ABSTRACT

Title of Dissertation: UPSTREAM EVENTS IN ETHYLENE SIGNAL TRANSDUCTION IN *ARABIDOPSIS*.

Jason Alan Shockey, Doctor of Philosophy, 2004

Dissertation Directed By: Associate Professor Caren Chang Department of Cell Biology and Molecular Genetics

Ethylene gas has profound effects on the growth and development of higher plants. The understanding of how plants can sense this gas, and react in the appropriate manner is important for both agricultural purposes as well as the basic understanding of plant biology. While many components of this signaling pathway have been identified using classical genetics, we have little understanding of how these components work together. My work has focused on the understanding of early events in ethylene signal transduction.

The interaction between the ETR1 ethylene receptor and the CTR1 Raf-like kinase was the first clue that the ethylene signaling pathway diverged from that of the yeast HOG1 osmo-sensing pathway. In this thesis, I examined the functional relevance of this interaction in the regulation of CTR1's activity. My work suggests that although CTR1 demonstrates the novel interaction with two-component receptors, the biochemical regulation of CTR1 may be similar to that of Raf1.

Recent studies have suggested that histidine kinase activity of ETR1 may not play a major role in ethylene signal transduction, despite the remarkable degree of sequence

conservation with functional histidine kinases from bacteria and yeast. In order to better understand the role of this highly conserved domain, either in ethylene signaling or other possible functions, I utilized biochemical assays, protein interaction studies and transgenic plants. My work indicates that phospho-relay plays no observable role in most ethylene responses, but plays an important role in recovery from ethylene treatment.

Important members of this signaling system may yet be unidentified. A gene previously identified in the Chang lab, D2, was shown to have a probable role as a scaffolding protein in ethylene signaling using multiple reverse genetic techniques. This gene is unique to plants and cyanobacteria, as is the ethylene binding fold suggesting the two may have evolved together.

The emerging paradigm of the ethylene signaling system reveals the pathway to be much more complex than originally thought.

UPSTREAM EVENTS IN ETHYLENE SIGNAL TRANSDUCTION IN *ARABIDOPSIS*

By

Jason Alan Shockey

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Advisory Committee:
Dr. Caren Chang, Chair
Dr. Eric H. Baehrecke
Dr. Carol L. Keefer
Dr. Richard C. Stewart
Dr. Shunyuan Xiao

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Dedication

To Jack and John.

It’s been a long, strange ride.

Dum spiro, spero!

Acknowledgements

First and foremost, I would like to thank my advisor, Caren Chang, without whom this work would have never been accomplished. Richard Stewart provided CheA protein and assistance with the histidine kinase assays. I would also like to thank Yafan Huang and Joe Kieber for the *ctr1-8* clone and their sharing of unpublished results with me. The seedling growth rate assay in chapter 3 was performed in Tony Bleecker's lab by Brad Binder, both of whom I would also like to thank. I also owe Vinayaka Kotraiah and Steve Mount for assistance with the baculovirus expression system. Many thanks to Adrienne Kish for her help in creating the phylograms reported in chapter 4. Finally, my friends and family for the support and love they gave me when I needed it (and when I didn't).

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List of Abbreviations

ACC, 1-aminocyclopropane 1-carboxylic acid
AD, Activating Domain
AHP, *Arabidopsis* Histidine-containing phosphotransmitter
ARR, *Arabidospsis* Response Regulator
BD, Binding Domain
CAPS, Cleaved Amplified Polimorphic Sequence
CIP, Calf Intestinal Phophatase
Col, Columbia
CTR, Constitutive Triple Response
EBF, EIN3-binding F-box
EER, Enhanced Ethylene Response
EIL, EIN3-Like
EIN, Ethylene INsensitive
EIR, Ethylene Insensitive Root
EREBP, Ethylene Response Element Binding Protein
ERF, Ethylene Response Factor
ERS, Ethylene Response Sensor
ETO, EThylene Overproducer
ETR, EThylene Resistant
FRET, Förster Resonance Energy Transfer
GFP, Green Fluorescent Protein
GIN, Glucose INsensitive
GST, Glutathione S-transferase
HK, Histidine Kinase
HK+R, Histidine Kinase and Receiver domain
HLS, HookLeSs
HOG, High Osmolarity Glycerol
ISR, Induced Systemic Resistence
JA, Jasmonic Acid
JAR, JAsmonate Response deficient
MAPK, Mitogen-Activated Protein Kinase
MAPKK, MAPK Kinase
MAPKKK, MAPKK Kinase
MBP, Maltose Binding Protein
MMK, *Medicago* MAP Kinase
MS, Murashige and Skoog basal medium
NPA, 1-naphthylphthalamic acid
NPR, Nonexpresser of PR genes
NR, Never Ripe
PCR, Polimerase Chain Reaction
PR, Pathogen Response
RCN, Roots Curl in NPA

RR, Response Regulator protein
RT-PCR, Reverse Transcriptase PCR
SA, Salicylic Acid
SAR, Systemic Acquired Resistance
SCF, Skp1-Cullin-F-box ubiquitin ligase
SIMK, Stress-actIvated MAP Kinase
SIMKK, SIMK Kinase
SLN, Synthetic Lethal of N-end rule
SSK, Suppressor of Sensor Kinase
TCH, TouCH sensitive
TILLING, Targeting Induced Local Lesions IN Genomes
WEI, Weak Ethylene Insensitive
YPD, tyrosine (Y) Phosphatase Dependant

Chapter 1: Introduction

All organisms need the ability to sense external stimuli and be able to react in an appropriate manner. In this manner plants are no exception. Indeed, as plants are sessile organisms their need of this ability may be even greater than that of animals. One of the main ways a plant can disseminate a response is through the use of phytohormones. Of the known phytohormones, the signal transduction pathway for ethylene is the best understood. That being said, there are still large gaps in our understanding of this pathway.

Contrary to what the Nobel Prize committee believes, ethylene was identified as a gaseous hormone in 1901, 97 years before the Nobel prize was awarded for nitrous oxide as "the first discovery that a gas can act as a signal molecule in the organism" (Kende, 1998; Neljubov, 1901). Despite being the simplest olefin possible, ethylene gas has profound effects on the growth and development of higher plants**.** These numerous responses include induction of ripening in climacteric fruits, leaf expansion, promotion of seed germination, promotion or inhibition of flowering, abscission of various organs, and senescence (Abeles *et al.* 1992). Environmental stresses such as wounding, pathogen attack, and flooding can induce ethylene production; this stress-induced ethylene in turn can lead to certain defense responses such as accelerated senescence, abscission of infected organs, or induction of specific defense proteins (Abeles *et al.,* 1992). Ethylene is also produced by plants for normal, non-stress processes such as fruit ripening. The biosynthetic pathway of ethylene and aspects of regulation have long been established (Kende, 1993; Johnson

and Ecker, 1998), however our understanding of the mechanisms of its perception and signal transduction remains incomplete.

During the past decade and a half, considerable progress has been made in the genetic and molecular dissection of the ethylene-response pathway (Johnson and Ecker, 1998; Kieber, 1997). Briefly, a number of loci involved in ethylene signaling have been identified based largely on the isolation of ethylene-response mutants in *Arabidopsis* (Johnson and Ecker, 1998; Kieber, 1997; Bleecker *et al.,* 1988; Guzmán and Ecker, 1990; Roman *et al.,* 1995). The primary way in which these mutants have been isolated is using the classic "triple-response" (Fig. 1-1). The triple-response is named after the three effects ethylene has on most dicotyledonous species' etiolated seedlings, a shortening and thickening of the hypocotyl, shorting and thickening of the root, and exaggeration of the apical hook. Many of these mutant genes have been cloned, defining a pathway from ethylene perception to changes in gene expression (Fig. 1-2). Ethylene is perceived by a family of receptors that are similar to bacterial two-component histidine kinase receptors. Acting downstream of the ethylene receptors is a negative regulator of ethylene responses, CTR1, which has similarity to the Raf family of mitogen activated protein kinase kinase kinase (MAPKKKs) (Kieber *et al.,* 1993). There is limited evidence of a functional MAP kinase cascade after CTR1 (Quaked *et al.,* 2003). Functioning downstream of CTR1 (or the MAPK cascade) is EIN2 (Alonso *et al.,* 1999) which displays sequence homology with the N-ramp family of metal ion transporters. Epistatic analysis has placed a battery of transcription factors (EIN3, EIL1, 2, 3 and ERF1) downstream of EIN2. The identity of five additional genes, *EIN5, EIN6, EIN7, WEI2*, and *WEI3* remain yet to be

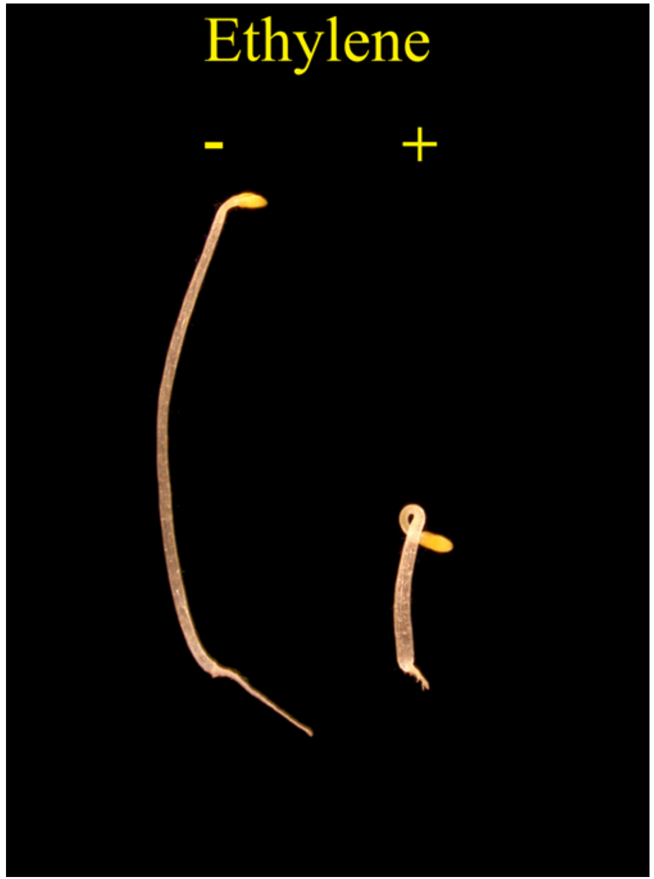


Figure 1-1. The triple-response of etiolated *Arabidopsis* seedlings. Dark grown Col-0 seedling in the presence or absence of ethylene. Note the shortened, thickened hypocotyl, the pronounced apical hook, and the shortened root for the ethylene treated seedling.

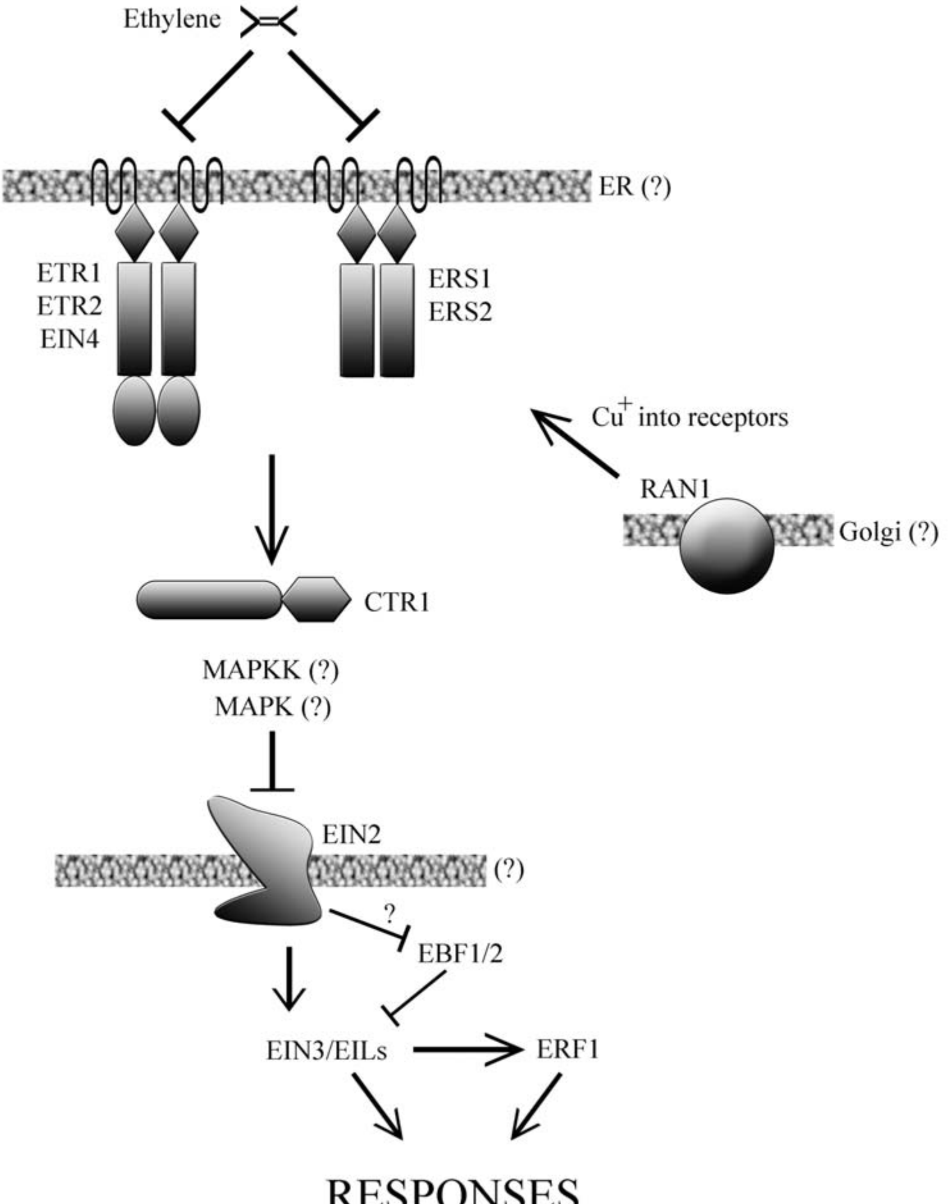

Ethylene
ER (?)
ETR1
ETR2
EIN4
ERS1
ERS2
Cu+ into receptors
RAN1
Golgi (?)
CTR1
MAPKK (?)
MAPK (?)
EIN2
(?)
?
EBF1/2
EIN3/EILs
ERF1
RESPONSES

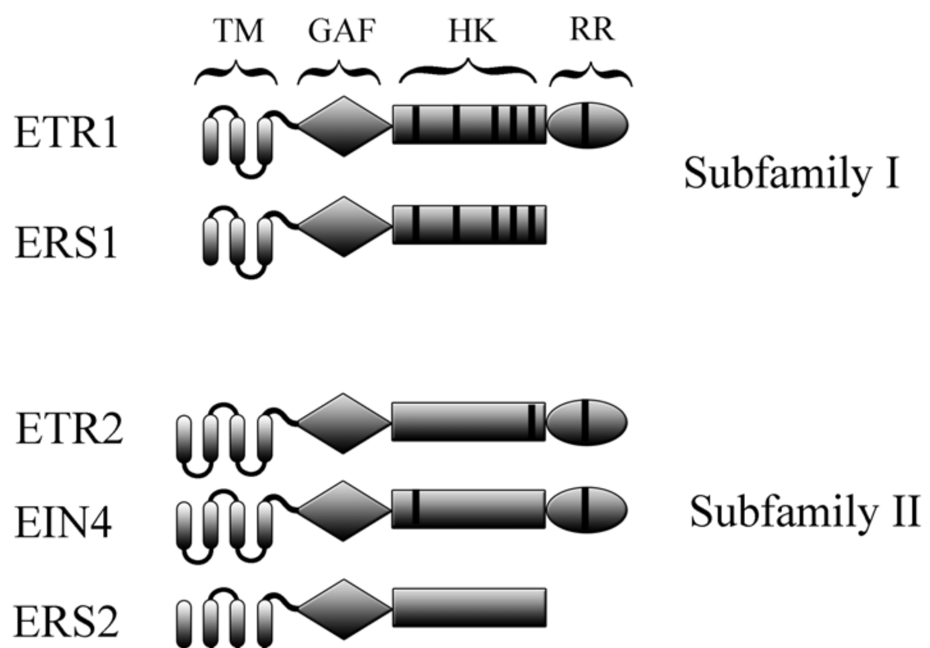
Figure 1-2. A current view of the ethylene signal transduction pathway based on cloned *Arabidopsis* genes. Ethylene gas is perceived by a family of ethylene receptor homodimers: ETR1, ERS1, ETR2, EIN4 and ERS2. The membrane-localized ethylene-binding sites require a copper cofactor, and the delivery of copper may depend upon the copper transporter RAN1. In the absence of ethylene, the receptors repress responses possibly through direct activation of the downstream negative regulator CTR1. CTR1 is thought to function as a MAPKKK in a MAP kinase module, potentially consisting of SIMKK and MPK6/13. Binding of ethylene, on the other hand, inhibits receptor activation of CTR1, perhaps through either promotion or inhibition of histidine autophosphorylation followed by phosphotransfer to an attached (or as yet unidentified) receiver. In the absence of activated CTR1, the EIN2 integral membrane domain, which has similarity to Nramp metal-ion transporters, activates the carboxyl-terminal domain of EIN2, which is comprised of a novel hydrophilic sequence. The carboxyl-terminal domain of EIN2 activates EIN3, which is a member of the EIN3/EIL family of transcription factors. EBF1/2 in the absence of ethylene ubiquinate EIN3 constitutively, and EIN3 is thus targeted for degradation. Ethylene turns this targeted proteolysis off, allowing EIN3 to proceed. EIN3 is a positive regulator that induces expression of an EREBP transcription factor gene called *ERF1*. ERF1 is a positive regulator that binds to the 'GCC box' promoter element of ethylene-regulated genes.

reported (Roman *et al.* 1995; Alonso *et al.,* 2003). In addition to these proteins which seem to fit in a linear pathway, several accessory proteins which act on the pathway have also been isolated (Hirayama *et al*., 1999; Larsen and Chang, 2001; Potuschak *et al.*, 2003; Guo and Ecker, 2003; Gagne *et al*., 2004).

The Ethylene Receptor Family

Three of the five members of the ethylene receptor family (*ETR1, ETR2*, and *EIN4*) were initially identified as missense mutations which conferred dominant insensitivity to ethylene. The first of these, *ETR1*, was cloned and found to encode a membrane protein with strong homology with the bacterial family of two component signaling proteins. The ethylene receptor family is characterized by an amino-terminal ethylene-binding domain, followed by a 'GAF'-related domain (Aravind and Ponting, 1997) and a histidine protein kinase-like domain (Bleecker, 1999) (Fig. 1-3). Some family members also possess a carboxyl-terminal receiver domain, which represents the second half of the two-component system. The GAF domain, which has been discerned in diverse proteins including cGMP phosphodiesterases (Aravind and Ponting, 1997), has an unknown function in the ethylene receptors.

The well studied two-component signaling systems are responsible for the interpretation of many environmental stimuli in most prokaryotic organisms (Stock *et al.*, 2000; Chang and Stewart, 1998). The two-component systems were named as such for the simple reason that the vast majority of these systems contain just two parts: A histidine kinase (HK) "sensor" and a response regulator (RR) responsible for output (Stock *et al.*, 2000; Chang and Stewart, 1998). The two-component HK "sensor" itself has two distinct domains. One, obviously, is the histidine kinase



	ETR1	ERS1	ETR2	EIN4	ERS2
ETR1	100/100	68/80	38/57	38/58	36/55
ERS1	68/80	100/100	43/62	35/54	37/56
ETR2	38/57	43/62	100/100	50/66	49/64
EIN4	38/54	35/54	50/66	100/100	45/61
ERS2	36/55	37/56	49/64	45/61	100/100

Figure 1-3. The *Arabidopsis* ethylene receptor family. The five receptors fall into two subfamilies (ETR1/ERS1 and ETR2/EIN4/ERS2) based on their gene (not shown) and protein structures. The percentage identity/ similarity of amino acids between domains of the receptors are shown. All members have an amino-terminal ethylene binding domain containing three (or four) transmembrane subdomains. The putative cytoplasmic portion is comprised of a 'GAF' related domain of unknown function, a histidine protein kinase domain and a receiver domain (which is lacking in ERS1 and ERS2). The conserved sequence motifs of histidine kinases and receiver domains, including the His and Asp phophorylation sites, are indicated by bars. The ETR2 subfamily contains an additional hydrophobic sequence at the amino-terminus, and lacks many of the histidine kinase sequence motifs.

domain itself, and is the domain that has the highest homology between the many HKs. When active, the HK utilizes ATP and phosphorylates itself, unlike the Ser/Thr and Tyr kinases which typically phosphorylate other proteins. The second and more diverse domain is the "input" domain and is responsible for the HK "sensor" actually sensing what it needs to. These domains are quite distinct, as domains for sensing different stimuli are in fact, different. Examples of HKs include both those as integral membrane proteins and those who are located in the cytosol (Stock *et al.*, 2000; Chang and Stewart, 1998).

The response regulators are also comprised of two domains. One domain is the so called receiver domain, which acts to "receive" the phosphate from the HK. The second, and most important domain from a specificity stand point, is the output domain. While this output domain is often a DNA binding motif, this is not always the case; prokaryotic examples include demethylases and proteases (Stock *et al.*, 2000; Chang and Stewart, 1998). In at least one eukaryotic examples (discussed below) the RR is involved in the regulation of a MAPKKK's activity (Maeda *et al.*, 1995; Maeda *et al.*, 1994).

Not all two-component systems have two components. Several examples exist in prokaryotes, such as *B. subtilis* sporulation regulation and *B. pertussis* virulence, in which the "two-component" systems actually contain four (Stock *et al.*, 2000; Chang and Stewart, 1998). Instead of the normal his (H) to asp (D) phosphotransfer, a phosphorelay of H to D to H to D occurs (Posas *et al.*, 1996; Uhl and Miller, 1996; Ninfa *et al.*, 1993; Burbulys *et al.*, 1991; Yang and Inouye, 1991). A significant portion of two-component like proteins found in eukaryotes are hybrid-

type HKs. That is, they have a receiver domain on the same protein as the HK, indicating that this multi-step phosphorelay plays a role in eukaryotic two-component systems. One signaling cascade where this has been shown is the HOG1 pathway in *S. cerevisiae* (Posas *et al.*, 1996).

The ethylene receptors identified in plants fall into two subfamilies based on sequence and structural similarities independent of whether there is a receiver domain. In *Arabidopsis*, there appears to be a total of five ethylene receptors; subfamily I is comprised of *ETR1* (Chang *et al.,* 1993) and *ERS1* (formerly called *ERS*) (Hua *et al.,* 1995; Alonso *et al.,* 2003), and subfamily II is comprised of *ETR2* (Sakai *et al.,* 1998), *EIN4* (Hua *et al.,* 1998) and *ERS2* (Hua *et al.,* 1998) (Fig. 1-3). In contrast to the *ETR1* subfamily, members of the *ETR2* subfamily lack most or all of the functional motifs of histidine kinases, and possess an extended amino-terminus containing a hydrophobic subdomain (Fig. 1-3). One member of each subfamily (*ERS1* and *ERS2*) lacks a receiver domain.

The most conserved portion among the ethylene receptor family members is the amino-terminal domain. This domain was found to be sufficient for saturable and reversible ethylene binding in ETR1 and ERS1, arguing strongly that ETR1 and ERS1 are ethylene receptors (Schaller and Bleecker, 1995; Rodriguez *et al.,* 1999, Hall *et al.,* 2000). Members of the *ETR2* subfamily are likely to be ethylene receptors as well, based on sequence similarity and mutant phenotypes comparable to those of the *ETR1* subfamily (Sakai *et al.,* 1998; Hua *et al.,* 1998; Hua and Meyerowitz, 1998). Notably, a similar sequence found within the genome of the cyanobacterium *Synechocystis* also encodes a protein that binds ethylene, suggesting a bacterial origin

for the ethylene receptors (Bleecker, 1999; Rodriguez *et al.,* 1999). Indeed, cyanobacteria such as *Synechocystis* and *Anabaena* are the only organisms outside of plants where this motif can be found, lending credence to the idea that the ethylene receptors have a plastid origin (Mount and Chang, 2002).

