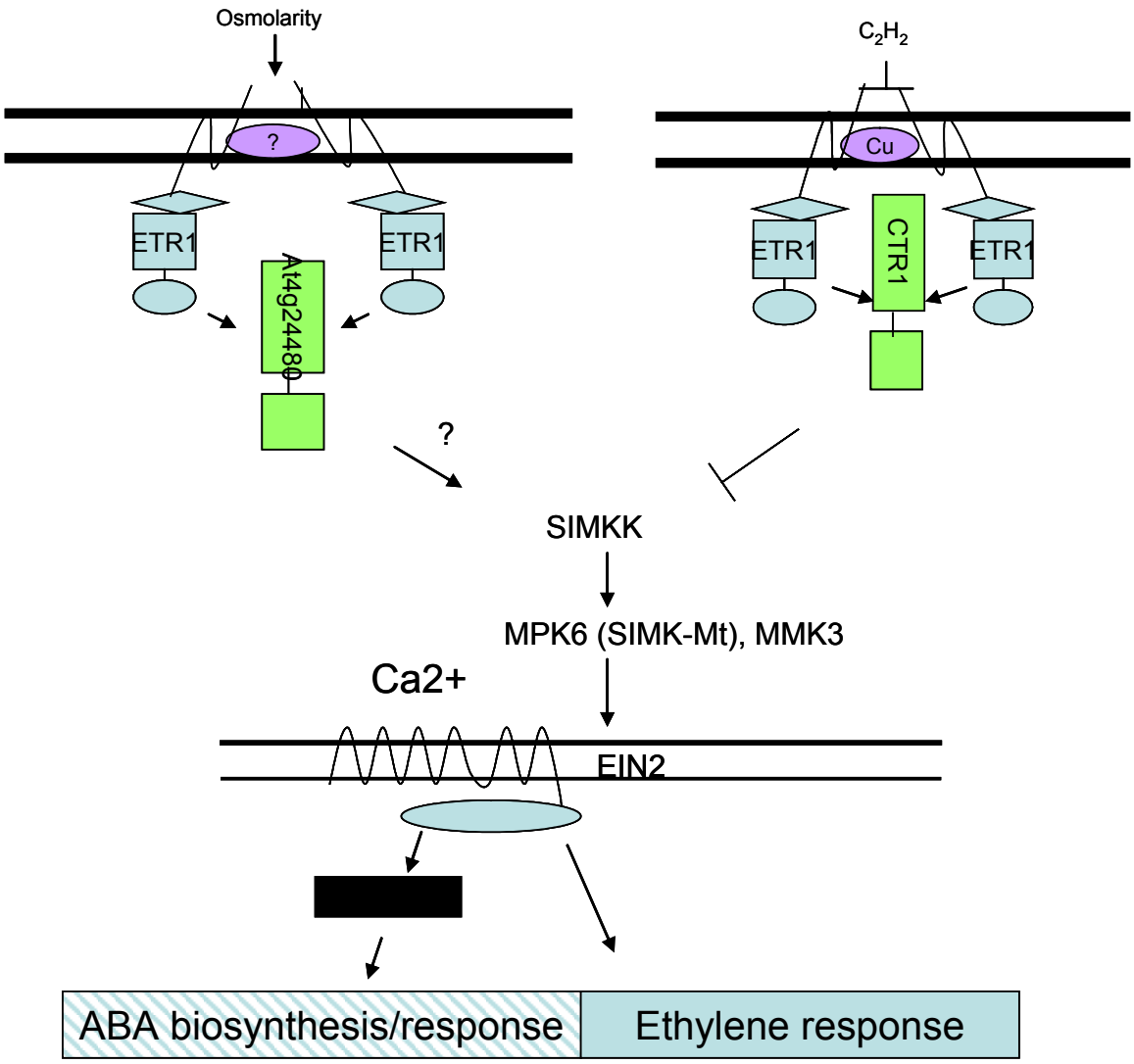


Figure 44. Hypothetical model for ethylene/osmosensing signal transduction.
In this model, ETR1 is predicted to act as an osmosensor in addition to an ethylene receptor. The signal produced through the MAPKKK cascade involving At4g24480, is mediated by EIN2. The black box underneath EIN2 represents an as yet undefined pathway through which a positive or negative regulation of ABA biosynthesis/response occurs, depending on the signal involved and stage of development of the plant.