In addition to binding ethylene (Schaller and Bleecker, 1995), ETR1 has been found to be a membrane-associated disulfide-linked homodimer (Schaller *et al.,* 1995). It was also discovered that high-affinity ethylene binding requires a copper cofactor (two Cu (I) molecules per ETR1 dimer) (Rodriguez *et al.,* 1999). This is in agreement with earlier predictions that reversible ethylene binding requires a transition metal (Burg and Burg, 1967). The binding was assayed using membrane extracts of yeast cells expressing ETR1 (Rodriguez *et al.,* 1999). Interestingly, silver (Ag(I)), which has long been known to inhibit ethylene-responses, was also found to enhance the binding of ethylene, suggesting that Ag(I) blocks ethylene signaling at a step other than ethylene binding (Rodriguez *et al.,* 1999), perhaps by dissociating ethylene binding and signal output.

Copper's role in ethylene perception was implicated further by the cloning of *Arabidopsis RAN1*. The RAN1 product has high similarity to copper transporting P-type ATPases, and was shown to rescue a copper transport defect in yeast (Hirayama *et al.,* 1998; Woeste and Keiber, 2000). In plants, RAN1 potentially serves to produce functional ethylene receptors via intracellular delivery of copper (Hirayama *et al.,* 1998; Woeste and Keiber, 2000). This is supported by epistasis analysis, which indicates that RAN1 acts upstream of ethylene perception and by phenotypic similarities between *RAN1*-cosuppressed plants and ethylene receptor loss-of-function

mutants (which are discussed below) (Hirayama *et al.,* 1998; Woeste and Keiber, 2000). Furthermore, the addition of copper partially rescues the *ran1* mutant phenotype. *ran1* mutants were first isolated based on their responsiveness to the ethylene antagonist *trans*-cyclooctene; thus reduced copper levels can apparently lead to relaxed ligand specificity (Hirayama *et al.,* 1998; Woeste and Keiber, 2000).

The dominant mutations conferring ethylene-insensitivity isolated in *ETR1*, *ETR2* and *EIN4*, each causes an amino acid substitution typically within one of the three amino-terminal membrane-spanning segments (Chang *et al.,* 1993; Sakai *et al.,* 1998; Hua *et al.,* 1998). Identical mutations introduced into *ERS1* and *ERS2* conferred similar dominant ethylene insensitivity to wild type (Hua *et al.,* 1995; Hua *et al.,* 1998). A study of those dominant *ETR1* alleles showed that mutations in the first two hydrophobic domains eliminate ethylene binding, whereas a mutation in the third hydrophobic subdomain does not reduce binding (Hall *et al.,* 1999). Thus, gain-of-function insensitivity can result from either the inability to bind ethylene (and thus, the concomitant inability to shut off output signaling) or the uncoupling of ethylene binding from receptor output signaling. Further evidence for this idea comes from a screen for weak insensitive plants that was successful in isolating an *ERS1* mutant (Alonso *et al.,* 2003). This weak mutation was actually outside of the ethylene binding motif, and is believed to separate ethylene binding from ethylene signaling. *etr1-2* also retains the ability to bind ethylene, yet a plant carrying this alleles is also insensitive (Hall *et al.,* 1999).

A critical question concerning receptor action is the relationship between signal perception and receptor output signaling. Analysis of loss-of-function mutants

has shown that all five *Arabidopsis* receptors are negative regulators of ethylene responses (Hua and Meyerowitz, 1998; Zhao *et al.,* 2002; Wang *et al.*, 2003). These mutants were isolated by intragenic reversion of dominant mutations or identification of a T-DNA insertion (Hua and Meyerowitz, 1998; Zhao *et al.,* 2002; Wang *et al.,* 2003). Individual loss-of-function mutants displayed essentially wild-type phenotypes, revealing that the receptors are largely redundant. However, triple loss-of-function mutants of either sub-family two, or one member of subfamily one and two members of subfamily two, displayed constitutive ethylene responses similar to the *ctr1* mutant, arguing that the receptors normally act to repress responses in the absence of ethylene. Interestingly, a double knockout of *ETR1* and *ERS1* confers a stronger constitutive response than a plant missing four receptor (Zhao *et al.,* 2002;Wang *et al.,* 2003). This indicates that *ETR1* and *ERS1* have a unique role in ethylene signaling. From the loss-of-function phenotypes, we can deduce that the dominant receptor mutations, which confer insensitivity to ethylene, are gain-of-function alleles (as opposed to dominant negative alleles). The fact that the receptors are negative regulators tells us that ethylene binding serves to shut off receptor signaling.

Exactly what receptor 'output' or catalytic activity is has not been firmly established. The similarity to two-component regulators suggests a His-to-Asp phosphotransfer, and indeed, ETR1 has been shown to possess histidine autokinase activity *in vitro* (Gamble *et al.,* 1998). However, the second step of the two-component mechanism – transfer of the phosphate to a receiver domain – has yet to be demonstrated. On the contrary, mutations that disrupt histidine kinase activity *in*

vitro do not appear to disrupt *in vivo* functions of the ethylene receptors (Gamble *et al.,* 2002, Wang *et al.,* 2003). Truncated ETR1 (1-349) was able to confer complete insensitivity when carrying the *etr1-1* mutation in Col-0, although interestingly, it conferred only partial insensitivity in an *ETR1* null background. So while it seems the HK domain of ETR1 seems to play no role in normal signaling, it does seem to have some function. In fact, there are many things about the ethylene receptors that are "contrary" to expectations. One truly intriguing discovery was that in *Arabidopsis*, ETR1 is localized to the ER, not the plasma membrane which had been the presumed location (Chen *et al.,* 2002). While unexpected, the binding of ethylene at an internal location would pose no great hurdle for ethylene, which is lipid soluble. Why the cells would retain the ethylene receptor in an internal membrane is currently a mystery however.

The topic of redundancy seems to fall into the realm of the perplexing as well. While it was initially thought that the receptors were either redundant or had unique properties, the reality may be someplace in between. The receptors seem to have some amount of complete redundancy in that single knock-outs of any receptor yield practically no difference from wild-type (Hua and Meyerowitz, 1998; Zhao *et al.,* 2002; Wang *et al.,* 2003). On the other hand, subfamily I seems to play a much larger role, as the subfamily I double null had a more severe phenotype than the subfamily II triple null (Zhao *et al.,* 2002;Wang *et al.,* 2003). Subfamily II receptors expressed under the control of a subfamily I promoter were unable to alleviate this phenotype. So why are subfamily I receptors more important? One initial idea was that subfamily II would signal through subfamily I to CTR1, which subfamily one can

strongly interact with. There is even limited evidence that suggests subfamily II receptors may function through the subfamily I receptors (Cancel and Larsen, 2002). However, it has been shown recently that ETR2 can interact weakly with CTR1 in yeast two-hybrid assays. More importantly, *in vivo* evidence strongly suggests an interaction between subfamily two and CTR1 (Cancel and Larsen, 2002; Gao *et al.,* 2003).

Gene copy number has an important role as well: what was once considered "complete" dominant insensitivity can be titrated out with higher gene number, such as that resulting from a cross to a natural *Arabidopsis* tetraploid (Bensheim) (Hall *et al.*, 1999). Apart from reduced insensitivity, the plants behave normally. *Arabidopsis* does not however seem to use this as a compensatory mechanism. In plants lacking one of the receivers, RNA expression of the remaining four is unchanged (Cancel and Larsen, 2002; Zhao *et al.,* 2002).

The crystal structure of the ETR1 receiver domain provides some insight into the possible function of the ethylene receptors (Grantz *et al.,* 1998; Müller-Dieckmann *et al*., 1999). The overall tertiary structure of the ETR1 receiver domain is highly conserved with that of prokaryotic receiver proteins (Müller-Dieckmann *et al*., 1999). The crystal structure shows that the ETR1 receiver domain can dimerize, and that the aspartate which is predicted to receive the phosphate lies on that dimerization face. This would suggest that in an un-phosphorylated state, the receiver domain would be dimerized, whereas in a phosphorylated state the receiver would be monomeric. While mutagenesis has not focused on the receiver domain, mutations that disrupt histidine kinase activity *in vitro* have been reported to have wild-type

function *in planta* (Gamble *et al.,* 2002, Wang *et al.,* 2003). This indicates that two-component activity may be dispensable for gross ethylene responses. Further analysis of the structure of the receiver domain of ETR1 shows, that while no sequence similarity exists, the receiver's structure is similar to that of Raf1 (Müller-Dieckmann *et al*., 1999). This has great implications for the signaling component immediately downstream of the ethylene receptors, the Raf-like kinase CTR1.

CTR1

A screen for *Arabidopsis* plants showing a constitutive response to ethylene (specifically a strong triple response in the absence of exogenously applied ethylene) yielded several mutants, all of which were overproducers of ethylene save one, *CTR1*. When cloned (indeed, it was the first ethylene response gene cloned), *CTR1* showed strong homology to the Raf-family of ser/thr kinases (33% identity overall, 41% identity over the kinase domain) (Kieber et al., 1993). The region of strongest homology consisted of the last third of *CTR1*, and was homologous to the kinase region of Raf1. The first two thirds of the protein are a predicted auto-regulatory domain, with strong homology to other plant homologues, and weak homology to some Raf-like kinases in animals. When the ethylene receptors were cloned, it was at first perplexing as to why a "prokaryotic" signaling system (two-component receptors) would be in the same pathway as a "eukaryotic" signaling system (a MAP kinase kinase kinase). However the elucidation of the HOG1 pathway in *S.*

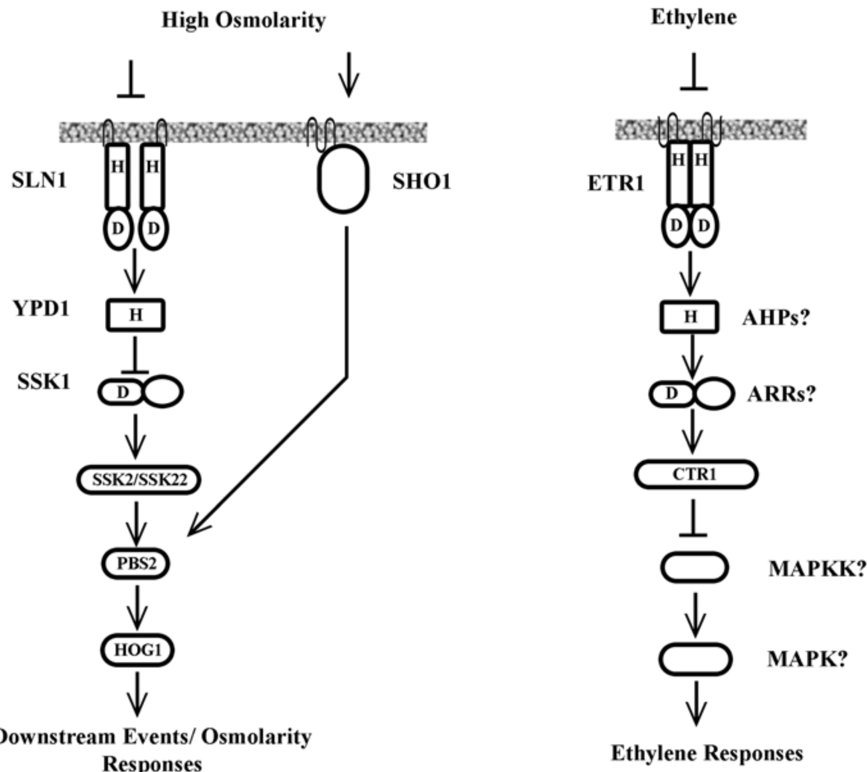


Figure 1-4. Parallels with the HOG1 pathway. Parallels with the yeast HOG1 osmosensing pathway were suspected before the ETR1/CTR1 interaction was demonstrated. ETR1 and SLN1 are both hybrid HKs who require MAPKKKs downstream.

cerevisiae described another eukaryotic signaling pathway which contained both "prokaryotic" and "eukaryotic" systems (Fig. 1-4) (Posas *et al.*, 1996).

In the HOG1 pathway, SLN1 acts as a hybrid histidine kinase, autophosphorylating and then transferring the phosphate to its own receiver domain. After SLN1 has transferred its phosphate to its receiver domain, the phosphate is then transferred to YPD1, a histo-phospho transfer domain protein. The phosphate is then transferred to the response regulator protein SSK1, which does not affect transcription, but rather modulates the activity of the MAPKKK SSK2 (Maeda *et al.,* 1994; Maeda *et al.,* 1995). The fact that three of the five ethylene receptors in *Arabidopsis* are also hybrid histidine kinases led to speculation that the ethylene signaling pathway may be very similar to the HOG1 pathway in yeast.

If the two-component mechanism is conserved, than a separate response regulator protein should act downstream of receptors that lack an attached receiver domain, and a His-containing phosphotransfer intermediate might act downstream of receptors that possess a receiver (Parkinson and Kofoid, 1992; Wurgler-Murphy and Saito, 1997). A number of response regulators (Sakakibara *et al.,* 1998; Imamura *et al.,* 1998; Urao *et al.,* 1998) and His-containing phosphotransfer proteins (Suzuki *et al.*1998) have been identified in plants, but to date, there is no evidence for their involvement in ethylene signaling. Quite the opposite, the regulatory domain of CTR1 was shown to associate with the presumed cytoplasmic portions of ETR1 and ERS1 in yeast and *in vitro* (Clark *et al.,* 1998). This novel interaction raises the

possibility that the receptors regulate CTR1 directly rather than through a response regulator as in the HOG1 pathway.

Of the many alleles of *CTR1* found up until now, all but one have been mutations in conserved kinase motifs or caused disrupted translation (premature stop codons or large insertions). The fact that disruption of kinase activity gives a constitutive ethylene response tells us that CTR1 is a negative regulator of ethylene responses, and thus should be inactivated by the ethylene receptors upon ethylene binding. Interestingly, EDR1 one of CTR1's closest homologues in *Arabidopsis* (63% identity) is also a negative regulator of its pathway (for EDR1 disease resistance) (Tand and Innes, 2002). Work with transgenic CTR1 and *in vitro* assays has shown that CTR1 has intrinsic protein kinase activity (Huang *et al.,* 2003). Interestingly, removal of the N-terminus of CTR1 did not increase kinase activity, which is a clear departure from Raf1. The *ctr1-8* mutation, the only mutation in *CTR1* which does not (at the primary sequence level) disrupt the kinase, gives a constitutive phenotype only slightly weaker than strong *ctr1* alleles. *ctr1-8* is a missense mutation in the N-terminus, and has been shown *in vitro* to possess wild-type levels of kinase activity (Huang *et al.,* 2003). So while *CTR1-8* retained wild-type kinase activity, its phenotype was that of a mutation disrupting the kinase. The solution for this apparent dichotomy came when *ctr1-8* was used in a yeast two-hybrid assay with *ETR1*. The mutation in *ctr1-8*, while having no effect on kinase activity, completely disrupted the interaction with ETR1. This both confirmed the interaction and expanded upon it: the interaction with ETR1 is essential for CTR1's kinase activity. Further evidence came with the overexpression of wild-type and

ctr1-8 N-terminals in *Arabidopsis*. The overexpression of the wild-type gave a strong constitutive phenotype, presumably by titrating out binding sites for the endogenous CTR1, whereas the *ctr1-8* remained unaffected (Huang *et al.,* 2003). Additional evidence for the interaction between the receptors and CTR1 came out of the receptor localization studies. It was shown using a CTR1 antibody that CTR1 was strongly associated with the ER membrane, much like ETR1 was shown to be (Gao *et al.,* 2003). In a *ctr1-8* plant, CTR1 was localized to the cytosol, indicative of the loss of the interaction. In addition, removal of more than one receptor also resulted in the loss of CTR1 at the membrane, even in plants missing only subfamily II receptors, providing indirect evidence of interaction of CTR1 with all the ethylene receptors (Gao *et al.,* 2003). This is again, another clear departure from Raf1, which, depending on the signaling state, moves between the cytosol and membrane (Stokoe *et al.*, 1994; Roy *et al*, 1997).

Search for a MAP kinase kinase and MAP kinase involved in ethylene signaling began as soon as *CTR1* was cloned. Despite there being around 10 MAPKKs and 20 MAPKs in *Arabidopsis*, none were ever identified in exhaustive mutagenesis screens for ethylene response mutants. Nevertheless it was shown (using ERK antibodies to phosphorylated and unphosphorylated forms) that in response to ethylene, MAP kinase activity does go up in plants (Novikova *et al.*, 2000). A full ten years after the cloning of *CTR1*, two MAP kinases, *SIMK* and *MMK3*, and one MAPKK, *SIMKK* were found to be activated in *Medicago* in response to ACC (Quaked *et al,* 2003). The *Arabidopsis* homolog of *SIMKK*, when overexpressed, gave a clear constitutive ethylene response. This is somewhat surprising (and is quite

controversial), as CTR1 is a negative regulator of ethylene responses, and *SIMKK* acts as a positive regulator. The exact relationship between *CTR1* and *SIMKK* has yet to be completely understood. To add further complexity to an already complex story, dephosphorylation of CTR1 may play a significant role in this kinase cascade. The *eer1-1* mutation in *Arabidopsis* confers an enhanced ethylene sensitivity, and alters *RCN1* a gene encoding a PP2A "A" subunit (Larsen and Chang, 2001; Larsen and Cancel, 2003). As it has been shown that another subunit of PP2A, the C subunit, interacts with CTR1, the *eer1-1* results suggest an involvement of PP2A in ethylene signaling.

Signaling to the Nucleus

In *Arabidopsis*, signal propagation from CTR1 to the nucleus requires *EIN2.* Nearly all *ein2* loss-of-function mutants (24 out of 25 alleles) are completely insensitive to ethylene, unlike other recessive ethylene-insensitive mutants, which are only partially insensitive (Alonso *et al.,* 1999). Mutants of *EIN2* have been also isolated in screens for delayed senescence, resistance to auxin transport inhibitors, or insensitivity to cytokinins or abscisic acid (Alonso *et al.,* 1999). A possible explanation for this is the ease with which complete ethylene insensitivity can be obtained at this locus (reflecting the importance of ethylene signaling in these other processes). Alternatively, *EIN2* has direct involvement in other signaling pathways. However, that may not be the case, as *EIN2* mutants confer such a strong insensitivity; it is the only non-receptor insensitive mutation that makes a subfamily I double null completely insensitive (Hall and Bleecker, 2003). The non-ethylene

phenotypes identified in *EIN2* mutants could be easily explained by ethylene insensitivity. When cloned, *EIN2* was found to code for a novel protein with an amino-terminal integral membrane domain that has similarity to the Nramp family of metal-ion transporters (Alonso *et al.,* 1999). EIN2 is membrane-associated, but lacks detectable metal transport activity. The amino-terminal domain may serve as a sensor of divalent cations or as a membrane anchor (Alonso *et al.,* 1999). Overexpression of *EIN2*'s hydrophilic carboxyl-terminal domain (in an *ein2* mutant background) confers a number of constitutive ethylene responses, and it appears that ethylene regulation of this activity requires the EIN2 amino-terminal domain (Alonso *et al.,* 1999). Ethylene-response phenotypes from overexpression of the EIN2 carboxyl-terminal domain are suppressed in an *ein3* mutant background. The *Arabidopsis EIN3* gene codes for a nuclear-localized DNA binding protein that acts downstream of EIN2 (Chao *et al.,* 1997). The *Arabidopsis EIN3* family includes three *EIL* (*EIN3-Like*) genes, two of which (*EIL1* and *EIL2*) are capable of rescuing the *ein3* mutant (Chao *et al.,* 1997). Overexpression of *EIN3* or *EIL1* also results in constitutive ethylene responses (Chao *et al.,* 1997).

Ethylene signaling produces dramatic changes in gene expression. Promoter analysis of ethylene-induced genes has led to the identification of ethylene-responsive *cis*-acting elements (Deikman *et al.,* 1998), as well as *trans*-acting DNA-binding proteins that recognize these sequences (Leubner-Metzger *et al.,* 1998). The latter include the ethylene-responsive element binding protein (EREBP) family first identified in tobacco and related to the *AP2* family of transcription factors (Ohme-Takagi *et al.,* 1995). A breakthrough in understanding the transcriptional cascades

involved in ethylene responses was the discovery that EIN3 is an activator of an *Arabidopsis EREBP* gene homolog, *ERF1* (*Ethylene-Response-Factor1*), thereby establishing a transcriptional hierarchy in ethylene signaling (Solano *et al.,* 1998). EIN3 binds as a dimer to a specific *ERF1* upstream sequence that has similarity to a previously identified ethylene-responsive element in plants (Solano *et al.,* 1998). ERF1, in turn, binds (as do other EREBPs) to a *cis*-acting ethylene response element known as the 'GCC box' (Solano *et al.,* 1998). Overexpression of *ERF1* confers a subset of constitutive ethylene responses (Solano *et al.,* 1998).

A unique aspect of *EIN3* regulation seems to be at the protein level rather than RNA. *EIN3* mRNA levels do not change in response to ethylene, but protein levels rapidly rise (Potuschak *et al.*, 2003; Guo and Ecker, 2003; Gagne *et al.*, 2004). As it turns out, EIN3 protein is constantly made, and then targeted for degradation by the SCF complex, mediated by an interaction between EIN3 and EBF1/2 (Potuschak *et al.*, 2003; Guo and Ecker, 2003; Gagne *et al.*, 2004). Single knockouts of the *EBF1* gene conferred a slight hypersensitivity to ethylene. Interestingly, Guo and Ecker (2003) report that a double knockout gives a phenotype comparable to a *ctr1* mutation, while Gagne *et al*. (2004) report a double mutant which has a phenotype dramatically more severe than any previously reported *ctr1* allele. As plants lacking EBF1 or 2 have been shown to accumulate EIN3 protein above normal amounts when gassed with ethylene, the phenotype reported by Gagne *et al.* (2003) makes more sense. How this targeted degradation of EIN3 is controlled and if EIL1 and 2 are controlled in a similar manner have yet to be shown.

Other Plants

One of the main questions that *Arabidopsis* geneticists have to answer is if the work they are doing is relevant to crop plants. In the case of ethylene, our faith in this little weed seems well placed. Ethylene receptor gene homologs have been reported in a number of other plants, including tomato, rice, broccoli, apple, *Rumex palustris,* muskmelon, tobacco, and pear (Lashbrook *et al.,* 1998; Tieman and Klee, 1999; Zhou *et al.,* 1996; Wilkinson *et al.,* 1995; Payton *et al.,* 1996; Sato-Nara *et al.,* 1999; Vriezen *et al.,* 1997; Yau and Yip, 1997; Chen *et al.,* 1998; Lee *et al.,* 1998; Xie *et al.,* 2003; El-Sharkawy *et al.*, 2003). Of these, the tomato family is the most characterized: *LeETR1, LeETR2* and *LeETR3* belong to the *ETR1*-like subfamily, while *LeETR4, LeETR5*, and *LeETR6* belong to the *ETR2*-like subfamily, and all but *LeETR3* possess a receiver domain (Lashbrook *et al.,* 1998; Zhou *et al.,* 1996; Wilkinson *et al.,* 1995; Tieman and Klee, 1999; Klee and Tiemann, 2002). The *LeETR3* gene corresponds to the dominant ethylene-insensitive *Never ripe* (*Nr*) mutant whose phenotype includes an extreme delay in fruit ripening (Wilkinson *et al.,* 1995; Lanahan *et al.,* 1994). A mutation identical to that in *etr1-1* was introduced into *LeETR4* and *LeETR5*, and these altered tomato genes conferred dominant insensitivity in transformed *Arabidopsis* (Tieman and Klee, 1999).

Detailed studies have shown that all five receptor genes of tomato have distinct expression patterns throughout development suggesting that the receptors have different tissue- and stage-specific roles in ethylene signaling (Lashbrook *et al.,* 1998; Tieman and Klee, 1999; Zhou *et al.,* 1996; Payton *et al.,* 1996). Differential expression was also observed in other plants (Sato-Nara *et al.,* 1999; El-Sharkawy *et*

al., 2003). Some receptor genes are inducible; for example, an *ERS1* homolog in *Rumex palustris* is induced by flooding (Vriezen *et al.,* 1997), and *LeETR3* is induced by ethylene in ripening fruit (Lashbrook *et al.,* 1998). These findings support the idea that there is complex regulation of ethylene responses at the level of ethylene sensitivity. Evidence for real biological distinctions between climacteric fruit bearing plants and non-fruit bearing annuals like *Arabidopsis* exists not only in the differential receptor expression seen in tomato (and lacking in *Arabidopsis*), but in the importance of receptors as well. In tomato, a knock-out of one receptor, *LeETR4*, conferred marked hypersensitivity to endogenous ethylene, while in *Arabidopsis* single knock-outs of any receptor cause little effect (Tieman *et al.*, 2000). A knock-out of *NR* in tomato also shows an induction of *LeETR4* mRNA expression, a functional compensation not seen in *Arabidopsis* (Tieman *et al.*, 2000; Cancel and Larsen, 2002; Zhao *et al.*, 2002)

In addition to ethylene receptors, other members of the ethylene pathway originally identified in *Arabidopsis* have been isolated in tomato. In *Arabidopsis*, a single *CTR1* gene plays a central role in the pathway; knock-outs of this gene confer a strong constitutive response. Interestingly, in tomato, there exists three distinct homologs of *CTR1*; *LeCTR1*, *LeCTR3*, and *LeCTR* all of which can rescue a *ctr1* mutation in *Arabidopsis* (Leclercq *et al.,* 2002; Adams-Phillips *et al.*, 2004). In addition, it appears *LeCTR4* has two alternate splicing forms, in *Arabidopsis CTR1* has no known splicing variants (Adams-Phillips *et al.*, 2004). One splicing form, *LeCTR4 sv1*, has a premature stop codon before the kinase domain. This is of special interest as a potential regulator of ethylene responses, as it has been shown in

Arabidopsis that over expression of the N-terminus alone can confer constitutive ethylene responses (Huang *et al.*, 2003). As in the receptors, tomato *LeCTR*s seem to have distinct expression patterns not seen in *Arabidopsis*, indeed *AtCTR1* is non-inducible while *LeCTR1* is. Whether or not these distinct expression patterns have a biological role has yet to be elucidated.

Whether a functional MAP kinase cascade exists in the ethylene response pathway is a question whose solution was long thought answered in *Arabidopsis* before all plants, due to its genetic trailblazing. It therefore came as a surprise when a MAPKK and MAPK involved in ethylene responses were originally isolated in *Medicago* (Quaked *et al.*, 2003). Homologs of the genes found in *Medicago* were isolated and found to behave in the same way in *Arabidopsis*. The presence of these proteins in other plants has not been reported as of yet, however their existence is not in doubt.

Downstream elements are also present in tomato in greater number then in *Arabidopsis*. There are three *EIN3* homologs in tomato, all of which can complement the *ein3-1* mutation in *Arabidopsis* (Tieman *et al.*, 2001). Antisense knockouts of anyone confer a weak insensitivity, while reductions in all three produce the strong insensitivity exhibited by the single knockout in *Arabidopsis*.

Ethylene Signaling in Defense Responses

A large body of work has contributed to the accumulating evidence that ethylene signaling has a substantial role in disease resistance. Expression of the dominant *Arabidopsis etr1-1* gene in wild-type tobacco conferred susceptibility to what is otherwise a non-pathogenic soil fungus (Knoester *et al.*, 1998). In tomato,

disease symptoms were analyzed in the ethylene-insensitive *Nr* mutant as well as in a 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase mutant; although both mutants displayed the normal primary reaction (bacterial spots and lesions) to several pathogens, secondary responses of systemic acquired resistance (SAR) (leaf necrosis and organ abscission) were substantially reduced (Lund *et al.*, 1998).

A major advance was the discovery that ethylene acts in jasmonic acid (JA)-dependent pathways that are distinct from the salicylic acid (SA)-dependent SAR pathway (Pieterse *et al.*, 1999). Induced systemic resistence (ISR) results from colonization of roots by non-pathogenic rhizobacteria, enhancing resistance to other pathogens. Analyses of the *Arabidopsis* mutants *etr1*, *jar1* (jasmonate response deficient), and *npr1* (a downstream regulator in SAR) showed that all are deficient in ISR even though growth of the eliciting pathogen was the same as in the wild type (Pieterse *et al.*, 1998). Further analyses showed that ethylene acts downstream of jasmonic acid, and upstream of NPR1, in the ISR pathway (Pieterse *et al.*, 1998). Ethylene signaling is also required for upregulation of the antimicrobial defensin gene *PDF1.2* (Penninckx *et al.*, 1996; Alonso *et al.*, 1999). Plants undergoing ISR do not display induction of *PDF1.2*, pointing to the existence of a separate pathway in which ethylene is involved (Penninckx *et al.*, 1998). A study of *PDF1.2* induction in *etr1-1* and *coi1-1* (JA insensitive) mutants indicated that ethylene and JA are concomitantly required in this second pathway (Penninckx *et al.*, 1998). A second piece of evidence for this is the expression of *ERF1*. Expression of *ERF1* increases in response to both ethylene and Jasmonic acid, and evidence suggests that activation of *ERF1* requires both (Lorenzo *et al.*, 2003).

Results of similar studies using soybean (*Glycene max*) mutants are less clear-cut. Ethylene-insensitive soybean mutants gave either a slight advantage or seemed to confer greater susceptibility when presented with a wide range of pathogens (Hoffman *et al.*, 1999). This suggests that ethylene has different effects depending upon the particular pathogen. Current opinion is that ethylene/jasmonate regulated responses show the most effectiveness against necrotrophic pathogens and herbivorous insects (Pieterse and Van Loon, 2004).

Ethylene signaling in other processes

Several papers have utilized ethylene-response mutants to analyze the involvement of ethylene perception/signaling in processes other than defense responses. For example, the tomato *Nr* mutant was used to elucidate ethylene's role in crown gall morphogenesis (Aloni *et al.*, 1998). Analysis of *etr1*, *ein2*, and *eto1* mutants provided genetic evidence that ethylene promotes root hair elongation in *Arabidopsis* (Pitts *et al.*, 1998). Nodulation was found to be unaffected by ethylene-insensitive mutations in soybean (in contrast to results in *Medicago truncatula*) (Schmidt *et al.*, 1999). Ample evidence suggests that ethylene plays a role in thigmomorphogenesis based on induction of ethylene biosynthesis by touch stimulation, yet thigmomorphogenesis and *TCH* (*touch*) gene expression are independent of *ETR1* or *EIN2* (Johnson *et al.*, 1998; Arteca *et al.*, 1999).

A connection between ethylene response and auxin transport in roots was found through the cloning of *Arabidopsis EIR1* (Luschnig *et al.*, 1998). Mutants of *eir1* were isolated by either root-specific ethylene-insensitivity or agravitropism (Roman *et al.*, 1995; Luschnig *et al.*, 1998). Evidence indicates that *EIR1* codes for

an auxin transporter, hinting that ethylene may inhibit root growth by increasing internal concentrations of auxin. This is reminiscent of the ethylene and auxin connection seen in the *Arabidopsis hls1* (*hookless1*) mutant (Lehman *et al.*, 1996). *HLS1* is a repressor of auxin-induced cell expansion in the apical hook, and the mutant phenotype can be phenocopied by inhibiting auxin transport in the wild type (Lehman *et al.*, 1996).

A significant amount of data suggests the ethylene-response pathway may intersect with the glucose-sensing pathway. Some of the phenotypes displayed by the *Arabidopsis* glucose-insensitive mutant *gin1* are similar to ethylene responses (Zhou *et al.*, 1998). Wild-type plants treated with the ethylene precursor ACC can overcome glucose repression. The mutants *ctr1-1* and *eto1-1* (an ethylene overproducer) are likewise resistant to glucose. *etr1-1*, in contrast, is hypersensitive to glucose. Double mutant analysis shows *GIN1* to be epistatic to *etr1*. It seems that when activated by high levels of glucose, *GIN1* represses a subset of ethylene responses, which include promotion of seed germination and greening and expansion of cotyledons (Zhou *et al.*, 1998).

Conclusion

While the general framework of the ethylene signaling pathway has been elucidated, what we have found has led to more questions. Indeed, it seems as though the ethylene signal transduction pathway is full of unexpected surprises and novel mechanisms. While in the early days of genetic dissection it seemed as though existing paradigms would provide an easy road map, the current view of the pathway is clouded and obscured with the fog of truly novel mechanisms.

Chapter 2: Regulation of the CTR1 Raf-like Kinase

Introduction

Since its initial discovery, the *CTR1* locus has been known to be a negative regulator of ethylene responses (Kieber *et al*., 1993). Once cloned, the CTR1 kinase domain was shown to have high similarity to that of the Raf family of mitogen-activated protein kinase kinase kinases (MAPKKKs), which have an amino-terminal regulatory domain and a carboxyl-terminal kinase domain (Kieber *et al*., 1993; Huang *et al*., 2003). The amino-terminal "domain" of CTR1 comprises two-thirds the length of CTR1 and is highly divergent from that of Raf (Huang *et al*., 2003) (Fig. 2-1). A critical yet unanswered question is how the ethylene receptors signal to CTR1. Known paradigms do not seem to apply in this case, as MAPKKKs are not typically regulated by two-component receptors. The demonstration that CTR1 could associate with ETR1 and ERS1 in the yeast two-hybrid assay and *in vitro* suggested that the ethylene receptors may directly regulate CTR1's function (Clark *et al*., 1998). CTR1 interacts weakly, if at all, with the subfamily 2 receptors (Cancel and Larsen, 2002; Wen and Chang unpublished), although there is indirect evidence that such an interaction occurs *in planta* (Gao *et al.,* 2003).

Evidence to support the idea that CTR1 is directly regulated by the receptors was provided by the *ctr1-8* allele, which is the only missense mutation known to lie in the amino-terminal domain of CTR1 (encoding a glycine to glutamate substitution at residue 354) (Huang *et al*., 2003). *ctr1-8* plants possess a constitutive ethylene-

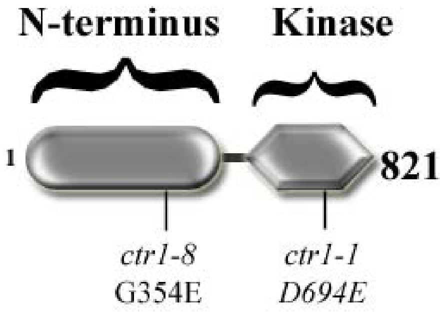


Figure 2-1. The CTR1 protein. 821 amino acids overall, the putative regulatory N-terminus extends to residue 532, with the remaining 289 amino acids forming the kinase domain which has a high degree of homology with the Raf family of MAPKKKs. Two important mutations, *ctr1-8* (G354E) and *ctr1-1* (D694E) are labeled.

response phenotype resembling that of loss-of-function *ctr1* mutants, which specifically lack kinase activity (Huang *et al*., 2003). The mutant CTR1-8 protein exhibits wild-type levels of kinase activity *in vitro*, suggesting that the *ctr1-8* mutation impairs regulation of the kinase domain, causing it to be inactive. Interestingly, the *ctr1-8* mutation was found to disrupt the interaction between CTR1 and the ETR1 receptor in the yeast two-hybrid assay, suggesting that physical association of CTR1 with ETR1 is required to keep CTR1 active (Huang *et al*., 2003). Furthermore, over-expression of the CTR1 amino-terminal domain (but not the CTR1-8 amino-terminal domain) resulted in constitutive ethylene responses (Huang *et al*., 2003), indicating that the over-expressed amino-terminal domain may interfere with the association of wild-type CTR1 and the receptors.

Another unresolved question about *CTR1* is thus: How does it signal to downstream elements? With the high homology to MAPKKKs, it has been assumed since its cloning that *CTR1* would be at the head of a MAP kinase cascade. 10 years of no evidence for that idea did nothing to tarnish it, and as it turns out, a MAP kinase cascade may in fact exist, but not like any other known. A MAPKK and two MAPKs have been identified as possible downstream targets of CTR1 in *Arabidopsis* and *Medicago* (Ouaked *et al*., 2003). But unlike CTR1, which is a negative regulator, this MAPKK and the two MAPKs are positive regulators. And thus, the plot thickens.

In order to examine the regulation of *CTR1*, and thus gain insight into its function, I addressed the following three questions. One, is there spatial regulation of CTR1? Raf-1 is known to have an inactive cytostolic state, and an active membrane

associated state (Stokoe *et al.*, 1994; Roy *et al*, 1997). As CTR1 is known to be able to associate with integral membrane proteins (ETR1 and ERS1), I wished to examine if CTR1 has a similar spatial regulation, that is differing sub-cellular localization in the absence or presence of ethylene.

Second I asked if CTR1 could be activated by ETR1 *in vitro*. While genetics has given us many clues into how CTR1 may be activated, biochemical evidence is lacking. An *in vitro* assay of CTR1 activity and activation would be a useful tool in dissecting how CTR1 is activated. Towards that goal, I wished to create an *in vitro* assay for CTR1 activation.

Finally I asked, what inter- and/or intra-protein interactions mediate regulation of CTR1 kinase activity? The N terminus of CTR1, while having no homology to RAF's N terminus, has been assumed to be a regulatory domain for CTR1's kinase. In addition to examination of the role of the interaction with ETR1 in CTR1's function, I wished to specifically examine the role of CTR1's N terminus.

Results

Spatial regulation of CTR1

In quiescent cells Raf is cytostolic and membrane bound in activated ones (Marshall, 1995; McCormick, 1995). Prior to the brute-force experimentation done by the Schaller lab localizing ETR1 to the ER, and showing that CTR1 remains bound to ETR1 (Chen *et al.,* 2002; Gao *et al.,* 2003), it was commonly held that CTR1 would behave in a manner similar to Raf. An important bit of knowledge that was missing is the binding state of ETR1/CTR1 in the presence or absence of

ethylene. In order to examine this, we decided to use an *in planta* assay using GFP tagged CTR1. The idea was fairly simple, express CTR1-GFP in *Arabidopsis ctr1-3* (a knockout) plants, and look at the GFP localization in the presence and absence of ethylene.

In order to begin experimentation before transgenic plants could be made, we used particle bombardment of onion epidermal cells to examine the localization. Several technical difficulties became apparent. The first was a problem inherent in particle bombardment, that of low efficiency. The probability of a particle with our DNA passing through the cells nucleus is much lower than that of the rest of the cell. One way to increase the rate of transformation is of course to bombard with more particles more times over the onion epidermis. This however pays tribute to the laws of diminishing returns. Indeed, there is an inflection point where the number of viable cells decrease due to the physical damage involved in the bombardment procedure. To get around this we would often do multiple bombardments per DNA sample on different onion samples to make sure we had good samples for every experiment.

The second technical difficulty was due to the physiology of ethylene biosynthesis. As ethylene is produced in response to tissue damage, we found that our onion epidermal cells were making large amounts of ethylene. The tissue damage had two sources, neither of which we could control for: the bombardment itself and the removal of the epidermal cells from the onion layers. To stop this production of ethylene, the onion cells were kept on media (both before and after bombardment) containing the ethylene biosynthesis inhibitor, AVG. AVG inhibits ACC synthase

which converts S-adenysol methionine to ACC, the immediate precursor to ethylene. For our samples that were to be plus ethylene we simply added ACC and AVG. Thus, the AVG would block ethylene production due to wounding, and the ACC was rapidly turned into ethylene by ACC oxidase.

The third technical difficulty proved harder to over come. When we expressed CTR1-GFP, we saw a band surrounding the periphery of the cell independent of the ethylene (Fig. 2-2). In fact, GFP alone gave a similar pattern (Fig. 2-2). Thinking about the physiology of onion cells quickly showed why: the vacuole in onion cells fills the vast majority of the cell's volume. Thus, the cytosol in onion cells is a thin shell trapped between the vacuole and plasma membrane. This prompted us to try and do these experiments in *Arabidopsis* protoplasts. Apart from the inherent difficulties involved with the production of *Arabidopsis* protoplasts, we ran across further difficulties. For one, the vacuole in protoplasts, while still smaller than in onion cells is still quite significant. Second, the auto-fluorescence of the cells was quite high due to the chlorophyll, which reduced our signal strength.

As the cytological localization of CTR1 was encountering difficulties, we decided to approach the problem from another direction. The question we were trying to address was not the subcellular localization of CTR1 *per se*, rather if the interaction with ETR1 was dynamic, and if so what state was the interaction in under what ethylene conditions. As the subcellular localization of CTR1 (and any change thereof) was proving problematic, we decided to utilize Förster Resonance Energy Transfer, or FRET.

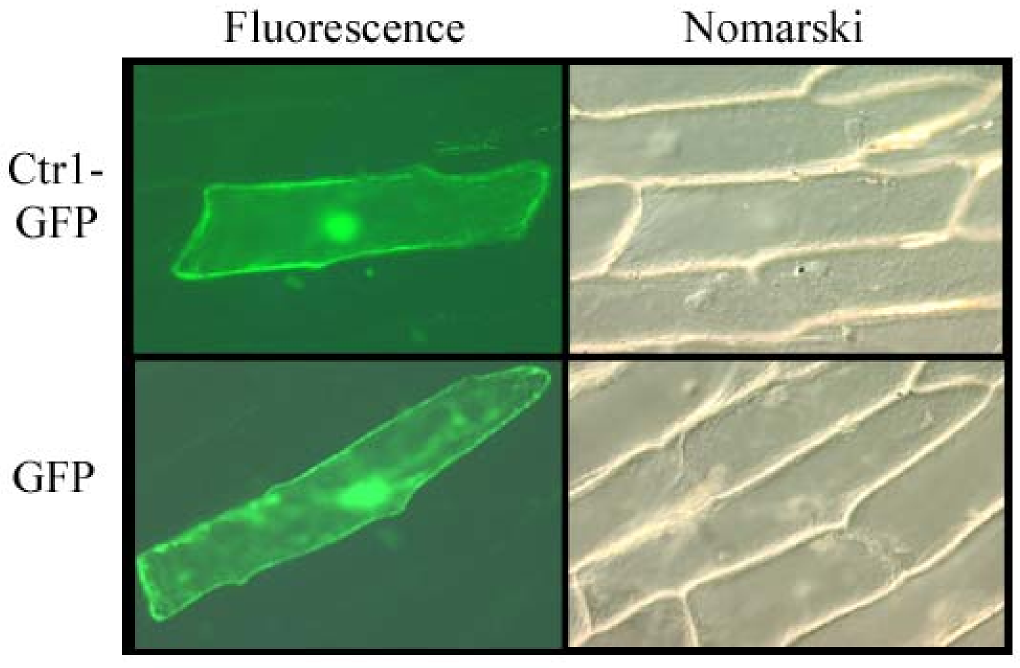


Figure 2-2. Localization of CTR1-GFP in onion cells. Onion epidermal cells were made to transiently express either CTR1-GFP or GFP alone following particle bombardment. No appreciable difference was seen between CTR1-GFP and GFP alone in cells exposed to ethylene and those not exposed. As a reference, the same samples were observed with Nomarski optics.

FRET occurs when excited-state energy from one fluorophor is transferred to another (Hink *et al.*, 2002). What this means experimentally is that when you excite the donor fluorophor, and the two are within 10nm, the energy will be transferred and you will see emission from the acceptor. Thus co-expression of a CTR1-GFP and an ETR1-BFP, when the two are bound, would provide a FRET signal, allowing us to determine if the two proteins were bound irrespective of our ability to determine if GFP signal was specific to the plasma membrane. Similar experiments have worked in *Arabidopsis*, showing an interaction between the light signaling proteins PHYB and CRY2 (Más *et al.*, 2000)

Initially we tried using a mix of two plasmids in the bombardment mixture, but quickly realized the likelihood of getting a cell co-transformed with both plasmids was extremely low. We therefore constructed a plasmid containing both CTR1-GFP and ETR1-BFP.

When we ultimately got this plasmid successfully into onion cells, we were unable to detect FRET under any circumstances. This may have been due to several reasons. One, the GFP and BFP resulted in inappropriately folded proteins, or proteins which were sterically hindered in binding. Second, the expression of ETR1-BFP may have been too low. Examination of ETR1-BFP constructs gave a very low signal for BFP.

Hindsight tells us this project was not likely to answer the questions we were asking of it. Assuming we were able to get the technical hurdles worked out, we would have found FRET under all conditions tested, as Gao *et al.* (2003) have shown that CTR1 remains bound to the ER membrane. Alternatively, there is the possibility

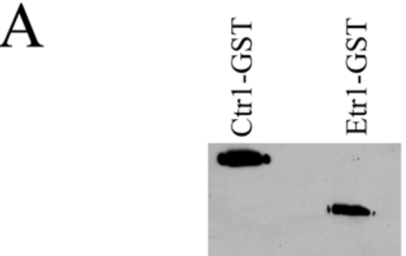
that we would have found that FRET was present under one condition (ethylene/no ethylene) and not the other. This would have put our results in direct conflict with that of Gao *et al.* (2003). However, as they have not shown that CTR1's staying at the ER is due to a direct interaction between ETR1 and CTR1, there is the possibility that while CTR1 stays at the ER, the interaction with ETR1 (and the other receptors) is dynamic.

In vitro activation of the CTR1 kinase

The novel interaction between the two-component like ethylene receptors and the MAPKKK CTR1 suggested that the receptors directly activated CTR1. While we did not believe that there was an enzymatic activation (such as phosphorylation of CTR1 by ETR1), the possibility of a structural interaction regulating CTR1's activity was not without precedent. In the HOG1 pathway in *S. cerevesiae* the response regulator SSK1 interacts with, and regulates, the MAPKKK SSK2. The genetic evidence for such a situation in *Arabidopsis* was strong, however, direct biochemical evidence was lacking. In order to obtain that evidence, we decided to examine the *in vitro* activity of CTR1.

In order to examine full length CTR1 activity, we expressed the full length CTR1 fused to GST, using a baculovirus expression system (Fig. 2-3A) (Kidd and Emery, 1993). The reasons for using the baculoviris system were two-fold. All attempts to express full length CTR1 or N-terminal deletions proved toxic to both bacteria and yeast, and prevented accumulation of the transgenic protein to any real amount. Second was that baculovirus is known to produce proteins that are folded and modified in a manner closer to that of the higher eukaryotes. Similar to Huang *et*

A



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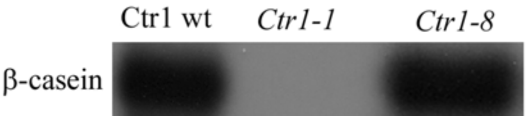


Figure 2-3. CTR1 has kinase activity that can be disrupted by the *ctr1-1* mutation, but not the *ctr1-8* mutation. A) A western blot on purified ETR1-GST and CTR1-GST. After purification, protein samples were run out using SDS-PAGE, and found to yield single bands of the right size. A western blot was conducted using α-GST antibodies to confirm the bands observed on the SDS-PAGE gel were in fact the GST-fusions. B) Using β-casein as a non-specific substrate for ser/thr kinase activity. Both CTR1 and *Ctr1-8* proved able to phosphorylate β-casein, while *Ctr1-1* was unable to. ETR1 also proved unable to phosphorylate β-casein (not shown).

al. (2003), we found that incubation of GST-CTR1 (1-822) with γ^{32}P-ATP and a non-specific kinase substrate (in our case β-casein) produces two radioactive bands, corresponding to β-casein and GST-CTR1 itself (Fig. 2-3B). We also demonstrated that CTR1-8 retained wild-type levels of kinase activity, and that CTR1-*1* was lacking any real kinase activity above background. Removal of the CTR1 N-terminal domain does not affect the kinase activity of baculovirus-expressed CTR1 (Huang *et al*., 2003), raising doubt as to its function as an autoinhibitory domain.