cytoplasmic calcium (Knight *et al*., 1997) and that calcium is essential for morphological responses to ethylene and for the induction of chitinase through the ethylene dependent pathway but not for the ethylene-independent pathway (Raz and Fluhr, 1992). Indeed, the similarity of the EIN2 N-terminus to the Nramp proteins suggests that a second messenger in the form of a divalent cation exists in the ethylene signal transduction pathway and that the N-terminus of EIN2 may sense this divalent cation (Alonso *et al*., 1999). An increased flux of calcium by osmotic stress could trigger osmotic response through activation of a specific transcriptional cascade mediated by EIN2.

An intriguing dilemma is how *At4g24480* mutants could be similar to *ein2* in response to ABA and osmotic stress, but not exhibit sensitivity to ethylene in etiolated seedlings. The answer to this dilemma may clarify the lack in detail of the model in Figure 44 downstream of EIN2 possibly reflecting the function of the EIN2 protein. EIN2 is proposed to act as a bifunctional signal transducer since it has been shown that the N-terminus is required for ethylene responsiveness, while the C-terminus is sufficient to activate the pathway (Alonso *et al*., 1999). In light of this, it is conceivable that EIN2 could normally regulate the seedling triple response in *At4g24480* mutants because it is able to correctly respond to ethylene, but is not receiving the proper signal to activate osmotic stress responses. If this were true, *At4g24480* mutants might be expected to respond to ethylene normally in other ways including responsiveness to glucose/sucrose and in terms of adult plant phenotypes. These phenotypes need to be better characterized in the *At4g24480* T-DNA lines. For example *At4g24480* mutants demonstrate what could be considered a hypersensitive response to glucose/sucrose

based on the observation that both cotyledons are always bleached, while in wild-type controls, typically only 1 cotyledon is bleached. If a lower concentration of glucose and sucrose were used, any hypersensitivity to sugars might be more accurately quantified.

The *ctr1-1 and ctr1-8* mutants were also characterized for sensitivity to glucose, sucrose, ABA and osmotic stress in order to glean insights into possible mechanisms of interaction of the ethylene signaling pathway with other signaling molecules. In adult plants and etiolated seedlings, and in response to glucose and sucrose, *ctr1-8* appears to show a weaker constitutive ethylene phenotype than *ctr1-1*. However, when grown in the light, the roots produce many more root hairs than *ctr1-1*, exhibiting a stronger ethylene phenotype than *ctr1-1*. Interestingly, while chitinase is constitutively expressed in the *ctr1-1* mutant in the absence of ethylene, chitinase is not expressed in *ctr1-8* in the absence of ethylene (i.e. the same as wild-type expression, data not shown). This observation could simply reflect the weaker phenotype of *ctr1-8*, perhaps due to leakiness of the mutation, or alternatively may help to explain the paradox of response degree of light grown roots. Since roots are normally in the dark, it is possible that a light regulated factor, not normally expressed in roots, becomes activated in the light and acts in a synergistic manner with *ctr1-8* to signal through the ethylene pathway.

Preliminary data suggests that *ctr1-8* mutants respond differently under osmotic stress and possibly in response to ABA than *ctr1-1* mutants in seeds and roots. It is difficult to discern whether this is due to the physiological nature of the mutation in the light grown roots, or if it possibly represents a different mechanism for regulation of CTR1 activity under osmotic stress conditions that does not require direct association

with the receptor. Alternatively, it is possible the *ctr1-8* mutants are over-producing ethylene when grown under these experimental conditions and are reacting in a similar manner as *eto1* mutants (which also exhibit a constitutive triple response).