Since it is known that many protein kinases are activated by phosphorylation (Deak and Templeton, 1997), we investigated whether phosphorylation is involved in activation of CTR1. Pretreatment of purified GST-CTR1 with calf-intestinal phosphatase (CIP) prior to the kinase assay dramatically decreased both phosphorylation of β-casein and CTR1's auto-kinase activity (Fig. 2-4). This indicates that phosphorylation plays a role in activation of CTR1 *in vitro*. How this could happen *in vivo* is not clear, possibly by a yet unidentified protein in the ethylene signaling cascade, or more likely, the recruitment of two CTR1s to an ethylene receptor complex promoting CTR1s autokinase activity. Incubation of de-phosphorylated CTR1 in the presence of ATP for longer than the scope of the kinase assay does show re-activation of CTR1, indicating, along with the observed auto-kinase activity, that CTR1 may be responsible, in part, for its own activation.

As we were able to inactivate CTR1 *in vitro*, we were now free to add proteins into the mix to determine if any were able to activate it. GST-ETR1, GST-ERS1, GST-ETR1 receiver domain, and GST alone were all expressed in the baculovirus

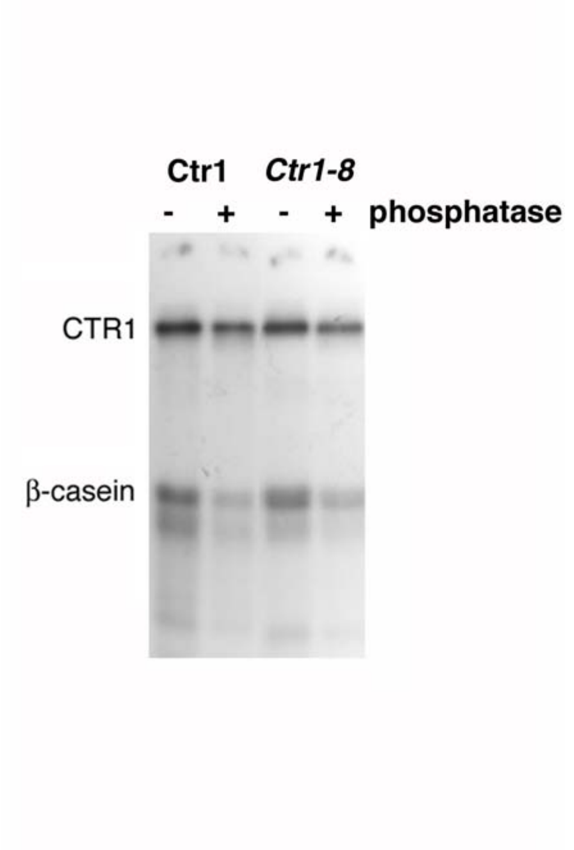
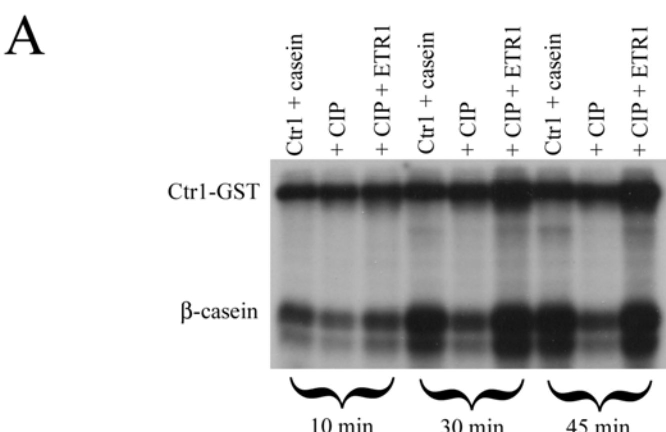
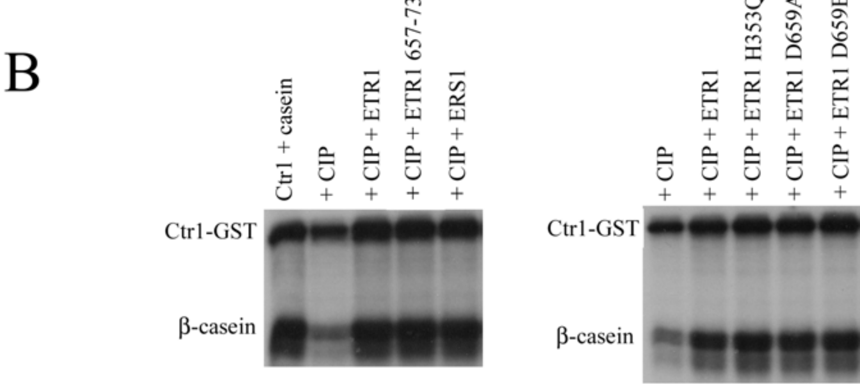


Figure ctr 2-4. CTR1 kinase activity is dependent on autophosphorylation. Treatment of CTR1-GST with CIP prior to *in* vitro kinase assays was able to reduce the kinase activity. *Ctr1-8* was similarly affected by CIP. Table 1 provides a quantification of the reduction of β-casein phosphorylation by CTR1 before and after treatment with CIP.

Table 1. Relative phosphorylation in the kinase assay
Protein samples were treated with or without calf intestinal phosphatase (CIP) and assayed for kinase activity as in Figure 3. The minus CIP results are normalized to 100%. The data for CTR1 and β-casein are from 5 and 8 independent experiments, respectively.

Protein	CIP	
	–	+
GST-CTR1	100%	59.5 ± 9.5%
β-casein	100%	46 ± 13.4%


A
Ctr1 + casein
+ CIP
+ CIP + ETR1
Ctr1 + casein
+ CIP
+ CIP + ETR1
Ctr1 + casein
+ CIP
+ CIP + ETR1
Ctr1-GST
β-casein
10 min
30 min
45 min


B
Ctr1 + casein
+ CIP
+ CIP + ETR1
+ CIP + ETR1 657-738
+ CIP + ERS1
Ctr1-GST
β-casein
+ CIP
+ CIP + ETR1
+ CIP + ETR1 H353Q
+ CIP + ETR1 D659A
+ CIP + ETR1 D659E
Ctr1-GST
β-casein

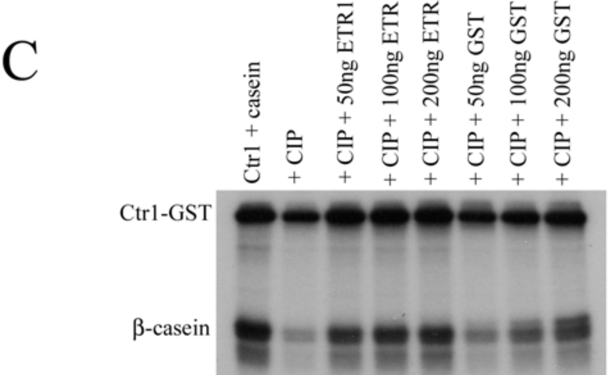

C
Ctr1 + casein
+ CIP
+ CIP + 50ng ETR1
+ CIP + 100ng ETR1
+ CIP + 200ng ETR1
+ CIP + 50ng GST
+ CIP + 100ng GST
+ CIP + 200ng GST
Ctr1-GST
β-casein

Figure 2-5. CTR1 deactivated by CIP, can be reactivated. A) Addition of ETR1-GST to the reaction mix with CIP treated CTR1 can reactivate CTR1. Three different time points were checked, and 45min was used for the remaining experiments. B) Other receptors, and truncations or mutants of ETR1 are just as able to reactivate CTR1. ERS1-GST was expected to behave similarly to ETR1-GST, as it has been shown to interact with CTR1 as well. The truncation of ETR1 is the receiver domain, and has also been shown to interact with CTR1. The mutations were all specific mutations that would disrupt the ability of ETR1 to autophosphorylate or transfer the phosphate to the receiver domain. The fact that ETR1 D659E- GST was able to reactivate CTR1-GST raised flags, as in the yeast two-hybrid the D659E mutation disrupts the interaction with CTR1. C) GST alone was able to reactive CTR1-GST. Increasing amount of GST alone was able to increasingly reactivate CIP treated CTR1-GST, much as ETR1-GST was.

system. Unfortunately, addition of any GST-fusion (or GST alone) protein was able to re-activate CTR1 (Fig. 2-5). There was no appreciable difference between the reactivation potential of any of the added proteins. This result, while unexpected, did tell us something. The fact that the addition of GST alone was able to reactivate CTR1, tells us that one of two things are probably occurring. One possibility is that GST-CTR1 was activated by dimerization, since GST, which was fused to the amino-terminus of CTR1, is known to dimerize (Ji *et al*., 1992; Maru *et al*., 1996). In plants, there is evidence that the ethylene receptors exist as homodimers (Schaller *et al*., 1995; Müller-Dieckmann *et al*., 1999). Thus, dimerization of CTR1 via binding to ethylene receptor dimers might similarly promote CTR1 autokinase activity (Schaller *et al*., 1995; Müller-Dieckmann *et al*., 1999). While Huang *et al*., (2003) showed that purified GST-CTR1 is a monomer, they found that CTR1 autophosphorylation is at least partially an intermolecular event involving *trans* phosphorylation. Activation of CTR1 by dimerization might explain why GST-CTR1 protein did not appear to be autoinhibited and why it had a similar level of activity as a truncated version lacking the amino-terminal domain.

Alternatively, the addition of free GST may work to activate CTR1 by driving the equilibrium of the system away from CTR1 protein being bound together. If close association sterically hindered the autoactivation of CTR1 protein, a large surplus of GST alone could shift a significant portion of the CTR1-GST fusions to being bound with a free GST, as opposed to a second CTR1-GST. This could free up that CTR1protein to autoactivate.

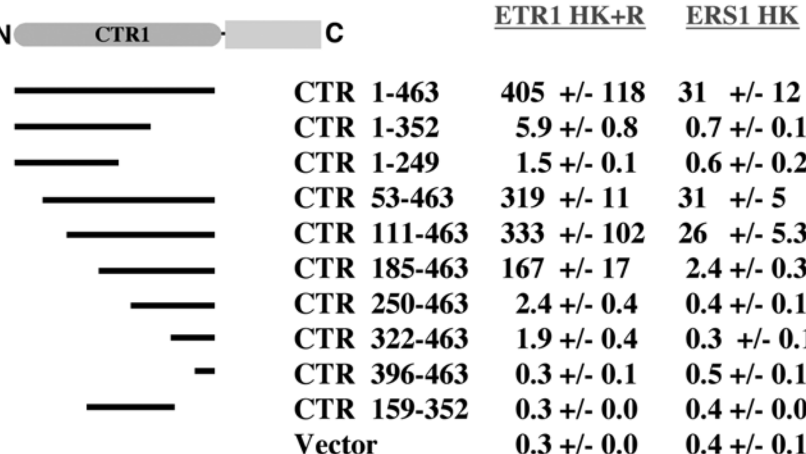


Figure 2-6. Truncations of CTR1 show the region of the N-terminus important for the interaction with ETR1. By making truncations on both the N-terminal end, and C-terminal end of the N-terminus, a region between 185-463 essential for the interaction between CTR1 and ETR1 was identified. CTR1 truncations are expressed as AD fusions using pACTII, while the receptors are expressed as DB fusions using pLex-A. Yeast two-hybrid assays were carried out in yeast strain L40. Interaction is shown as β-galactosidase activity as in Clark *et al.*, 1998.

The removal of the GST moiety with thrombin digestion resulted in the digestion of CTR1 as well. The potential re-activators are to be re-cloned as 6X-his fusion proteins and retested in Paul Larsen's lab at UC Riverside. Examination of the potential complex formation via GST is being explored *in planta* currently.

Inter- and intra-protein interactions may mediate regulation of CTR1 kinase activity

In order to better understand the interaction between CTR1 and ETR1, truncated forms of the CTR1 N-terminal domain were constructed and tested for their ability to interact with ETR1 (293-729) using the yeast two-hybrid assay. Figure 2-6 shows β-galactosidase reporter activity found with these constructs. CTR1 53-463 was that used by Clark *et al*. to show CTR1's interaction with ETR1 (293-729) and ERS1 (261-613). The inclusion of residues 1-52 did not increase this interaction significantly (Fig. 2-6). C-terminal truncations further than residue 463 reduced the activity approximately 100-fold, while truncations from the N-terminus had little to no effect up to amino acid 111. Interestingly, truncation to amino acid 185 reduced the interaction with ETR1 approximately two fold, while the same truncation reduced the interaction with ERS1 100 fold. These results identified a region from amino acids 185-463, which is important for CTR1's interaction with ETR1, while CTR1's association with ERS1 seemed to be more sensitive to deletion, with a larger region (residues 111-463) being necessary for interaction.

The region 185-463 contains within it the location for the *ctr1-8* mutation (Huang *et al*, 2003). This led us to speculate that the *ctr1-8* may interfere with the

interaction with ETR1. Using the yeast two-hybrid assay, we showed that mutant CTR1-8 N (53-568) completely disrupted interaction with ETR1 (293-729) (ETR1/CTR1 LacZ units 78±5, ETR1/CTR1-8 0.11±0.01 units) (Fig. 2-7), as shown for CTR1 residues 7-560 (Huang *et al*., 2003). In addition, we found that CTR1-8 N (53-568) failed to interact with ERS1 (261-613) (Fig. 2-7). As the *ctr1-8* N-terminus (53-568) is unable to interact with ETR1 (293-729) and ERS1(261-613), this gives us some insight into why *ctr1-8* plants have a phenotype similar to those plants lacking a functional CTR1 kinase. The observation that *ctr1-8* disrupted the interaction with ETR1 (but failed to examine ERS1) was independently made by Huang *et al*. (2003) using CTR1 (7-560).

By investigating biochemical aspects of CTR1 further, we have uncovered additional possible mechanisms of CTR1 regulation. Given that dissociation of CTR1 as in the *ctr1-8* mutation (Huang *et al*., 2003; Gao *et al.,* 2003) from the receptors results in loss of CTR1 activity, we asked how the inactivation of CTR1 might occur. Many protein kinases are regulated by autoinhibitory domains (Soderling, 1990; Tu *et al*., 1997). In the case of Raf-1, an amino-terminal autoinhibitory domain regulates the kinase domain of the same molecule by direct interaction (Heidecker *et al*., 1990; Rapp *et al*.; Stanton *et al*., 1989). Although CTR1's carboxyl-terminal kinase domain is highly similar to that of Raf, the amino terminal portion is substantially diverged and shows little similarity to known proteins other than plant CTR1 homologs (Ichimura *et al*., 2002; Jouannic *et al*., 1999). We were therefore interested in seeing whether the amino-terminal domain

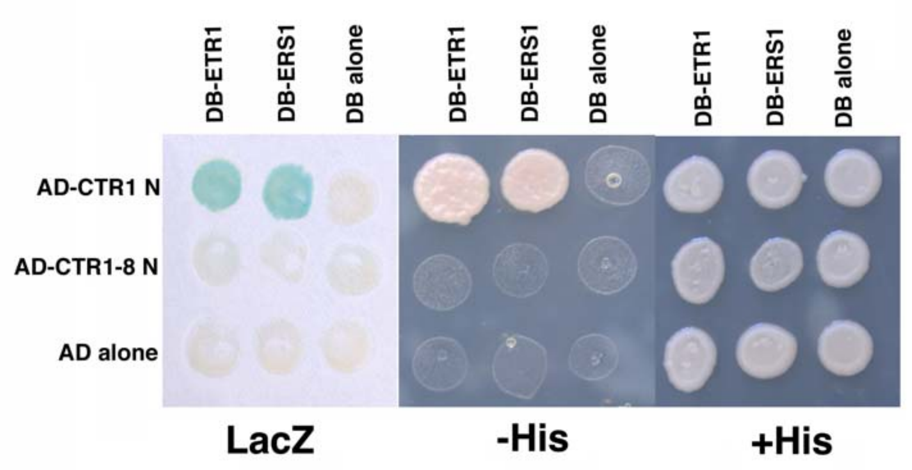


Figure 2-7. The *ctr1-8* mutation disrupts interaction of CTR1 with both ETR1 and ERS1. The amino-terminal domains of wild-type CTR1 and CTR1-8 were each tested for interaction with the receptors ETR1 and ERS1 in the yeast two-hybrid assay. Interaction is indicated by LacZ activity (blue cells) and growth on medium lacking histidine (-His). Growth of cells on media containing histidine (+His) is shown. CTR1 and *ctr1-8* are expressed as AD fusions using pACTII, while the receptors are expressed as DB fusions using pLex-A. Yeast two-hybrid assays were carried out in yeast strain L40.

and the kinase domain of CTR1 can physically associate. This was tested by both an *in vitro* pull-down assay and far-western blotting (Fig. 2-8). (Use of the two-hybrid assay was precluded by poor expression of the CTR1 kinase domain in yeast [Huang *et al*., 2003; Shockey and Chang, unpublished].) For both the *in vitro* pull down and far-western blot, the CTR1 amino-terminal domain (residues 53-568) was expressed in *E. coli* as a fusion with maltose binding protein (MBP) (MBP-CTR1 N) and tested for the ability to associate with a radiolabeled CTR1 kinase domain (538-821) synthesized by *in vitro* translation. In both assays, MBP-CTR1 N gave a clear signal, whereas MBP-ETR1 (293-738) and MBP alone gave very weak signals (Fig. 2-8) comparable to that obtained with amylose resin alone (which was used to purify the MBP fusion proteins) (not shown). This finding raises the possibility that interaction of the CTR1 amino-terminal domain with its own kinase domain results in autoinhibition of kinase activity as shown for Raf-1.We then tested the *ctr1-8* mutation for disruption of the interaction between the amino-terminal and kinase domains, we found that the mutation had no appreciable effect (Fig. 2-8). The maintenance of this interaction in the CTR1-8 protein has several implications. First, it argues that the *ctr1-8* mutation does not cause a gross structural defect in the amino-terminal domain. Second, it is consistent with the possibility that the *ctr1-8* mutant phenotype could be due to a specific disruption of the association with ETR1. Third, it raises the possibility that the loss of signaling by CTR1-8 could be due to autoinhibition of the kinase domain instead of (or in addition to) a spatial requirement to localize with ethylene receptor complexes at the ER. This would infer a mechanism in which, while detached from the ethylene receptors, CTR1-8 would

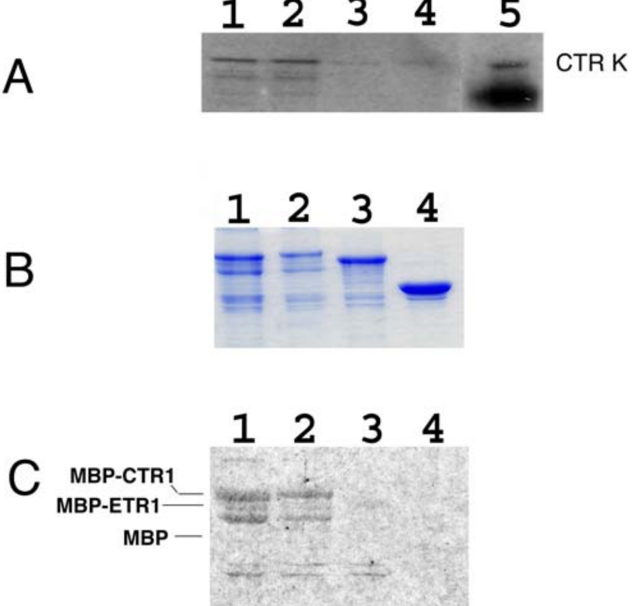


Figure 2-8. The amino-terminal domain of CTR1 and CTR1-8 can physically associate with the kinase domain. A) *in vitro* pull-down assay. Radiolabeled CTR1 kinase (residues 538-821) was synthesized using the TnT T7 Quick Coupled Transcription/Translation System (Promega) and used as the probe. Lanes: 1, MBP-CTR1$^{53\text{-}568}$; 2, MBP-CTR1-8$^{53\text{-}568}$; 3, MBP-ETR1$^{293\text{-}738}$; 4, MBP alone; 5, CTR1 kinase domain (CTR1 K) probe produced by *in vitro* transcription/translation. Unincorporated ^{35}S-Methionine is seen as a strong signal near the bottom.

B) SDS-PAGE gel stained with coomassie blue, showing the MBP fusion proteins used in A and C. Lanes are the same as in A.

C) Far-western blot. Radiolabeled CTR1 kinase (residues 538-821) was used again as the probe. Lanes are the same as in A.

adopt a kinase inactive conformation, with an interaction between the amino-terminus and kinase domains.

Discussion

This possible mechanism for CTR1-8 regulation is consistent with the regulatory mechanisms found in Raf, i.e. Raf is cytostolic in quiescent cells, and membrane bound in activated ones (Marshall, 1995; McCormick, 1995). However, in the case of wild-type CTR1 protein, Gao *et al*., (2003) have shown that it remains bound to the same membrane fraction as the ETR1 receptor, unlike CTR1-8, which is consistent with the data that show CTR1-8's inability to bind to ETR1 and ERS1. If we take into account all the current data on CTR1, the current working model is shown in Figure 2-9. We propose that in the absence of bound ethylene, a CTR1 molecule is bound to an ethylene receptor dimer through the regulatory domain, driving activation of CTR1. When CTR1 is active, ethylene responses are repressed. When ethylene is bound by the receptors, an unidentified mechanism causes ETR1 to undergo a conformational change, allowing the CTR1 regulatory domain to associate with the CTR1 kinase domain, inhibiting activity. This repression of CTR1 kinase activity allows ethylene responses to proceed. In the *ctr1-8* mutant, CTR1 might not bind as well to the receptors, and therefore takes on the inactive conformation, thus conferring the constitutive ethylene responses displayed by *ctr1-8* plants.

The proposed model provides a simple working hypothesis for how CTR1 might be regulated by the ethylene receptors. The actual mechanism is likely to be much more complex and to involve additional components such as protein

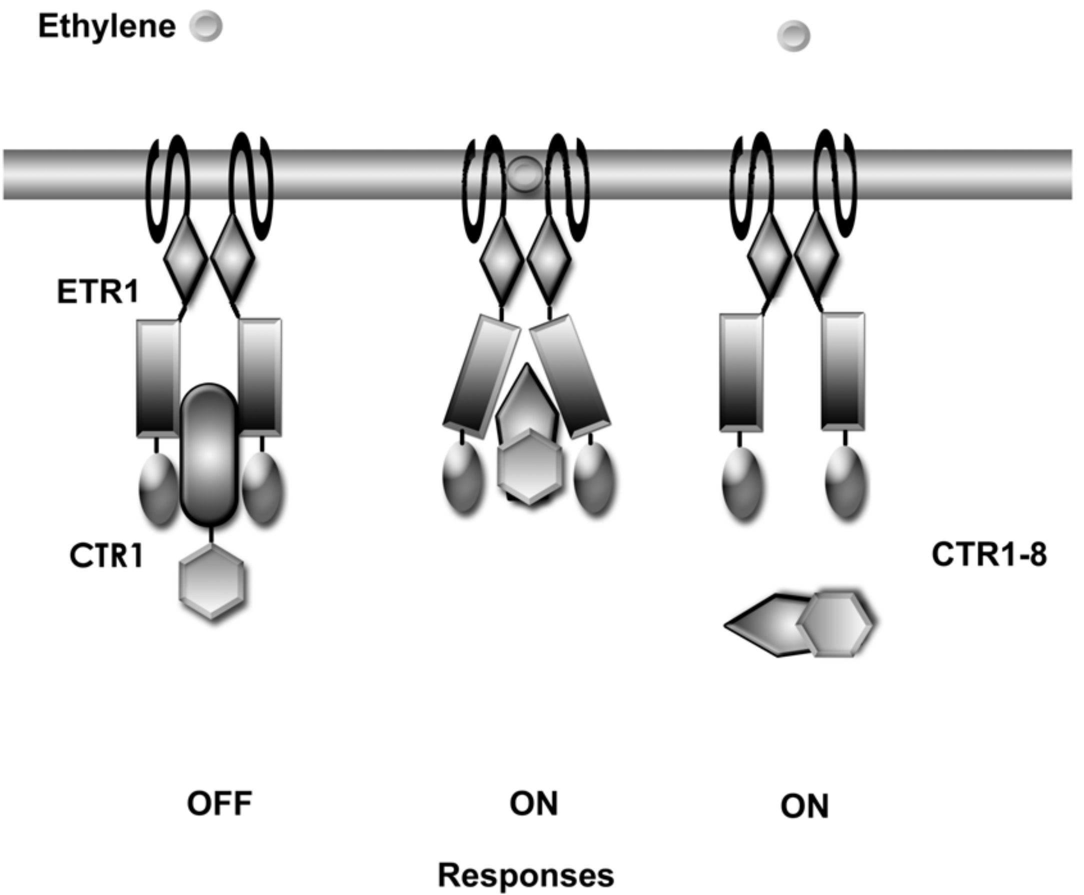


Figure 2-9. Proposed model for the regulation of CTR1 activity. In the absence of ethylene, CTR1 is bound as a dimer to ETR1. This interaction somehow activates CTR1 kinase activity, which is known to inhibit ethylene responses. When ethylene is bound, ETR1 undergoes a structural shift that releases CTR1, which results in disruption of the kinase activity. Inhibition of CTR1 activity may involve association of the CTR1 amino-terminal domain with the CTR1 kinase domain. In the absence of CTR1 kinase activity, ethylene responses are allowed to proceed.

phosphatases and perhaps scaffolding proteins. Indeed, the *eer1* mutation which confers enhanced ethylene responsiveness (Larsen and Chang, 2001) is in the gene for the protein phosphatase 2A A subunit (Larsen and Cancel, 2003). Interestingly, most of the features tested thus far for CTR1 are similar to those of Raf and related protein kinases, suggesting that activation of CTR1 may be similar to the activation of Raf, but involving a novel association with two-component receptor homologs.

Materials and Methods

Cloning of vectors – All vector cloning was done by PCR amplification of the target sequence, followed by integration into pGem-T vector (Promega). Using the M13 forward and reverse sequences in the pGem-T vector, the insert would be sequenced to make certain it harbored no PCR based mutations. The insert would then be cut out using the appropriate restriction sites and subcloned into the vector of interest. Both the CTR1 and *ctr1-8* for baculovirus expression were PCR amplifyed from cDNA clones using the 5’ primer cccgggatggaaatgcccggtagaag and the 3’ primer gaattcttacaaatccgagcggttgg, which generated a Sma1 and an EcoR1 respectivly. The CTR1 fragments were subcloned into pACG3X using Sma1 and EcoR1. All ETR1 fragments and mutations were cloned into pAcHLT-A using EcoR1 and BamHI. ETR1 164-738 was generated using the 5’ primer gaattcaagactacacttgttg and 3’ primer ggatccttacatgccctcgtac amplifing from a cDNA. Both the D659A and D659E were generated using the same primers and mutated cDNAs. ETR1 615-738 was generated with the same 3’ primer and the 5’ primer gaattcatggatgagaacgggg. The ERS1 for baculovirus expression was cloned into pAcHLT-A using Xho1 and BamH1, and was amplified from cDNA using the 5’

primer ctcgaggaagaaccactcttgt and 3' primer ggatcctcaccagttccacggtctgg. CTR1 deletions for the yeast two-hybrid were generated with region specific primers and cloned into pACTII using Sma1 and Xho1. CTR-N (53-568) MBP fusions for bacterial expression were cloned into pMAL-C2 (New England Biolabs) with the 5' primer gaattcaaggcggagagaggcggatttg and the 3' primer tctagaagcacggtggacagtgccaaag, and the restriction enzymes Xba1 and EcoR1. The CTR1 kinase used for *in vitro* transcription/translation was the same as in Clark *et al.*, 1998. The GFP constructs were the kind gift of Paul Larsen.

Particle bombardment – 60mg of ethanol treated tungsten M-25 particles were vortexed with 50µg of DNA, 50µl of 2.5M $CaCl_2$, and 20µl of 0.1M spermidine for 3 minutes. After gentle centrifugation, pellets were resuspended in 70% ethanol, sonicated for 10 seconds, pelleted again, and finally resuspended in 100% ethanol. Coated particles were gently spread on a macrocarrier just prior to bombardment. Onion epidermal sheets were spread on 1X MS media (if supplimented using 100µM ACC and/or 10µM AVG) and bombarded using a BioRad Biolistic PDS-1000 and 1100psi rupture discs. Cells were incubated in the dark o/n and imaged on a variety of fluorescence microscopes.

Protoplast isolation and transformation – Protoplasts were isolated and transformed using the protocol of Kovtun *et al.* 2004.

Baculovirus expression and protein purification - Proteins were each expressed with an amino-terminal GST tag from vector pAcG3X or pAcHLT-A (PharMingen) in insect cells and purified using glutathione sepharose beads according to the manufacturer's specifications (PharMingen).

In vitro ser/thr kinase assay - For the kinase assay, the GST-CTR1 (attached to the beads) was incubated with 1 μg dephosphorylated β-casein (Sigma Chemical) (as a non-specific substrate) in the presence of CKA buffer (20mM Tris-HCl pH 7.5/ 5mM $MgCl_2$/ 0.5mM DTT/ 150mM KCl/ 50mM non-radioactive ATP/ 1μCi [γ-^{32}P] ATP (5.5nM) for 45 min. at 22°C. Reactions were terminated by adding protein sample buffer. Proteins were then separated by 12% SDS/PAGE, fixed, dried and viewed by autoradiography or phosphorimaging. Quantification of the signal was performed using ImageQuaNT 5.0 (Molecular Dynamics) and a STORM 840 (Molecular Dynamics).

Dephosphorylation - The purified proteins (attached to the beads) were incubated in the presence of 50mM Tris-HCl pH 8.0/ 100mM NaCl/ 10mM $MgCl_2$ either with (+) or without (-) 40 units of calf intestinal phosphatase (CIP) for 30 min. at 30°C. The beads were then pelleted and washed twice with CKA buffer minus ATP.

Yeast two-hybrid assay - The two-hybrid assay was performed as previously described (Clark *et al*., 1998) except that cells were spotted onto plates from overnight liquid cultures. β-Gal assay was performed as desribed in Clark *et al.*, 1993.

Yeast transformation – Yeast transformation was performed by a lithium acetate based method described in Chen *et al.*, 1992.

Bacterial expression and purification - MBP-fusion proteins were expressed and purified from *E. coli* as previously described (Clark *et al*., 1998).

in vitro transcription/translation - Radiolabeled CTR1 kinase (residues 538-821) was synthesized using the TnT T7 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's specifications in the presence of [^{35}S]-methionine.

in vitro pull downs - 5µg of MBP-fusion protein bound to 20 µl of amylose-containing beads was incubated with 10 µl of the radiolabeled probe in the presence of GS buffer (71.7mM K_2HPO_4/ 28.3mM KH_2PO_4/ 150mM KCL/ 1mM $MgCl_2$/ 10% Glycerol/ 0.5% Triton X-100/ 1mM DTT) with 5%BSA at 4°C for 90 min. The amylose-containing beads were then pelleted by centrifugation and washed 4 times with ice-cold GS buffer lacking BSA, resuspended in protein sample buffer and separated by 12% SDS/PAGE. After fixation, gels were soaked in Amplify (Amersham) and subjected to autoradiography.

Far-western - MBP fusion proteins were expressed and purified as above. The proteins were separated by 12% SDS/PAGE and transferred to nitrocellulose. The blot was blocked for 1 hour at 4°C with 2% milk powder in AC buffer (20mM Tris-HCl pH 7.6/ 100mM NaCl/ 0.5mM EDTA/ 10% glycerol/ 0.1% Tween-20). After blocking, the blot was probed with radiolabeled peptide produced as above, except purified over a Sephadex G-25 column. Probing was carried out in 2% milk powder/AC buffer/ 1mM DTT for 2 hours at 4°C. The blot was then washed 4 times with ice-cold AC buffer and subjected to autoradiography.

Chapter 3: The role of the receiver domain of the *ETR1* ethylene receptor

Introduction

In order to begin to understand upstream events in ethylene signaling, knowing what the ethylene receptors "do" is of vital importance. Study of the HOG1 pathway in yeast (Fig. 1-4) was of particular interest to researchers in ethylene signaling, mainly due to the similarities found between the two pathways. The main characteristics shared by the HOG1 pathway and the ethylene pathway were; 1) both have hybrid HKs that are membrane bound; and 2) they both are functionally connected with a MAP kinase cascade. Though the ethylene receptor *ETR1* and the osmosenser SLN1 were cloned around the same time, our understanding of the HOG1 pathway progressed much faster, due in no small part to the simplicity of the phenotype involved (death or life) and the many factors that make single cell organisms much faster when it comes to genetics (rapid life cycle and the sheer numbers that can be screened). Thus, using genetics, proteins involved in a multistep phospho-relay system were identified as components of the HOG1 pathway, and biochemical analysis verified the function of this phospho-relay in yeast (Posas *et al.*, 1996).

While the ethylene receptors all bear strong homology with the two-component histidine kinases, it has long been stressed by many in the field that this homology is not enough to define them as two component histidine kinases (Chang and Stewart, 1998). Early biochemical studies demonstrated that ETR1 expressed in yeast does indeed have histidine kinase activity *in vitro* (Gamble *et al.*, 1998).

However, this may be insufficient to define all the receptors as HKs. Recent controversial studies have suggested that *ETR1* may be unique among the receptors with its histidine kinase activity, as the other four receptors in *Arabidopsis* and two receptor homologs in tobacco seem to posses ser/thr kinase activity (Xie *et al.,* 2003; Moussatche and Klee, 2004; Zhang *et al.*, 2004).

To further complicate the story, transgene analysis has demonstrated that HK activity is not required for *ETR1* receptor function (Gamble *et al.,* 2002, Wang *et al.,* 2003). Despite this apparent disposability of the kinase activity, the domains themselves are less so. Work expressing truncated and mutant forms of *etr1-1* (a dominant mutation conferring ethylene insensitivity) indicated that removal of both HK and receiver domain does not disrupt the ability to confer ethylene insensitivity in wild-type (Gamble *et al.,* 2002). However, when the same construct was expressed in an *etr1* null mutant, only partial ethylene insensitivity was conferred. This would suggest that the HK domain, and possibly the receiver domain as well, play some role in signaling, but one that may be redundant in a wild-type background. Indeed, recent work from the Bleecker lab has demonstrated that HK activity in general, and the presence of the receiver domain specifically, is required for rapid growth rate recovery after ethylene treatment is terminated (Binder *et al.*, 2004a).

A disproportionate amount of work has been done on the *ETR1* HK domain, despite the strong homology of the *ETR1* receiver domain to the receiver domains of two-component response regulators. With the emerging evidence arguing that the HK activity plays only a subtle role in ethylene signaling, our understanding of the

highly conserved receiver domain is vital to our knowledge of how the receptors work.

I was very interested in determining whether *ETR1* does take part in a phospho-relay, and what role this activity (or lack thereof) plays in ethylene signaling. To gain an understanding of the role of the receiver domain of *ETR1* in ethylene signal transduction, the I asked several questions.

One was concerning the model of two-component phospho-transfer: does it apply to ethylene signaling? Histidine kinase activity in ETR1 was examined in order to determine if phospho-transfer to the receiver domain could be observed *in vitro.* Due to my inability to reproduce the published histidine kinase activity of ETR1, I was unable to directly explore this question.. The second question I asked was does the conserved Aspartate have a function at all? In order to address this question the role of the conserved aspartate in the receiver domain was examined using molecular and genetic techniques.

Results

The model of two-component phospho-transfer: does it apply to ethylene signaling?

The first step in a phospho-relay is the autophosphorylation of the HK. This step has been shown for *ETR1* in *Arabidopsis*; however the second step, phosphotransfer to an aspartate, was not observed (Gamble *et al.*, 1998). This however may be explained by the experimental design used to detect HK activity. Purified ETR1 (164-738) was incubated in the presence of ATP and divalent cations. The reactions were then separated by SDS-PAGE, blotted, and subjected to treatment

with water, acid, or base. The acid base treatments were included to determine where the proteins were phosphorylated, because phospho-histidine (or more specifically its phosphoamidate bond) is sensitive to acid and resistant to base. On the other hand phospho-aspartate (or more specifically acyl phosphate bonds) is sensitive to both, while phospho-tyrosine is resistant to both. Finally, ser/thr are base-labile and acid resistant, allowing for the determination of what residues have been phosphorylated based on pH stability (Duclos *et al.*, 1991)

Note that if a protein were to be phosphorylated twice (or a mix of different phosphorylated forms was present) then the acid/base stability would not properly reflect the actual phosphorylation state of the protein. In our case, if within the pool of ETR1 only a small portion had undergone the phosphorelay, it would not be noticed in the previous work. This is due to the relative acid/base stability of phospho-histidine and phospho-aspartate. When ETR1 would be treated with either acid or base, the phospho-aspartate would be destroyed, whereas the phospho-histidine would only be destroyed in the acid. Thus, the overall picture would be of a protein only phosphorylated on the histidine.

While Gamble *et al.* (1998) showed that ETR1 did possess *in vitro* HK activity; the question of functionality was still unanswered. Early work had been unable to determine if phosphotransfer took place due to the reason I outlined above. To get around this problem, I planned on taking advantage of the ability of many two-component proteins to work in *trans* (Posas *et al.*, 1996; Uhl and Miller, 1996; Ninfa *et al.*, 1993; Burbulys *et al.*, 1991; Yang and Inouye, 1991). One form of ETR1 would be expressed as a truncation lacking the receiver domain, the second would

contain both the HK and receiver domain, but would possess a mutation disrupting its ability to be phosphorylated on the canonical His residue (H353Q). As these proteins would be different sizes, they would be easily separated by SDS-PAGE. If the larger of the two ETR1 truncations were to be phosphorylated, taking into account the absence of autokinase activity, the phosphate would presumably have come from the other ETR1 HK. Acid/base stability would be used to show that the larger band would be due to phosphorylation on the aspartate. Experiments like this have been used to great effect in the HOG1 pathway (Posas *et al.*, 1996).

To carry out these experiments, it was first necessary to reproduce the HK activity previously described (Gamble *et al.*, 1998). The yeast strain (sc295) and expression plasmid (pEG [KT] ETR1 164-738) were the kind gift of the Schaller lab. Despite following the published protocol, and troubleshooting through personal communication with the Schaller lab, significant expression of ETR1 using this system did not occur in my hands. However, strong expression of ETR1 164-738 was obtainable using the baculovirus (BV) expression system (described earlier). As the BV expression system has been found to often provide post transcriptional modifications for higher eukaryotic proteins in a manner more like that of the native environment (Kidd and Emery, 1993), I decided to attempt to show HK activity with this protein.

ETR1's histidine kinase activity was not obtainable in my hands, despite a lengthy process of troubleshooting (Fig. 3-1). CheA protein was donated by the Stewart lab and tested alongside ETR1, showing that HK activity was possible using the conditions I tried. Acid/base stability confirmed the labeled CheA did in fact

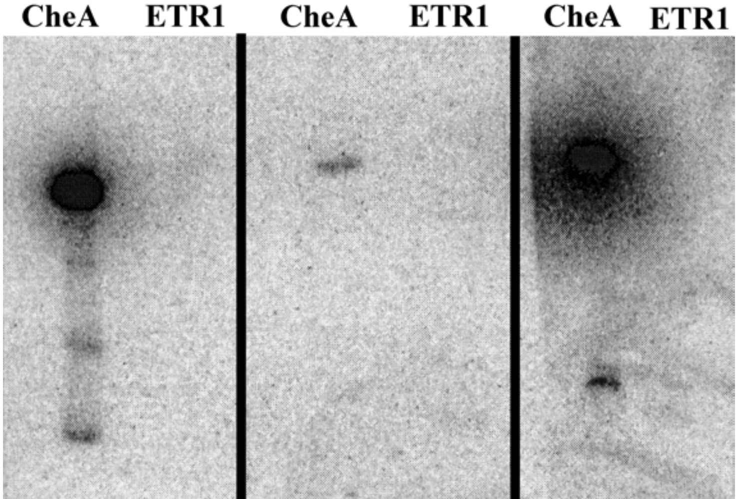


Figure 3-1. ETR1 HK activity was not observed. CheA and ETR1 were incubated in the presence of [γ-^{32}P] ATP. The products were seperated on a SDS-PAGE gel, blotted to a nylon membrane, and incubated in neutral, acid, or alkali solutions and placed on a phosphor screen over-night. CheA is readily phosphorylated on a histidine, while ETR1 shows no autokinase activity.

occur on a histidine. Unfortunately, this inability to reproduce ETR1's HK activity made it impossible to pursue my intended experiments to determine if *ETR1* phospho-transfer occurred *in vitro*. In order determine if a phospho-relay played a role in ethylene signaling, an alternative to biochemical analysis was then sought.

The conserved Aspartate: a function at all?

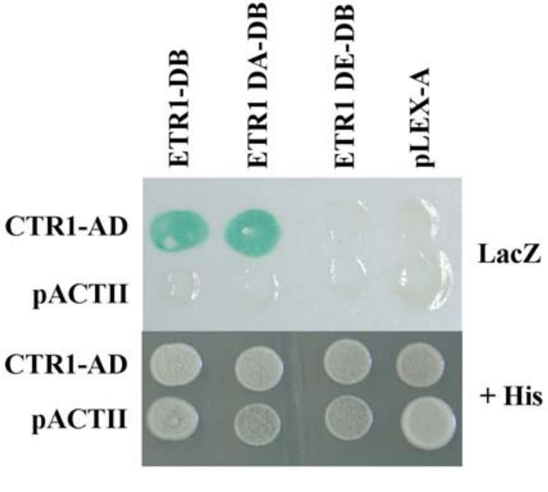
While biochemical analysis of phosphotransfer was not possible with ETR1, it was possible to examine the role of the conserved aspartate in the receiver domain using genetics. Using site-directed mutagenesis, mutations affecting the apparent phoshorylation state of *ETR1's* receiver domain could be tested both in the yeast two-hybrid and *in planta*. If *ETR1* is indeed able to act in a phospho-relay, elimination of its ability to transfer the phosphate to the receiver domain would enable us to determine what *ETR1's* function is under the HK-off state. This was easily accomplished by mutation of D659 to an A, as this should eliminate the carboxylic acid required for the linkage with the phosphate. To determine *ETR1's* effect when the receiver domain is phosphorylated, D659 was changed to E. It has been shown for several RRs that mutation of the aspartic acid to glutamic acid can mimic the phosphorylated state (Han *et al.,* 1992; Moore *et al.*, 1993; Klose *et al.*, 1993; Brown *et al.*, 1994; Lan and Igo, 1998).

Using site-directed mutagenesis, both mutations were made at the conserved aspartic acid (659) in the *ETR1* (164-738) yeast two-hybrid clone which had been used to demonstrate the interaction with the CTR1 N-terminus (CTR1-N). When tested against CTR1-N, ETR1 D659A interacted with the same affinity as wild-type. When the ETR1 D659E was tested against CTR1-N, the interaction was completely

abolished (Fig. 3-2). This exciting result suggested that the interaction between ETR1 and CTR1 may be dependent on the phosphorylation state of ETR1's receiver domain. Additional support for this model came from the crystal structure of the ETR1 receiver domain, where it was observed that the ETR1 receiver domain in an unphosphorylated state formed a dimer (Müller-Dieckmann *et al.*, 1999). Based on the dimerization interface, it is predicted that phosphorylation would disrupt this dimer, causing a conformational change which may interfere with the interaction with CTR1 (Müller-Dieckmann *et al.*, 1999).

To test this theory *in vivo*, the ETR1 D659A and D659E mutation were created in a plant expression vector using *ETR1's* native promoter. To determine the effect of these mutations, the sub-family I (*ETR1* and *ERS1*) double knock-out was transformed with both mutant forms and a wild-type control. This double knock-out was chosen for its very obvious severe phenotype that can be rescued with a single functional copy of *ETR1* (Zhao *et al.,* 2002;Wang *et al.,* 2003). Due to the severity of the phenotype, plants with the genotype *ers1-2*/*ERS1*; *etr1-6/etr1-6* were transformed. Resultant transformants were then genotyped for the *ers1-2* mutation, and homozygotes were isolated. Lines homozygous for both the transgene and the *ers1-2* mutation were used for further study.

At the gross phenotype level, neither the D659A and D659E mutations diminishes the ability of *ETR1* to rescue the double null to wild-type. This is evident in both dark and light-grown seedling phenotypes, as well as adult rosette size (Fig. 3-3). To determine if there were subtle quantitative effects (such as the mild hypersensitive response in an *etr1*-7 single null) dose response analysis was



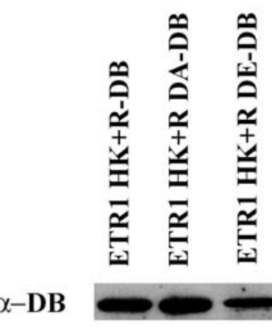


Figure 3-2. The *ETR1* D659E mutation disrupts the interaction between ETR1 and CTR1. The *ETR1* D659A mutation has no affect on the CTR1 ETR1 interaction, while the putative phospo-mimic, D659E, completely abolishes the interaction. . CTR1 is expressed as an AD fusion using pACTII, while the ETR1 receptors are expressed as DB fusions using pLex-A. Yeast two-hybrid assays were carried out in yeast strain L40. All three *ETR1*-DB constructs are expressed at equal levels as indicated by the western blot utilizing an anti-LexA antibody.

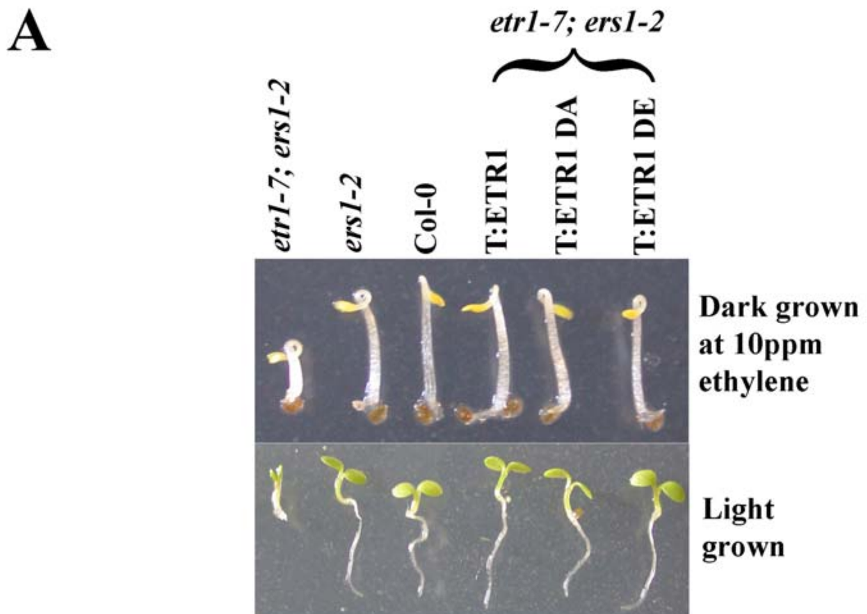


Figure 3-3. Mutations in the receiver domain have no obvious effect on *ETR1's* function. Both the D659A and D659E mutations did not disrupt *ETR1's* ability to rescue the *etr1-7; ers1-2* double mutants severe dark-grown, light-grown and adult rosette phenotypes. Seedlings were grown on MS in the dark or 24 hours light. Adult plant were grown under 14 hours light/ 10 hours dark. All plants were grown at 20ºC.

conducted with these lines. The response curves of multiple lines of the D659A and D659E mutants were indistinguishable from those of the wild-type control (Fig. 3-4). Adult responses were also unaffected in the D659A and D659E, as adult rosette senescence responses to ethylene were unaltered (Fig. 3-5). Three independently isolated lines for each of the three genotypes (wild-type, D659A, and D659E) were utilized and found to behave similarly in all tests (data not shown).