In summary, preliminary data suggests that *At4g24480* T-DNA mutants are aberrant in response to ABA and osmotic stress in the same developmental stage specific manner as *ein2*. This has led to the hypothesis that At4g24480 could function in response to osmotic stress, through components in the ethylene signal transduction pathway. Indeed, many more experiments would need to be undertaken to prove such a hypothesis. First, it is necessary to further characterize the *At4g24480* T-DNA and *ctr1-8* mutants. Dose response curves would be useful to better measure significant differences in sensitivity to glucose/sucrose, osmotic stresses ABA, and ethylene. In several experiments, trends seemed apparent but could not be quantified. In the case of NaCl tolerance, too high of a concentration of NaCl may have been used as all genotypes tested were inhibited by salt to varying degrees. Additionally, while etiolated seedlings of *At4g24480* do not appear to respond abnormally to ethylene, it is also possible that the response is not significant at the concentration of ethylene used. Second, an obvious question as to whether ETR1 could actually sense and respond to osmotic changes needs to be answered, and it would also be useful to know if At4g24480 associates directly with the receptor as it does with CTR1 or if it is activated in a different manner.

Experimental Procedures

Plant material

Arabidopsis thaliana ecotype Columbia plants were grown in a growth chamber under 16h day length at 22°C. Arabidopsis *At4g2448*0 T-DNA lines (S025685 and S123763) were obtained through the ABRC from the SALK Institute (Alonso *et al*., 2003*a*).

PCR analysis

Three primers for each T-DNA line (S123763 and S025685) were designed to verify the T-DNA insertion site and to determine if the insertion was homozygous at that site. A forward (F) and reverse (R) primer flanking the insertions were designed so that in wild-type, a product of about 900 bp would be obtained using this set of primers (set A) (Figure 26). A third primer (LB) was designed from the left border of the T-DNA to be used along with the reverse (R) primer from set A so that amplification would yield a product of 600 bp with this set of primers (set B) (Figure 26). In a heterozygous plant, a product of 900 bp would be obtained from set A primers and a product of 410 bp would be obtained from set B primers. In a homozygous plant, no product would be obtained with set A primers, but with set B primers, a product of 410 bp would be generated. Primer sequences were as follows: LB (left border primer) 5' TGGTTCACGTAGTGG GCCATC-3'; S123763 For 5'-CAAACCAATTCAATTTATATCCACC-3'; S123763 Rev 5'-GAAACTGCCGCGGAAAGAAGT-3'; S025685 For 5'-TGTGGACTTTCAGG GCATGGT-3'; S025685 Rev 5'- CCCACACGCTCTTTGATATG-3'. Seeds from putative homozygous plants were plated on 50ug/ul Kanamycin for further confirmation of homozygousity.

Sequence analysis

The promoter sequence of At4g24480 was scanned against the Plant cis-acting regulatory DNA elements (PLACE) database (Higo et al., 1999). Amino acid sequence identities were calculated using the ALIGN program (GeneStream Server, http://www.genestream.org).

Seedling Sensitivity Assays

Sterilized seeds were incubated for 4 days at 4 degrees Celsius and plated on 0.8% agar containing 10% sucrose, 6% glucose, 1 uM ABA, or 0.5M sorbitol at a density of 50 seeds/plate. The plates were placed in 16 hours of light and the percentage of germination, seed coat emergence, cotyledon expansion and true leaf formation was recorded each day for 10 days. Criteria for scoring each indicated developmental event were as follows: germination was defined as any portion of the plant which visibly protruded from the seed coat; seed coat emergence indicated that both cotyledons had emerged from the seed coat but were still vertical in orientation to each other; cotyledon expansion indicated that the cotyledons were approximately 180 degrees to each other and both were green; true leaf formation was recorded the day the true leaves became visible.

Root growth sensitivity assays

Sterilized seeds were incubated at 4 degrees in the dark for 4 days and then plated on sterile cellulose membranes (Bio-Rad) placed on medium containing MS salts, 1X B5 vitamins, and 1.2% Phytagar. The plates were incubated in the vertical position for 5 days in the light after which the membranes were transferred to plates containing MS

salts, 1X B5 vitamins, and 1.2% Phytagar, supplemented with either 160mM salt or 1uM ABA. Root length was marked on the plate at the time of transfer and was compared to root length from non-treated seedlings after 5 days to determine relative root growth.