One of the more interesting recent discoveries is that *etr1-7* (*ETR1* knock-out) plants show a marked delay in recovery time of growth rate after ethylene has been removed from the system (Binder *et al.*, 2004). In order to assess if this delay in recovery may, in part, be due to phosphorylation on the receiver domain, my D659A and D659E plants (along with their wild-type control) were tested using this assay. Both the D659A and D659E demonstrated a delay in recovery time which was not demonstrated by the wild-type transgene control (Fig. 3-6). Due to the inability to measure more then three seedlings at a time in this assay, small sample size can result in a high degree of background and variability. More experimentation on my lines is required in order to make a good statistical analysis, however it should be pointed out that these preliminary results mesh well with the results by Binder *et al.*, 2004.

It had been shown that the dominant insensitivity of *etr1-1* does not require the HK or receiver domain in a wild-type background, but in an *ETR1* knock-out plant deletion of the HK and receiver in the *etr1-1* transgene only conferred partial insensitivity (Gamble *et al.*, 2002). To determine if the conserved aspartate of the receiver domain was required for full insensitivity, I mutated my constructs (*ETR1* wt, D659A, and D659E) so that they also carried the *etr1-1* mutation. These

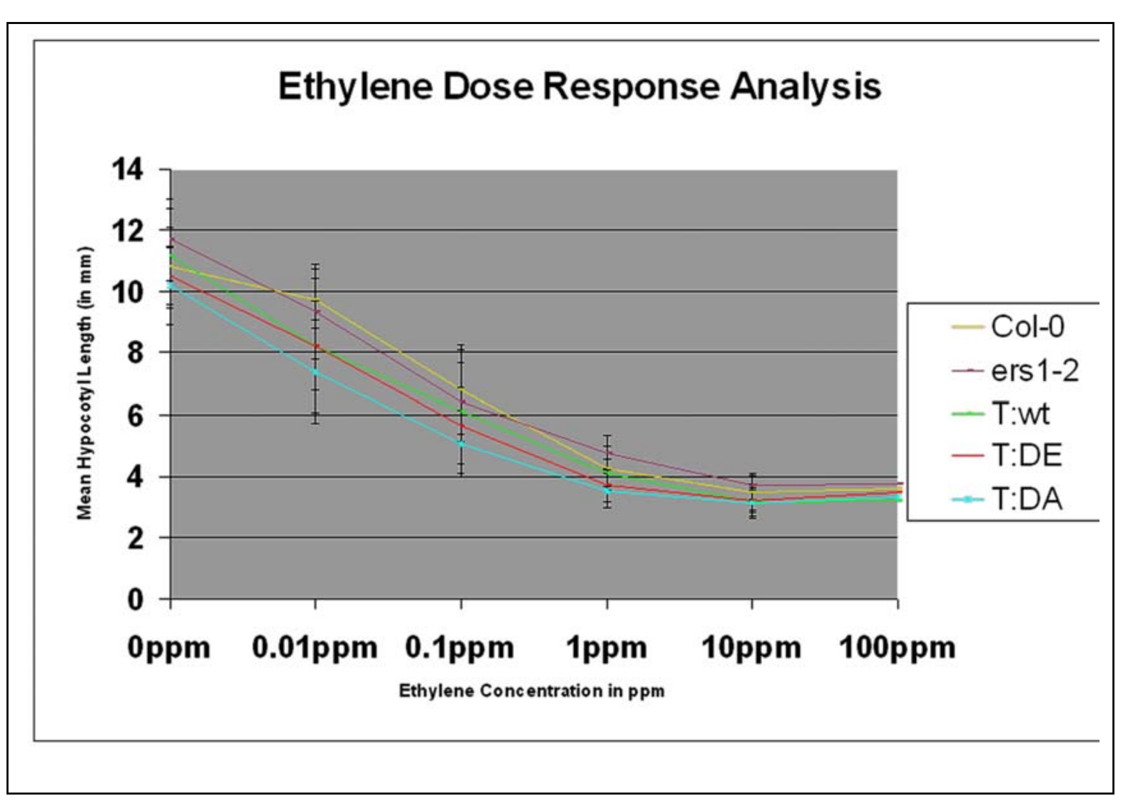


Figure 3-4. Dose response analysis shows no statistically significant difference between the D659A and D659E mutations. Both the D659A and D659E mutations behave the same as the wild-type control in a quantitative ethylene dose response analysis based on hypocotyl length. Seeds were sown on MS plates and incubated for 4 days at 4ºC. Plates were then places in mason-jars with the appropriate concentration of ethylene and incubated at 20ºC for 4 days in the dark. Seedling hypocotyl length of 15-20 seedlings was measured. The graph presented is the mean of three experiments showing standard deviation.

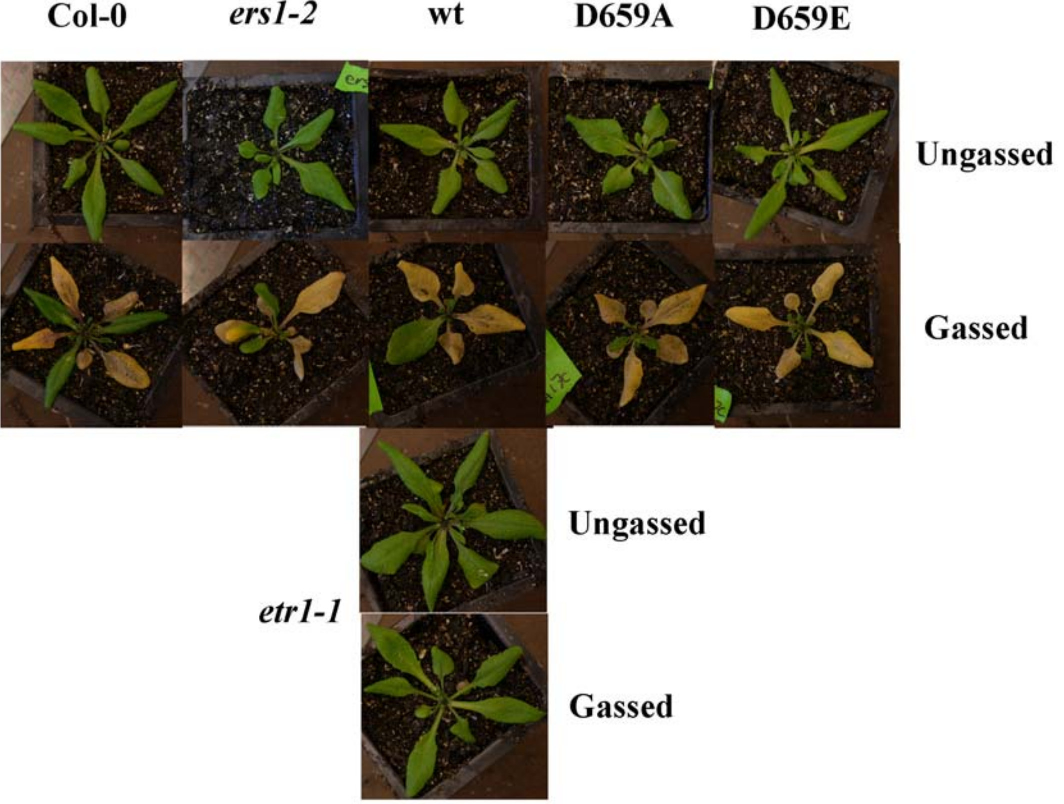


Figure 3-5. The D659A and D659E show normal adult responses to ethylene. After flowering, *Arabidopsis* rosette leaves will rapidly senesce in response to ethylene. Adult plants were exposed to 100ppm ethylene for 72 hours. Stems and flowers were removed just prior to photographing for visual reasons.

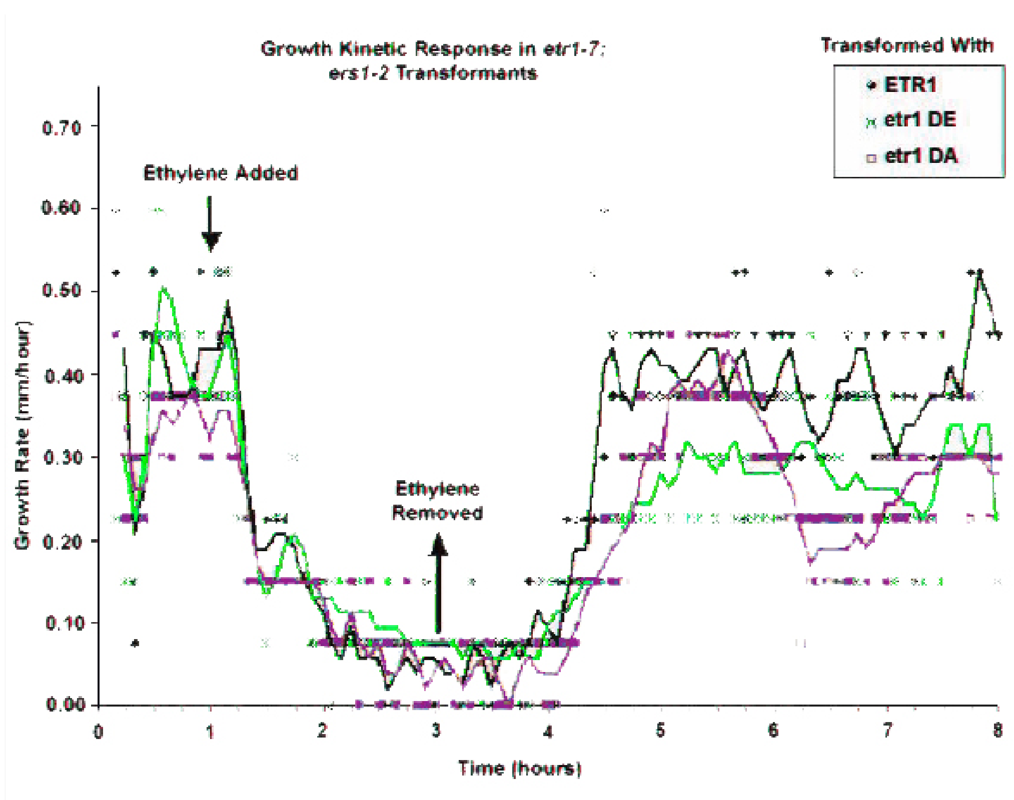


Figure 3-6. Mutations in the receiver domain affect recovery after ethylene treatment. Hypocotyl growth rate was determined using a close focus IR camera as detailed in Binder *et al.*, 2004. Both the D659A and D659E mutations delayed the growth rate recovery of seedlings following an ethylene dosage.

transgenes were then transformed into Col-0 (wild-type), *etr1-7*, and the *etr1-7; ein4-4; etr2-2* triple null mutant. This triple mutant was chosen as it is lacking all receptors containing a receiver domain. The resulting transformed lines were made homozygous, and the homozygous lines were subjected to dose response analysis. Unlike the results reported by Gamble *et al.* (2002), our transgenes conferred complete insensitivity across all doses in all three backgrounds (data not shown). This would suggest that the partial insensitivity previously reported was not due to the conserved aspartate of the receiver domain, rather the complete removal of the HK and receiver domains.

Discussion

Despite the difference in the yeast two-hybrid assay, both the *ETR1* D659A and D659E restored the growth properties of the *ETR1*/ *ERS1* double mutant to wild-type. Traditionally all ethylene phenotypes that are tested are the result of long term exposure to ethylene. However, response to ethylene at the molecular level is much faster; maximal induction of *ERF1* expression occurs within 30 minutes of ethylene exposure (Solano *et al.,* 1998). Concerns over plant's ability to adapt to constant ethylene led the Bleecker lab to look for ways to assess plant's response to ethylene in real time, as opposed to morphological characteristics after 3-4 days of constant exposure. The system they devised consists of a gas flow chamber (so the ethylene levels can be quickly changed) into which a plate with the seeds to be tested have been placed in a horizontal line. A CCD camera with a close focus lens (which image the growing seedlings using an IR light source) is aligned with the seedlings, and the whole apparatus is placed in a darkroom. As the seedlings grow, their actual growth

rate (converted to mm/hour) is determined every 5 minutes. Ethylene gas can be added in various concentrations (or removed) and the growth rate measured. This system has been used to great affect in the study of light signaling in *Arabidopsis* (Parks and Spalding, 1999; Folta and Spalding, 2001) and recently in the study of ethylene (Binder *et al.*, 2004a; Binder *et al.*, 2004b).

What the Bleecker lab has found is that there is a rapid reduction in growth rate within 15 minutes, much faster than maximal expression of *ERF1* is achieved (Binder, O'malley *et al.*, 2004). This rapid reduction in growth rate is not due to the repression of the proteolitic degradation of *EIN3* (Potuschak *et al.*, 2003; Guo and Ecker, 2003; Gagne *et al.*, 2004), as it is *EIN3*/*EIL1* independent (Binder, Mortimore, *et al.*, 2004). This rapid response may be due to a yet unidentified primary response not involved in transcription or transcription factors in any fashion.

As both the D659A and D659E transgenic seedlings behaved the same, this would suggest one of two things: 1) that phosphorylation on the receiver domain is not the determining factor in the ethylene recovery time, rather a phospho-relay system, which would be disrupted by both D659A and D659E. Or 2) the D659E mutation does not actually mimic the phosphorylated state and behaves to block phospho-transfer to the receiver as D659A would. The latter, taking into account the evidence against a phospho-relay system in ethylene signaling, seems more probable, especially since some prokaryotic RRs do not mimic the phosphorylated form with a D to E mutation (Bourret *et al.*, 1990). Despite any existing questions about why the D659A and D659E substitutions behave in a similar fashion, the fact that they do not

rescue the delay in recovery demonstrates some sort of role for that residue in recovery (ETR1 659).

The role of the conserved aspartate, or lack of one, remains elusive. While the D659A and D659E behave differently in the yeast two-hybrid assay, in every way tested they behave identically in plants. The simplest explanation is that in the yeast two-hybrid assay the truncated fusion protein with the D659E folded differently than the D659A, while in the plant the full length protein folds properly and the D659E does not mimic the phosphorylated state. Another explanation could be that in the yeast two-hybrid assay the loss of interaction with the receiver domain has a much stronger effect then it would in the plant. This second possibility does have some evidence for it, as in the yeast-two hybrid assay CTR1 can interact with just the HK of ETR1 (Clark *et* al, 1998) In any case, it is apparent that the ability to receive a phosphate on the receiver domain of ETR1 does not play a role in the general ethylene response, whereas the conserved aspartate does seem to play a role is recovery from ethylene. Our understanding of how the ethylene receptors function seems to be cursory at best, with subtle layers of complexity unconceived of ten years ago.

Materials and Methods

Protein expression - MBP-fusion proteins and baculovirus GST-fusion proteins were expressed and purified as in Chapter 2.

HK assay – Assay conditions were originally attempted as in Gamble *et al.*, 1998, alterations of buffer used this as the baseline. Reactions were seperated out via

SDS-PAGE (12% gels), and blotted to Immobilon-P PVDF membranes (Millipore). To examine pH stability blots were washed in either 3N NaOH, 2N HCl, or deionized water for 2 hours. Blots were subjected to autoradiography.

Plasmid construction – Baculovirus plasmids were those described in chapter 2. Yeast two-hybrid vectors were those described in Clark *et al.*, 1998 except for the D659A and D659E ETR1 HK+R. These were constructed via site-directed mutageneisis by the Quick Change XL system (Stratagene). pXW11 (ETR1 HK+R) was used as the template. The mutagenic primers were: D659A sense 5' gtggtcttcatggcugtgtgcatgcccggg, D659A antisense 5' cccgggcatgcacacagccatgaagaccac, D659E sense 5' gtggtcttcatggaagtgtgcatgcccggg, and D659E antisense 5' cccgggcatgcacacttccatgaagaccac. Plant expression vectors were constructed so as to include the native promotor (3kb 5' of the gene) and the NOS termination signal. A Pst1 Sma1 fragment of the ETR1 genomic clone was ligated into the Pst1 Sma1 of pBJ36. ETR1 (and ETR1 D659A and ETR1 D659E) in HBT95 (the gift of Ruth Stadler) was cut with BstX1 and Stu1 and ligated into the BstX1 and Stu1 of the ETR1 genomic in pBJ. The entire ETR1 fragment (including promoter and terminator) was cut out with Not1 and ligated into the Not1 sight of the pML-BART transformation vector. *Etr1-1* versions of these vectors were created by site-directed mutageneisis using the Quick Change XL system (Stratagene). Mutagenic primers used were the sense primer 5' gttaataagatgagttgctccataaagaacgataaaagcaccaaactg and the antisense 5' cagtttggtgcttttatcgttctttatggagcaactcatcttattaac.

Dose response – Ethylene dose responses were conducted as desribed in Larsen and Chang, 2001. Each genotype/dose had between 15 and 30 seedlings measured.

Plant transformation – Primary blots were clipped off of adult plants, which encourages growth of seconday bolts. 3-5 days after clipping plants are inverted in a dish containing the Infiltration media (IM) for 5min. 300ml of stationary phase agrobacterium (carrying the appropriate construct) is pelleted, and resuspended in 500ml IM. IM is 10mM $MgCl_2$, 5% sucrose, .044 μM benzylamino purine, and 0.03% silwet L77.

Yeast two-hybrid assay - The two-hybrid assay was performed as previously described (Clark *et al*., 1998) except that cells were spotted onto plates from overnight liquid cultures. β-Gal assay was performed as desribed in Clark *et al.*, 1993.

Western Blot – Westerns were performed as in Clark *et al.*, 1998.

Plant growth – Plants were grown at 20ºC for all conditions. Seeds were sown on 1X MS, pH 5.7 0.8% bactoagar. Seeds are cold-treated at 4ºC for 4 days and then incubated either in the dark of 24 hour light. After transfer to soil plants are grown in 14 hour light/day.

Chapter 4: Novel Protein Interactions with the Ethylene Receptors

Introduction

A complete understanding of the signaling mechanism between ETR1 and CTR1 could be difficult to achieve if additional unknown components are involved. Almost all of the genes identified in ethylene signal transduction were isolated using the very simple genetic screen based on the seedling triple response (Fig. 1-1). Using this easily identifiable phenotype, two main types of screens have been carried out: those looking for a lack of triple response in the presence of ethylene (= ethylene insensitive), or those looking for a triple response in the absence of ethylene (= constitutive response). While some novel genes have been isolated by looking for Weak Ethylene Insensitive (the *WEI* genes), or Enhanced Ethylene Response (the *EER* genes), these screens have been mostly used to saturation (Larsen and Chang, 2001; Alonso *et al.*, 2003). The *EIN2* gene, a central component of the ethylene response pathway, alone has over 40 alleles! To date, there are no known components acting between ETR1 and CTR1 from genetic screens.

In order to identify additional components of the ethylene response pathway, other methods apart from genetic screens must be utilized. One potentially powerful tool available to the molecular biologist is the yeast two-hybrid assay (Fields and Song, 1989; Fields and Sternglanz, 1994). The yeast two-hybrid assay utilizes the separate nature of protein domains. Most transcription factors consist of two distinct domains; a DNA binding domain (DB) and a transcriptional activation domain (AD).

The key to the yeast two-hybrid assay is that these two domains may be somewhat distant from one another and still retain their functionality. This allows the molecular biologist to create fusion proteins with the DB (usually the Gal4-DB) and with the AD (usually the LexA-AD). If the two proteins of interest interact, a functional transcription factor will be formed to drive the expression of a reporter gene(s), typically his3 or lacZ. In order to conduct a screen utilizing the yeast two-hybrid assay, two things are required; a "bait" protein fused with the DB domain, and a cDNA library fused to the AD. A yeast strain carrying the "bait" vector is then transformed with the library. The transformants are then screened for interacting proteins, usually by auxotrophic growth on His$^-$ media. 3-amino-triazole (3-AT) is often added to increase the stringency of the assay, as it competitively inhibits His3p. Several examples exist where the yeast two-hybrid assay has successfully led to the identification of novel components in a signaling pathway (Yang *et al.*, 1992; Clark *et al.*, 1993; Ni *et al.*, 1998; Choi *et al.*, 1999).

Another powerful methodology available to the molecular geneticist are the many techniques of reverse genetics. Indeed, with the explosion of sequenced genomes over the past seven years, reverse genetics is quickly gaining ground on traditional genetics as the provider of phenotypes (Henikoff *et al.*, 2004). Reverse genetics, at its simplest, is starting with a gene of interest and creating a phenotype by mutating or manipulating that gene. From the resulting phenotype it is possible to determine the function of that gene. Until recently the creation of the mutant had been a bottleneck for reverse genetic experiments in *Arabidopsis*. Thankfully, many methods currently exist, and we can optimistically expect more to be developed.

Currently, the list consists of knocking out genes via homologous recombination (which does not work in plants), sense over-expression, anti-sense suppression, RNAi, and in more "organized" communities focused on one organism, transposable element insertion pools, deletion lines, and point mutations identified through TILLING (Henikoff *et al.*, 2004).

To identify additional components of the ethylene signaling pathway, I utilized three approaches. Two approaches, yeast two-hybrid screen and examination of YPD1 homologs in *Arabidopsis* will be presented in full in appendix A for reasons detailed below. *ERS1* is an intriguing member of the ethylene receptor family. Using *ERS1* as the "bait" in a yeast two-hybrid screen, I looked for novel interacting factors. The HOG1 osmosensing pathway in yeast shares many features with the ethylene signaling pathway in plants. The yeast osmosensor, SLN1, like 3 of the 5 ethylene receptors in *Arabidopsis*, is a hybrid-type histidine kinase. Its downstream partner, YPD1, has 5 homologs in *Arabidopsis*. These proteins were examined to determine if they have a role in ethylene signal transduction. The yeast two-hybrid assay yielded five distinct clones, none of which were deemed acceptable for further study. Examination of the YPD1 homologs in *Arabidopsis* did not provide ample evidence that the AHPs (*Arabidopsis* Histidine-containing phosphotransmitter) have a role in ethylene signaling.

The third, and most exciting method, was the reverse genetic analysis of At3g29185. At3g29185 (hereafter referred to as D2, which was its original clone name in that screen) was originally identified in a yeast two-hybrid screen in our lab using *ETR1* (293-729) as the bait, and was of particular interest for two reasons: the

sequence is completely novel and it was also able to interact with CTR1 (308-569) (Ding, 2000). D2 function was analyzed using RNAi, TILLING, and the isolation of T-DNA insertions. The resultant mutants were examined for defects in ethylene perception.

Results

Reverse genetics analysis of At3g29185 (D2)

D2 is a single copy, expressed gene in *Arabidopsis*; the D2 gene product has a predicted single transmembrane domain based on TMHMM and PHD analyses (Fig. 4-1) (Krogh *et al.*, 2001; Sonnhammer *et al.*, 1998; Rost, 1996; Rost *et al.*, 1996). There are two alternatively spliced variant forms present in EST libraries (Fig. 4-2). Based on extensive BLAST and PSI-BLAST analysis, D2 has no homology to any protein of known function. Notably, the proteins it does share homology with are all in plants and cyanobacteria, indicating that the ancestral homolog may have entered plants during the endosymbiotic event that gave rise to chloroplasts (Fig. 4-3). An unrooted phylogram using minimum evolution shows D2's evolution to be along accepted taxonomy lines within plants, with dicot homologs more related to each other than to monocots and so forth (Fig. 4-4). This is of great interest to the study of ethylene signal transduction, as the fold that actually is responsible for the binding of ethylene has followed a similar evolutionary path (Mount and Chang, 2002).

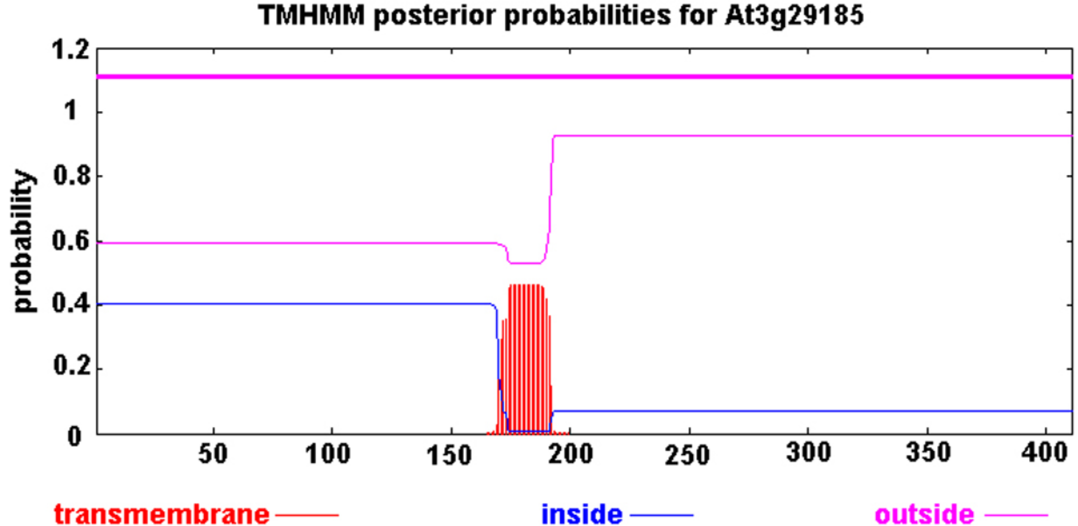


Figure 4-1. D2 contains a single transmembrane domain. The peptide sequence encoded by D2 was subjected to analysis by several prediction programs which gave mostly identical results. TMHMM analysis predicts D2 to have a single transmembrane domain as shown.

```
                 10        20        30        40        50        60
647    MAAATSFTCSLPFTPLSKSLKPIRSPILRSDYGTSRSFVIRSMTVQEDDKRTSDESMSID
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
563    MAAATSFTCSLPFTPLSKSLKPIRSPILRSDYGTSRSFVIRSMTVQEDDKRTSDESMSID
                 10        20        30        40        50        60

                 70        80        90       100       110       120
647    NLRGFVDLNVGKWTGSFHQFDGNGNLLHKIDTRLSASSYGEDELLSLNQSLYIKQPTSAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
563    NLRGFVDLNVGKWTGSFHQFDGNGNLLHKIDTRLSASSYGEDELLSLNQSLYIKQPTSAT
                 70        80        90       100       110       120

                130       140       150       160       170       180
647    SVSEEEEEEPEWVEYKIKETNMFTVDKYQQVRVALFISISQDTKFEFCDLSLKYLMTILI
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
563    SVSEEEEEEPEWVEYKIKETNMFTVDKYQQVRVALFISISQDTKFEFCDLSLKYLMTILI
                130       140       150       160       170       180

                190       200       210       220       230       240
647    SIISSLQIGFFPKERAFSLRYQTAGMLDTTLRQGVLGEDDTGEESPRNLKLPSRRPSLVC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
563    SIISSLQIGFFPKERAFSLRYQTAGMLDTTLRQGVLGEDDTGEESPRNLKLPSRRPSLVC
                190       200       210       220       230       240

                250       260       270       280       290       300
647    ENCLYSKEIDRRARAFHIMDPKGVLEMLIVFLEERGLENLAHPVLDNAQNDAERINPFLG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
563    ENCLYSKEIDRRARAFHIMDPKGVLEMLIVFLEERGLENLAHPVLDNAQNDAERINPFLG
                250       260       270       280       290       300

             L  310       320       330       340       350   K   360
647    TWKGRSVTKRSGVYGATLSEADTVAVLEMNDKGQVVQDISSTSDEKKVTTNVHWEGKMSK
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
563    TWKGRSVTKRSGVYGATLSEADTVAVLEMNDKGQVVQDISSTSDEKKVTTNVHWEGKMSK
                310       320       330       340       350       360

                370                                380       390
647    DLVTFAEGYQMTLLPG----------------------EFCWLESPSSRQRLIRTYDHEG
       ::::::::::::::::                      ::::::::::::::::::::::
563    DLVTFAEGYQMTLLPGGMYMGCPCDVSKCVADLKSFHLEFCWLESPSSRQRLIRTYDHEG
                370       380       390       400       410       420

      400       410
647    LAVSSTYFTETKM
       :::::::::::::
563    LAVSSTYFTETKM
                430
```

Figure 4-2. D2 has alternatively sliced forms. Alignment of peptide sequence of D2s two spliced forms show they are identical save for the second form containing an additional 22 amino acids. Blue text indicates the location of the transmembrane domain. Red text indicates the sequence of the two-hybrid clone with which D2 was originally isolated. TILLING point mutation are shown above the sequence (D2t A is a point mutation at near the 5' splice site of the first intron, D2t B is E355K, D2t G is S530L)

```
CLUSTAL W (1.82) multiple sequence alignment


Arabidopsis_thaliana      MAAA-------TSFTCS-LPFTPLS----KSLKPIRSPILRSDYGTSR--SFVIRSMTVQ 46
Lycopersicon              MATAPAIFVPSTSLASSNARKINLSSWNEKLVIPQNFPFWTPKYQSRKPPSCSGTNVTVV 60
Oryza_sativa              MAEALVAVLRLAASAAATARPQSRSGRHGSCAARVPCPGPSP-FRRGR----LCARAAVA 55
Nostoc_punctiforme        ------------------------------------------------------------
Anabaena_variabilis       ------------------------------------------------------------
Synechocystis_sp          ------------------------------------------------------------


Arabidopsis_thaliana      EDDKRTSDESMSIDNLRGFVDLNVGKWTGSFHQFDGNGNLLHKIDTRLSASSYGEDELLS 106
Lycopersicon              QGDRNTTELPMSIDALDSFIRLNLGNWTGSFHQFDGHGNLMHRITTKLAVGSYGEGELMS 120
Oryza_sativa              GPPEVDDDDAMTIDNLRRFFDVNVGKWNGAFYQFDAHGRVLQGISTRLSVSTYGEDDLIS 115
Nostoc_punctiforme        --MTNDKGQIFMKSQWECFLQN-LGVWEGSFSNFSPEGTLLNDTSSRLCLEGLNNNQTVR 57
Anabaena_variabilis       -----------MSSQWERLLLN-LGEWQGSFTRFSPQGQLLNDIPTVVSLTGLNNNQTVR 48
Synechocystis_sp          ----------MTMANWENFLKN-LGEWQGSFTRLSPQGEILSNTPSILTLEGLDDDKLVK 49
                                            :.   :* * *:* .:. .* ::    : :     .:.. :

Arabidopsis_thaliana      LNQSLYIKQPTSATSVSEEEEEEPEWVEYKIKETNMFTVDKYQQVRVALFISISQDTKFE 166
Lycopersicon              LLQTLYIKQPPSTTSCSGDD-CESEWFEYKIKETNMFTVDKYQ----------------- 162
Oryza_sativa              LLQSLYIKQASSQISFVDEE-DSEEWVEYKIKETNMFTVDKYQ----------------- 157
Nostoc_punctiforme        LTLSRSG----------------------------------------------------- 64
Anabaena_variabilis       QIIRQE------------------------------------------------------ 54
Synechocystis_sp          FRLRRYDNPDYQDP---------------------------------------------- 63


Arabidopsis_thaliana      FCDLSLKYLMTILISIISSLQIGFFPKERAFSLRYQTAGMLDTTLRQGVLGEDDTGEESP 226
Lycopersicon              --------------------QIGFFPKEKAYALRYQTAGMLETVLRQGVLGEDDIGEESP 202
Oryza_sativa              --------------------QVGFFQEEKAFALRYQTAGMLETVLRAGVLGEDDTGEESP 197
Nostoc_punctiforme        ---------------------------KDDVIREFRSVGGGLLFFENGSFSE---GLIQL 94
Anabaena_variabilis       ---------------------------NTEKILEYSSLARTVLFFENGAFSQ---GSIQL 84
Synechocystis_sp          --------------------------PTQDYSQDYRSLGRQIIFFGTGAFSK---GPWQL 94
                                                          : : .     :  * :.:   *  .

Arabidopsis_thaliana      RNLKLPSRRPSLVCENCLYSKEIDRRARAFHIMDPKGVLEMLIVFLEERGLEN-LAHPVL 285
Lycopersicon              RNLKLPSKRPSIVCENCLYSLEKDRRVRAFHIMDPKGVLEMLLVFLEERGNGE-AIPPSF 261
Oryza_sativa              KNLKIPSRKPSIVCENCLYSREGNGRVRAFHIMDPKGVLDMLIIFHEKQGSEVPLMYSSD 257
Nostoc_punctiforme        GPFSEFGGELAFVHE--------NRRLRLVQLFDRNGHLNGLTLIREHLAGTP------- 139
Anabaena_variabilis       APFSEFGAELGLIHE--------NRRLRLVQLFDKNGQLDQITLIREHLAGTP------- 129
Synechocystis_sp          APFSEFGAEFGFVDG--------DRRMRFVQLYDKGLSLASLTFIREFRRGSD------- 139
                            :.  . . .::          : * * .:: *    *  : .: *

Arabidopsis_thaliana      DNAQNDAERINPFLGTWKGRSVTKRSGVYGATLSEADTVAVLEMNDKGQVVQDISSTSDE 345
Lycopersicon              DDFKEDTERILPHLGTWKGHSRTTRTGVYGATITEASTTAVLEINKDGQLIQDITSTSGA 321
Oryza_sativa              DADITNSDRIAPLLGRWEGRSVTKRSGVYGATLSEADTVVLLEKDRNGQLILDNMSTKSG 317
Nostoc_punctiforme        -VAERPLLQINDLLGEWRGQAVTIYRDLRPPDIYSTTLKIQLD--DAGR-LMQSTSFGER 195
Anabaena_variabilis       -AKENPPLQIDDLLGEWQGEAITIYPDWRSPDTISTNLKLQLD--ENGR-LIQTLNFAGR 185
Synechocystis_sp          -AQERPALKVEQLLGTWQCQVYTGYPDWREPELS--TMEISLS--QTGDSVEQRVTVQGQ 194
                                  ::   ** *. .  *   .   .          *.    *  : :  .

Arabidopsis_thaliana      KKVTTNVHWEGKMSKDLVTFAEGYQMTLLPG----------------------EFCWLES 383
Lycopersicon              TNITTNVHWTGTISNDLVTFDGGFQLTLLPGGIYMGYPSDVAKNVQESTAFHVEFCWLES 381
Oryza_sativa              SSTTTTVHWTGSANNNLLQFDGGYEMTLLPGGMYMGYPTDIGKIVNDMDSFHLEFCWMES 377
Nostoc_punctiforme        TITSTATIKG-SIVLFDQDPEKQVQVLLLPDG----ASATSPLKVQLRQPLFLEAGWLIQ 250
Anabaena_variabilis       TITSTARIKG-SIILFDQDPEKQVQVLLLPNG----ASATSLLKVQVRQSFILEVGWLIQ 240
Synechocystis_sp          TSVMQGKVMGDQIHFLGPNPSK---VLLLPDG----ASSCTPDRLQLGQPFSGEVGWLVR 247
                          .                        : ***.                      *  *:

Arabidopsis_thaliana      PSSRQRLIRTYDHEGLAVSSTYFTETKM--- 411
Lycopersicon              PGKRQRLIRTYDVEGFAVSSTYFIESKV--- 409
Oryza_sativa              PGKRQRLVRTYDSAGLAVSSTYFFETKV--- 405
Nostoc_punctiforme        SDLRQRMIRSYNDKGEWVSLTLVTEERV--- 278
Anabaena_variabilis       PNLRQRMVRSYSDKGEWVSLTLVTEQRVKTH 271
Synechocystis_sp          PNERQRLIRYYDNRGAWTHSAFVVEHRQ--- 275
                          .  ***..* *.  *  .  .  . * .
```

Figure 4-3. D2 has homologs only in plants and cyanobacteria. Alignment of D2 and its homologs indicate several stretches of highest conservation. Analysis was performed using ClustalW.

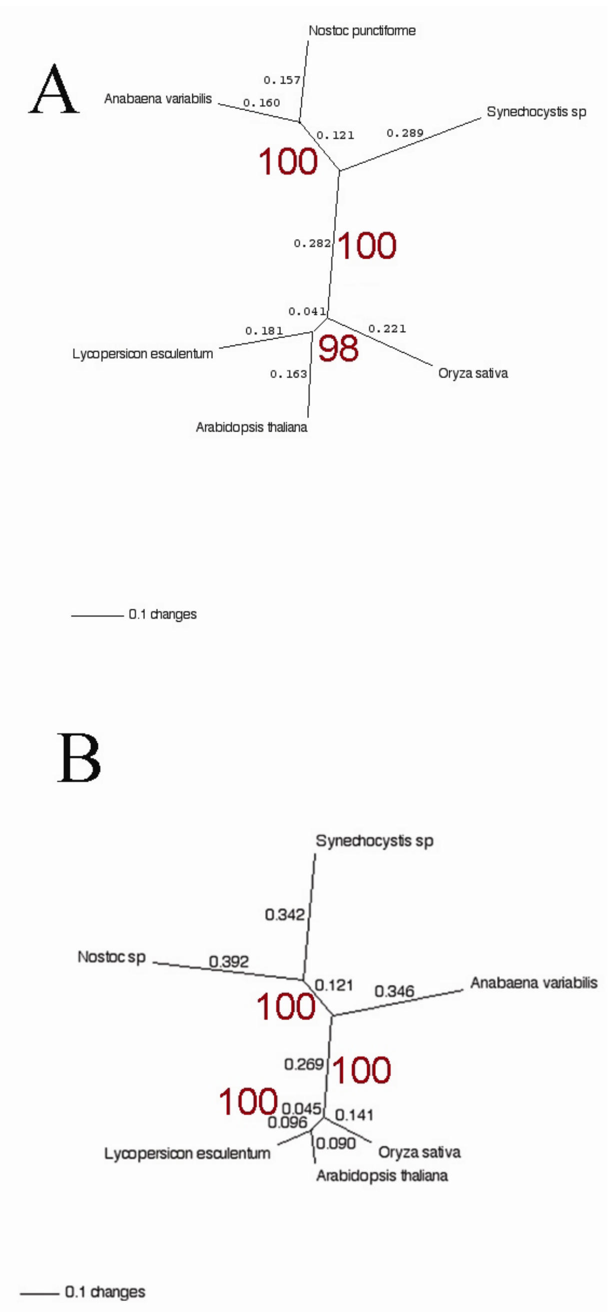


Figure 4-4. Unrooted phylogram shows D2's relation to its family members. A) Using the minimum evolution model, D2 and its closest relatives branch upon accepted evolutionary lines. B) Unrooted phylogram showing the relationship of the ethylene binding fold. Red numbers indicate bootstrap values for the adjacent branch.

As the ethylene receptors and D2 have similar origins, it is exciting to speculate that there may have been an ancient functional interaction. D2 was able to interact with both ETR1 and CTR1, suggesting D2 has gained the function of a scaffolding or regulatory protein in ethylene signaling (Fig. 4-5). Interestingly, D2 is also able to interact with the ETR1 (293-729) D659A, while losing the interaction with ETR1 harboring the phosphormimic mutation D659E. This parallels the interaction results for CTR1 and these *ETR1* mutations, suggesting that CTR1 and D2 together may interact with ETR1.

In order to determine the function of D2, reverse genetic techniques were employed. Rather than relying on a single method, multiple approaches were taken. After the completion of the *Arabidopsis* genome, several projects were initiated by the *Arabidopsis* community in order to aid in the determination of the function of all genes in *Arabidopsis*. The T-DNA insertion project of the Ecker Lab (http://signal.salk.edu/cgi-bin/tdnaexpress) and the TILLING collections (http://tilling.fhcrc.org:9366/) were of widespread importance in the isolation of mutant alleles for specific genes. The T-DNA project yielded two T-DNA insertions in D2 (one in the first exon and one in the 3' untranslated region) while TILLING provided six point mutations within D2. The location the three point mutations discussed below are shown in Figure 4-2.

In addition, I synthesized a RNAi construct utilizing the forced hairpin method, which has been shown to provide strong knock-out effects in *Arabidopsis* (Chuang and Meyerowitz, 2000). This RNAi construct was transformed into many

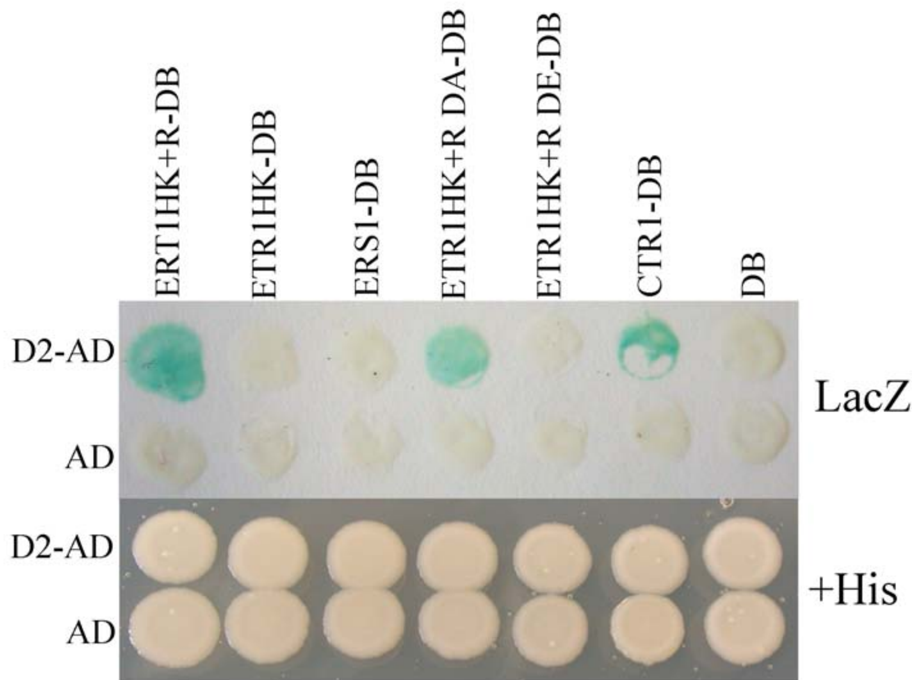


Figure 4-5. D2 is able to interact with *ETR1* and *CTR1*. A truncated form of D2 was isolated as a DB fusion protein from a cDNA-DB library. D2 was then tested against *ETR1*, *ERS1*, and various truncations and mutations of *ETR1*. Interaction is indicated by LacZ activity (blue cells). D2 is expressed as an AD fusion using pACTII, while the receptors are expressed as DB fusions using pLex-A. Yeast two-hybrid assays were carried out in yeast strain L40. Growth on medium containing histidine (+His) is included to demonstrate viability of transformants.

different ethylene mutant backgrounds in addition to the wild type. The rational for using different mutant backgrounds is that D2 may only have a subtle effect, and a greater difference may be observable in a plant already displaying altered ethylene responses (as it turned out, the transformations into the various mutant backgrounds proved superfluous).

Segregating mutants were screened initially for ethylene responses in dark-grown seedlings. No significant deviation from wild type was exhibited by any of the mutants. In order to perform quantitative dose-response analysis on these lines to determine if there was a subtle effect, non-segregating lines were required.

In order to identify the non-segregating lines, different methodologies were required for each type of mutant. The T-DNA insertion lines were supposed to carry the Kan^R selectable marker, which would have allowed me to use selection on kanamycin to find homozygous lines. However, neither T-DNA line demonstrated resistance to kanamycin. This was likely due to cosuppression of the Kan^R, which is a well known problem with these lines. In order to properly genotype these lines, I instead used D2-specific primers in conjunction with primers specific to the T-DNA insertion. Genotyping of the TILLING lines was relatively straight forward, as every point mutation created a CAPS (Cleaved Amplified Polimorphic Sequence)marker. The RNAi lines carried a $Basta^R$ selectable marker, so in order to find homozygotes I followed segregation of the $Basta^R$.

Difficulties in isolating homozygotes prompted me to grow seedlings on MS plates to conduct a mass genotyping. Serendipitously, I noted that for many of the lines, four day old light-grown seedlings displayed the extreme “cupped” [*sic*]

ethylene response (the ethylene "cupped" phenotype [Hall and Bleecker, 2003] is a misnomer, as a true Cupped [*CUC*] phenotype has fused cotyledons [Takada *et al.*, 2001], whereas the ethylene "cupped" does not). This extreme seedling-lethal phenotype was observed in lines from all three methods, and gave a similar light-grown phenotype (Fig. 4-6) which is very similar to the phenotype conferred by the *etr1 ers1* double null and the *ran1-3* mutant. Interestingly, only the T-DNA insertion in the exon gave this phenotype, the insertion in the 3' untranslated region behaves in a wild-type manner. In addition, of the six TILLING mutants isolated, only three gave this phenotype, potentially giving some insight into what residues are important for the function of D2. As the D2 RNAi construct conferred such a distinct phenotype, the "sensitized" backgrounds were not needed. Segregation analysis confirmed that this phenotype was recessive and followed Mendelian segregation (For D2B1, D2G1, and the SALK D2 line 4 χ^2 values were 0.896, 0.130, and 0.287, all of which were well below the critical value of 3.841 of one degree of freedom). There was no noticeable difference between the mutant backgrounds and wild-type (data not shown).

Examination of dark-grown seedlings (as stated above) did not give a consistent response (data not shown), which parallels with the leaky triple response demonstrated by the *etr1 ers1* double null. Since D2 seems to only display a strong ethylene phenotype in the light-grown conditions (and is lethal), it is not surprising that it has not been identified in the many mutants screens conducted in the field, as those screens have all been based on the triple-response. Senescence effects were unable to be tested as D2 mutants do not set true leaves before death, let alone flower.