Light-grown root assay

Sterilized seeds were incubated at 4 degrees in the dark for 3 days and then plated on sterile cellulose membranes (Bio-Rad) placed on medium containing MS salts, 1X B5 vitamins, with and without 1% sucrose, and 1.2% Phytagar. The plates were incubated in the vertical position for another 9 days in the light. Root length was recorded starting on the third day. 1% sucrose was used in the root complementation assay and root length was recorded after 8 days.

Triple response seedling assay

Sterilized seeds were incubated at 4 degrees in the dark for 3 days and then plated on sterile cellulose membranes (Bio-Rad) placed on medium containing MS salts, and 1.2% Phytagar with and without 10uM ACC. The plates were incubated in the vertical position for 5 days in the dark. Root and hypocotyl length was recorded.

DNA analysis

DNA extraction and Southern analysis were performed as described in Chapter 2.

CHAPTER V

SUMMARY

Ethylene, in addition to other plant hormones, governs a range of developmental processes in plants and acts as a signal molecule to elicit responses to internal and external stimuli. In *Arabidopsis thaliana*, the Raf-like kinase CTR1 acts through the ethylene signal transduction pathway as a negative regulator of ethylene responses. In this study, isolation and functional characterization of three *LeCTR* cDNAs and corresponding genomic clones from tomato has provided the first experimental evidence of a multigene family of *CTR1-like* genes which are functionally able to participate in ethylene signal transduction. Based on amino acid alignments and phylogenetic analysis, these tomato *CTR1-like* genes were more similar to Arabidopsis *CTR1* than any other *CTR1-like* sequences in the Arabidopsis genome. Structural analysis revealed considerable conservation in the size and position of the exons between Arabidopsis and tomato *CTR1* genomic sequences. Two *LeCTR4* splice variants were isolated, differing only in the processing of one intron. As a result, a stop codon is read through in frame in one of the variants (LeCTR4sv1) presumably rendering the protein truncated and likely non-functional. It is possible this represents a form of gene regulation outside of transcriptional control and could potentially provide an alternative means of regulation for other both LeCTR4 and other CTR1-like genes.

Complementation of the Arabidopsis *ctr1-8* mutant with each of the tomato *CTR* genes indicated that they were all capable of functioning as negative regulators of the

ethylene signaling pathway. Each construct's ability to complement the triple response, glucose and sucrose insensitivity, and root length in light grown seedlings was similar for each treatment with the consistent exception of complementation of the adult plant phenotype. At the early rosette stage, LeCTR3 appears to complement the ctr1-8 mutant to the greatest degree, while during later rosette development and inflorescence formation, LeCTR4 becomes indistinguishable from LeCTR3 and WT, while LeCTR1 still remains only mildly able to complement ctr1-8. This may indicate a developmental significance for LeCTR4 and will need to be examined further in tomato.

Quantitative real-time PCR was carried out to generate an expression profile for the tomato *CTR1* gene family. The tomato *CTR1* gene family was found to be differentially regulated at the mRNA level by ethylene and during stages of development marked by increased ethylene biosynthesis, including fruit ripening. In apparent contrast to Arabidopsis, modulation of expression by ethylene occurs at the levels of both receptors and CTRs in tomato, while only receptors respond transcriptionaly to ethylene in Arabidopsis. In Arabidopsis, CTR1 interacts directly with the ethylene receptors and is thought to be part of an ethylene-receptor signaling complex. It is likely that LeCTR1, LeCTR3, and LeCTR4 interact with the receptors as well, possibly each specific to a certain individual or subset of receptors. To date, ethylene responses appear to be mediated through a common, primary signal transduction pathway from receptors through EIN3 transcription factors and the method by which diverse downstream responses occur over a wide range of ethylene concentrations is still unclear. One model that has been proposed, which is based on kinetics associated with seedling-growth

response and B-chitinase induction, allows for one primary pathway and a set of secondary pathways, which operate over different ranges of signal input from the primary pathway (Chen and Bleecker, 1995). In this regard, based on signal input from the primary pathway, transcription from the primary component EIN3, could initiate a transcription factor cascade activating downstream targets in the secondary pathway. It is important not to underestimate the level of regulation that could exist between CTR1 and the ethylene receptor, resulting in differential signal amplification through the MAPKKK pathway. Certainly if either the upstream "donor" or the downstream "acceptor" of these signals is taken out of the picture, ethylene responses will be globally impacted, but this does not mean these components are not important in modulating the activity of specific downstream targets, particularly through a MAPKKK or transcriptional cascade. A combination of differential expression of receptors and multiple *CTR* genes in conjunction with differential interaction kinetics for LeCTR/receptor signaling complexes could represent a mechanism for further optimization of ethylene responses in tomato and other species likely to have *CTR1* gene families.

While *LeCTR1*, *LeCTR3* and *LeCTR4* have been shown to be functional through complementation of *ctr1-8* in Arabidopsis, it is important to characterize their individual functions in tomato. This may not be entirely straightforward, as it is possible they could functionally compensate for each other. Consequently, analysis of phenotypes resulting from both over-expression and reduced expression is being undertaken and should lead to insights regarding their individual functions. Alternatively, multiple

LeCTRs may need to be reduced before an effect is seen. Plans to characterize individual functions of the *LeCTR* genes through over-expression and reduced expression via RNAi in tomato were outlined.

The possibility of a multi-gene family of *CTR1-like* genes in other species besides tomato was examined through mining of EST and genomic sequence databases. The close relative to Arabidopsis, *B. oleracea,* has two *CTR1-like* sequences sharing a higher percentage of amino acid identity to *AtCTR1* than any other sequence in the Arabidopsis genome. Additionally, there seemingly exists a small family in potato, lettuce and soybean and rice. It is possible that there were multiple copies of *CTR1-like* genes in plants before monocots and dicots diverged, and while this family persisted in the *Brassicaceae*, Arabidopsis lost one or more of these members. The retention of multiple *CTR1-like* genes might reflect the impact that ethylene has on a process crucial to the survival of the plant. Alternatively, other CTR1-like sequences in Arabidopsis may have diverged rather quickly through subfunctionalization and do not resemble CTR1-like genes as defined by amino acid identity.

Based on nucleotide and amino acid identity, *At4g24480* is the next most similar to *AtCTR1* and thus could potentially represent a *CTR1-like* gene function in Arabidopsis. Arabidopsis plants carrying a T-DNA insert in the *At4g24480* locus were examined for abnormal ethylene response phenotypes as well as for sensitivity to other hormones, signal molecules and abiotic stresses. Two *AtCTR1* mutant alleles, *ctr1-1* and *ctr1-8*, containing mutations that disrupt kinase activity and receptor association, respectively, were examined for sensitivity to these same treatments in an effort to better

characterize ethylene hormone and non-hormone signaling interactions. Preliminary data suggested that while *At4g24480* mutants do not appear to show abnormal ethylene mediated triple response, they do exhibit abnormal sensitivity to abscisic acid and osmotic stress. *At4g24480* mutants respond to ABA and osmotic stress in the same manner as the ethylene insensitive *ein2* mutants in that they are sensitive during germination, but are insensitive in roots. This could indicate that At4g24480 and EIN2 are involved in the same signaling pathway. A model was proposed where ethylene receptor gene(s) encode proteins which serve as osmosensors, in addition to ethylene receptors, mediating osmotic responses through regulation of a MAPKKK cascade that signals to EIN2. Depending on the developmental stage of the plant, and the signal involved, this could result in positive or negative regulation of ABA biosynthesis and/or response.

Interactions between ethylene and other hormones and signaling molecules represent a complex signal transduction web, where depending on the developmental stage of the plant and environmental stimuli, positive and negative interactions take place to create appropriate responses. The complexity of this signal transduction and regulation needs to be better understood and considered, particularly from the standpoint of optimal genetic manipulations for crop improvement.

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