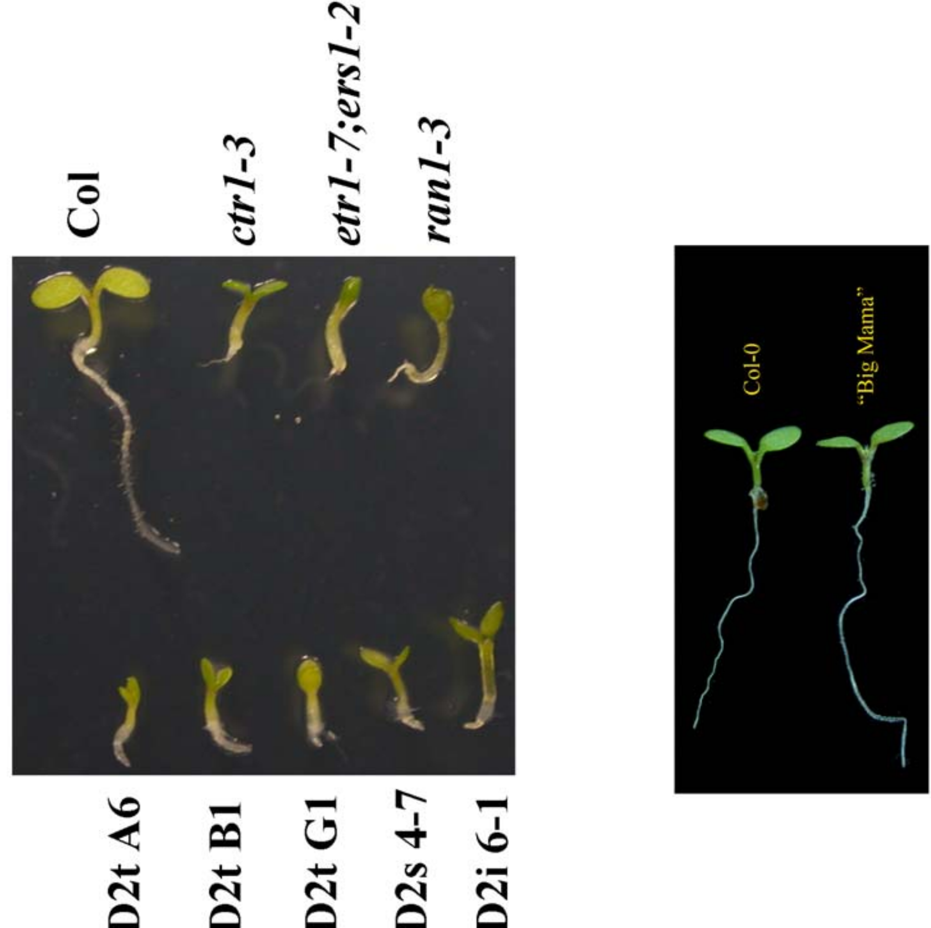


Figure 4-6. D2 mutants confer a light-grown phenotype similar to the *etr1 ers1* double null. A DNA insertion line (D2s), three TILLING point mutants (D2t), and an RNAi line (D2i) have severe light grown effects when compared to wild type. This severe phenotype is similar to that of the *etr ers* double null and the *ran1-3* mutant. A comparison of Col-0 and “Big Mama” (the background of the TILLING mutants) shows no light grown difference.

Currently the only evidence linking D2 directly to ethylene signaling is the yeast two-hybrid interaction and the cup-like phenotype exhibited by strong constitutive ethylene response mutants. The possibility does exist that D2's function may lie outside ethylene signaling, however experiments to place D2 in the pathway are currently underway.

Discussion

The fact that mutations in D2 give such an extreme ethylene response is very exciting. As D2 mutants only display their phenotype in the light, a significant oversight in the ethylene field becomes glaringly obvious. The over-dependence on the seedling triple response has delayed our identification of an important component of the pathway, and perhaps others. D2 is a completely novel gene, and understanding of its function will be an interesting challenge, but one that may be vital for our understanding of how plants can sense ethylene. Examination of its evolutionary origins may provide some clue.

While D2 seems to have similar evolutionary pattern as the ethylene binding fold, this in and of itself does not functionally link them. The molecular interaction and the very obvious phenotype suggest very strongly that D2 has a role in ethylene signaling in *Arabidopsis*, but why would cyanobacteria possess this protein? *Synechocystis* has a homolog of ETR1 that can bind ethylene (Rodriguez *et al.*, 1999). But why *Synechocystis* would have such a protein has been a standing question in the field, especially as cyanobacteria have no known responses to ethylene. The existence of a second protein coming from cyanobacteria seems to be more than coincidence. The simplest explanation is that in cyanobacteria the ethylene binding

fold is used to detect something else. A distinct possibility is copper because copper is required for ethylene binding, and the receptors bind copper in order to bind ethylene. In any case, the interaction of ETR1 and D2 may be a remnant of an ancient signaling motif not yet described.

But what important role could D2 be playing that would cause such a severe response, more severe than saturating levels of ethylene can produce? If D2 were indeed playing a scaffolding role keeping CTR1 at the ER membrane, there would still be the possibility of other MAPKKKs that D2 was responsible for keeping at the ER. The fact that *ctr1* null mutants can still respond slightly to ethylene (Larsen and Chang, 2001) suggests the existence of a *CTR1*-independent path in ethylene signaling which may be D2 dependent. Wild-type *Arabidopsis* has the ability to become adapted to high levels of ethylene. Disrupting the signaling pathway can produce ethylene responses well beyond levels achievable with ethylene alone, such as in the *etr1 ers1* double null. Further analysis of the D2 gene could provide great insights into not only ethylene signal transduction, but signaling in cyanobacteria.

Materials and methods

Plant transformation – Plants were transformed as in Chapter 3.

Plasmid construction – The C-terminal fragment of D2 isolated in the yeast two-hybrid screen was PCR amplified using the 5' primer 5'gaattcatcgatcagattggatttttcccaaag and the 3' primer 5' tctagagaattctaagtggaaagactt. PCR product was ligated into pGem-T, and sequenced. The D2 fragment was then cut out with EcoR1, and ligated into the EcoR1 site of pRNA69 and orientation was determined. A second copy of D2 was cut out of the pGem-T construct with Cla1

and Xba1 and ligated into the Cla1 and Xba1 site of the pRNA69/D2 construct with the correct reverse orientation. The whole 35s-D2 antisense-D2 sense- OCS terminator fragment was then cut out with Not1 and placed into the Not1 site of the transformation vector of pBart.

Chapter 5: Conclusion

Conclusions and perspectives: A new paradigm

When I began this work, many believed the framework of the ethylene signaling pathway to be well understood. Parallels with the yeast HOG1 osmo-response pathway were evident, and many (myself included) felt that the ethylene pathway would mirror the HOG1 pathway (Fig. 1-4). Both pathways were thought to herald a "New Paradigm" in signaling mechanisms (Chang, 1996). As it turns out, the ethylene pathway is indeed defining a new paradigm, but the model is not the one that was believed several years ago.

One way in which the ethylene pathway has begun to define a new paradigm is the role of the *CTR1* Raf-like kinase. Prior to my entering this field, the role of *CTR1* was believed to exist at the top of a MAP kinase cascade, somehow regulated by the ethylene receptors. At that time, the only other example of a MAP kinase cascade regulated by a two-component system was the HOG1 pathway (Maeda *et al.* 1994). Based on the HOG1 model, we would expect two additional proteins between the receptor HKs and the MAPKKK: a YPD1 homolog and a RR (Posas *et al.*, 1996). Work done by other labs, as well as by me, has indicated that the *Arabidopsis* homologs of YPD1 and RRs do not have a detectable function in ethylene signaling (To *et al.*, 2004; Tanaka *et al*., 2004). Perhaps a more important departure from the HOG1 pathway is the direct interaction of the ethylene receptors and the MAPKKK CTR1 (Clark *et al.*, 1998). This interaction was demonstrated in the Chang lab just

prior to my joining the lab, and examination of this interaction and its functional significance has dominated my graduate studies.

My early efforts to dissect this relationship involved the use of GFP-tagged CTR1. Based on current models for Raf1, we expected that CTR1 would display a dynamic localization dependent on its signaling state. I was unable to observe any change in CTR1 localization, or find evidence for the ETR1/ CTR1 interaction using FRET, due to the many technical difficulties encountered. It was shown some time later (using sucrose density gradients) that CTR1's localization does not change and is always at the ER with ETR1 (Gao *et al.*, 2003). Thus, in hindsight, my attempts were never going to work for two reasons: 1) CTR1 does not have a dynamic localization and 2) the localization to the ER would not have been easily determined in the cells used. CTR1's static localization is a clear departure from Raf1, and may be a result of its novel interaction with the two-component receptors.

In order to explore the novel interaction of a Raf-like kinase (CTR1) and two-component receptors, I then attempted to design an *in vitro* assay for CTR1. Based on genetic data it is predicted that the receptors activate CTR1. I was able to reproduce the ser/thr kinase activity of CTR1 previously reported (Huang *et al.*, 2003), and was able to show that de-phosphorylation of CTR1 with phosphatase was able to deactivate CTR1. While this deactivation was unknown for CTR1, it is a feature shared by many Raf-like kinases. Additional evidence for dephosphorylation playing a role in the regulation of CTR1 comes from the *EER1* gene, which encodes a subunit of the PP2A phosphatase and when disrupted conferers an ethylene hypersensitive phenotype (Larsen and Chang, 2001; Larsen and Cancel, 2003).

Addition of ETR1 or ERS1 was able to reactivate CTR1, however it was found that GST alone was also able to reactivate CTR1. While this result did not support the theory that the receptors directly activated CTR1, it did raise the possibility that either dimerization or binding of some factor could activate CTR1, as GST is known to dimerize (Maru *et al.*, 1996).

Using deletion analysis of the CTR1-N, I isolated a region that was required for the yeast two-hybrid interaction with *ETR1*. This segment of CTR1 contained the residue mutated in the *ctr1-8* mutant, which was shown to disrupt the interaction with *ETR1* and *ERS1*. Based on the similarities between the *ctr1-8* mutation and all other kinase deficient *CTR1* mutants, this suggested that the loss of interaction with the receptors was the functional equivalent to a *CTR1* kinase null mutation. My demonstration that the kinase domain of CTR1 can bind to both wild-type CTR1-N and the *ctr1-8* N-terminus suggests a model (Fig. 2-9) where the N-terminus of CTR1 acts to negativly regulate its activity, similar to that of Raf1. Thus, while *CTR1* remains similar to other Raf-like kinases with its regulatory N-terminus and its dependence on phosphorylation for activation, there are several ways in which it is distinct. While there are other examples of MAP kinases cascades regulated by two component systems, the ethylene pathway is distinct in that there is a direct interaction between the two-component-like receptors and *CTR1*, the Raf-like kinase. Though there is no evidence that this interaction itself is static, there is evidence that CTR1 does not move from the ER membrane where the receptors are located.

The function of the ethylene receptors is another component of the ethylene signaling pathway which is defining a novel paradigm. Although uncommon, several

examples of two-component systems in eukaryotes do exist. As mentioned, the HOG1 pathway in yeast was thought to be a close relation with the ethylene pathway, with its hybrid histidine kinase and MAP kinase cascade. Unlike the HOG1 pathway, there has been no report of a functional phosphorelay system, nor any evidence for YPD1 homologs or RRs in ethylene signaling. I conducted many experiments trying to determine if the *AHPs* (YPD1 homologs) had a role in ethylene signaling and attempted to show phosphotransfer to the receiver domain. Several other labs have also found no role for the *AHPs* in ethylene, and there is increasing evidence that most of the ethylene receptors are actually ser/thr kinases (Xie *et al.*, 2003; Zhang *et al.*, 2004; Moussatche and Klee, 2004). Two recent reports have observed that both histidine kinase activity and ser/thr kinases activity are possible with the same protein; ERS1 (a subfamily I receptor) and NTHK2 (a subfamily II receptor from tobacco) have both demonstrated dual activities, NTHK2 demonstrating a cation dependence on which activity was present.. While no biological relevance has yet to be attributed to these *in vitro* results, they are quite intriguing. The one receptor which has been reported to have only histidine kinase activity is *Arabidopsis ETR1*.

Taken with my results in mind, this dual kinase activity presents a tantalizing possibility. I have shown that mutations disrupting the ethylene receptor's ability to phosphorylate the receiver domain have no effect on steady state ethylene responses (triple response, seedling light-grown responses, rosette size, and senescence). These mutants do, however, have a delayed growth-rate recovery after exposure to ethylene, indicating that phosphorylation of that residue may be important in recovery time. Elevated intracellular levels of calcium are required for many ethylene responses

(although it is not clear if these go up through the direct action of ethylene) (Raz and Fluhr, 1992). If these elevated calcium levels were to "flip" the ethylene receptors to function as HKs (as in the case of NTHK2), the result could be the phosphorylation of the receiver domain. This could potentially function as a feedback loop as it seems phosphorylation of the receiver domain plays a role in recovery. This utilization of different cations, if true, would be a novel mechanism of signaling for two-component systems.

The final facet of the ethylene pathway's emerging paradigm is comprised of a potentially important player, D2. My analysis of the D2 gene indicates that it encodes a novel transmembrane protein only found in plants and cyanobacteria. This is particularly intriguing, as the protein fold that is responsible for ethylene binding is also found only in plants and cyanobacteria. As D2 can interact with both *ETR1* and *CTR1*, it is likely to play some role in the receptor/CTR1 complex. Reverse genetic analysis shows D2 mutants to have severe ethylene responses and to be seedling lethal. Additional work is required to determine what role D2 plays in the ethylene receptor complex.

In order to consolidate many of my findings I propose the model presented in figure 5-1. In this model CTR1's kinase is active in the absence of ethylene, suppressing ethylene responses. In addition to the interaction of CTR1 and ETR1, D2 is present as part of the complex and can interact with both ETR1 and CTR1. Upon binding of ethylene, ETR1 undergoes a conformational shift, which allows the kinase to interact with the N-terminus which turns the kinase off. This allows ethylene responses to proceed. D2 does not play a role in this conformational shift. At some

Figure 5-1. A proposed model for D2's role in the ethylene signaling complex.

point (exactly when is not clear), ETR1's HK becomes active. Phosphotransfer to the receiver domain causes CTR1 and D2 to dissociate from ETR1, however due to D2's transmembrane domain they stay localized near ETR1. Somehow ETR1's phosphorelay is important in recovery from ethylene.

Future directions and reflections on the past

When applying to graduate school I attended a seminar sponsored by the American Chemical Society, which was a collection of short talks by senior and junior graduate students. One line that stood out from that evening is this: "When doing research for a degree, know when to stop." At the time it seemed the kind of simple logic that should be obvious. As it turns out, knowing when to stop is not always that simple. There rarely is an endpoint in scientific research, and when one has a vested interest in a project, you are often thinking about "the next step". While several projects I have discussed in this manuscript hit dead ends (a fairly logical stopping point), many have lines of questioning still open.

My demonstration that the N-terminus of *CTR1* can interact with the kinase domain, while suggesting a regulatory role, does not provide complete evidence. Overexpression of the N-terminus confers a *CTR* phenotype arguing that the N-terminus regulates the kinase domain; however over expression of the *ctr1-8* N-terminus does not affect the plants. One interpretation of this may be that the over-expressed N-terminus is not directly interacting with the kinase, but rather titrating out CTR1 binding sites on the receptors, thus driving CTR1 into an unbound inactive state. As *ctr1-8* N-terminus cannot interact with the receptors, this would not affect

the pathway unless it were to interfere with the kinase domain, which may very well be sterically “protected” by other unidentified members of the complex.

To further define the role of the N-terminus in regulating the kinase, I would suggest initial examination of the many *CTR1* fragments produced in the lab. This has been largely impeded only by the labor intensive method of *in vitro* pull-downs. Yeast two-hybrid assays are not possible due to the cytotoxicity demonstrated by expression of the *CTR1* kinase. To get around this, the *ctr1-1* mutation could be introduced into the kinase domain in a yeast two-hybrid construct. Several questions could be quickly asked with this construct. 1) Does the kinase interact with a part of the N-terminus distinct from the binding area of the receptors? 2) By using a yeast three hybrid assay, can CTR1-N interact with both ETR1 and the kinase domain at the same time? If a distinct portion of the N-terminus is required for the interaction, overexpression of just that fragment may be able to inactivate the kinase at the membrane. If CTR1-N can not interact with both the kinase domain and ETR1 at the same time, *in vitro* assays could be used to determine which binds with a higher affinity, potentially providing valuable insight into the actual mechanisms of signaling by this complex.

Further analysis of the receiver domain lies in two distinct paths. On one, testing of additional mutants of the receiver domain may still find unique effects. A recent survey of mutations that can activate response regulators indicates that there are many substitutions that have been shown to activate response regulators (Smith *et al.*, 2004). While many substitutions do not have the same effect on all response regulators (the D to E is one of them) there are a handful that activate most. These

mutations could be put into *ETR1* in an effort to mimic the "activated" state of ETR1. The second "path" could explore the possible link between calcium levels, HK activity and recovery time. Seedlings supplemented with calcium, or grown on media containing a calcium chelator could be assayed for recovery time. In addition, existing plants containing mutations that would affect HK activity could be tested for normal ethylene responses in both extremes of calcium availability.

As very little is known about D2, there remain a great many obvious things to do to uncover the function of this gene. First and foremost is placing this gene within the ethylene-response pathway. For this, plants heterozygous for the severe D2 mutations need to be crossed to an *ein2-1* mutant. If the severe D2 mutant indeed interacts with ETR1 and CTR1 in the ethylene pathway, it should be supressed by this downstream ethylene-insensitive mutation. Overexpression of D2 may also provide some insight into its function. Perhaps most importantly, Eric Schaller (responsible for the localization of ETR1 and CTR1 to the ER) is very interested in the possibility that D2 plays a central role in receptor/CTR1 complexes, and we plan on a collaboration to determine if D2 does exist as part of the complex *in planta*.

Looking back over the past several years, I could write a list of the many things I have learned. A portion of that list would be very obvious things: how to do a Southern blot, PCR, etc. While those things are important to a research scientist, anyone who can follow a recipe can do those things. The most important things I have learned are the intangible things. The physical part of doing science is (relatively) easy. Knowing how to do science: - what are the appropriate controls to include, what does this experiment tell us, what does it not tell us, what is the next

step, - these were the real lessons to be learned. I made several errors in basic philosophy in my earlier years, often doing experiments without thinking long term, losing the forest for the trees. Consequently, much of my work may never be published. It is only in my last few years that I have started planning experiments based on the idea of a publication. Knowing when to drop a project was another hard-learned lesson. I devoted large portions of my time to projects which dead-ended, many of which I did not even discuss in this thesis. While many were considered “high risk”, some were not (such as the reproduction of ETR1’s HK activity), thus my dogged continuation of those projects long past the point of wisdom. One explanation may be the actual approach I took towards science. Almost all my work was “model based”, that is, I started with a model of how I believed the pathway worked and then I attempted to prove it. Perhaps a more productive approach could have been an unbiased, descriptive approach. Ultimately my most exciting work was the result of descriptive based science, work based on observations, and not predictions.

Appendix A

Yeast two-hybrid screen

The "bait" I decided to conduct a yeast two-hybrid screen with was the cytoplasmic portion of the *ERS1* ethylene receptor. The reason for this selection centered on the unique features of *ERS1*. Distinct among the five ethylene receptors, *ETR1* and *ERS1* contain all the residues necessary for a functional histidine kinase. A double knockout of subfamily I (*ETR1* and *ERS1*) is much more severe than a triple knockout of the three subfamily two receptors. As *ERS1* plays such an important role, its differences with *ETR1* (as *ETR1* and *ERS1* share 68% identity) warrant examination. Of the five ethylene receptors only two, *ERS1* and *ERS2*, are not hybrid histidine kinases, that is: they do not contain receiver domains (Fig. 1-3). The idea that they may have unique functions, or interacting partners (such as a response regulator) would seem to follow. Thus *ERS1* was an obvious screening choice for novel interacting factors: It was very important in conjunction with *ETR1*, yet was missing the receiver domain.

A yeast two-hybrid screen was carried out using ERS1 (261-613) and a cDNA-AD library made from etiolated seedlings. Out of greater than 3.7 x 10^6 transformants, 211 putative interactors were isolated on His^- media containing 10mM 3-AT. The plasmids containing these putative interactors were isolated, rescreened against the bait, and tested against a negative control. After this second round of screening twelve, putative components still interacted, and did not interact with the

negative control (human lamin protein). These remaining clones were then sequenced. The results, and their identities are listed in table II.

One of these, the UDP-glucosyl transferase, does make a certain amount of sense. As a membrane protein, *ERS1* may be subjected to glycosylation, so it would not be unexpected that there could be an interaction between *ERS1* and a UDP-glucosyltransferase. While in all likelihood functionally important, this protein was of little interest to me as it seemed to have little direct role to play in signal transduction, rather providing the receptor with the appropriate modifications.

Two of the five interactors sparked immediate interest: The lipase and the tetratricopeptide repeat (TPR) protein. The lipase was initially of interest due to the involvement of phospholipases in many signaling pathways, including that for absisic acid in plants (Wang, 1999). However, upon further analysis, it became apparent that At3g48690 belonged to a class of esterases/lipases which tend to function on soluble substrates, and not the phospholipases implicated in signaling pathways (Fojan *et al.*, 2000). TPR domains were of interest as they are important in protein-protein interactions. TPR proteins have been found to be important in chaperones, cell-cycle control, transcription, and protein transport (Marchler-Bauer *et al.*, 2003, Blatch and Lässle, 1999). However, a BLAST search with protein sequence outside the TPR motifs produced alignments with a heat-shock protein and Gar2 from *S. pombe*, a non-ribosomal nucleolar protein, precluding its logical inclusion in upstream events in ethylene signaling (Sicard *et al.*, 1998).

The remaining two putative interactors made little sense from the beginning. One, At2g27860 is a characterized gene named *AXS1*. *AXS1* is a UDP-d-

TABLE 2 Interacting Yeast Two-hybrid clones

Gene Name	Predicted Function	Number of clones isolated
At2g21170	Triosephosphate isomerase	8
At3g48690	Esterase / Lipase	1
At2g36800	UDP- glucosyl transferase	1
At4g30480	Tetratricopeptide repeat protein	1
At2g27860/ AXS1	UDP-d-apiose/UDP-d-xylose synthase	1

apiose/UDP-d-xylose synthase, an enzyme involved in nucleotide-sugar biosynthesis. The second was At2g21170, and accounted for 66% (8 out of 12) of the putative factors, the only clone isolated more then once. At2g21170 encoded a chloroplast form of triosephosphate isomerase. The triosephosphate isomerase is one of the enzymes dedicated to glycolysis, surely a hard leap to ethylene signaling! While one could look at the cross-talk between sugar and ethylene perceptions, and propose fanciful models where the ethylene receptors are directly responsible via and interaction with components of glycolysis, there is little evidence supporting such a claim: indeed epistasis analysis places the cross talk between the two pathways well downstream of *ERS1* (Zhao *et al.*, 1998).

After sequencing the twelve isolated clones the two-hybrid screen was dropped. The five proteins found all seemed to be false positives, or well beyond our current understanding. One concern was that *CTR1* has not yet been isolated using a yeast two-hybrid screen with either *ETR1* or *ERS1*, despite the strong interactions between *CTR1* and the subfamily I receptors (Clark *et al.*, 1998). There is actually a simple reason for this: expression of the CTR1 kinase domain is cytotoxic to bacteria. Thus any clone carrying *CTR1* would be drastically underrepresented in the library. And this may give us a clue as to why the two-hybrid screen has proved so unsuccessful in finding new components to the ethylene signaling pathway. All five of the receptors and *CTR1* have very low expression levels in the plant. Thus, we could infer that any component of the receptor complex may also be expressed at a low level. Thus, with a low expression level, we would expect that real components

to the complex would be hard to find with such a high background of highly expressed false interactors.

Examination of YPD1 homologs in *Arabidopsis*

When *ETR1* was cloned, the most surprising thing was its strong homology to two-component histidine kinases (Chang *et al.*, 1993). *ETR1* was the first two-component like protein was found in a eukaryote, notable as prokaryotes were long thought to be the sole possessors of two-component systems (Chang and Stewart, 1998). The same year, a second two component system was isolated in a eukaryote, SLN1, a member of the yeast HOG1 osmosensing pathway (Ota and Varshavsky, 1993; Maeda *et al.*, 1994). The HOG1 pathway and the ethylene signaling pathway as we know it share many similarities. For one, both systems have the typically prokaryotic two-component system upstream, and a very eukaryotic system (a MAP kinase cascade) downstream. Secondly, both have hybrid-type histidine kinase receptors. This would imply a multistep phosphorelay system (His to Asp to His to Asp). In yeast, we know that this multistep phosphorelay does exist, with the phosphate from the HK domain transferred to the attached receiver domain, and then transferred to a His-containing phosphotransfer protein, YPD1 (Posas *et al.*, 1996). The existence of YPD1-like proteins in *Arabidopsis* would provide logical suspects for components of the ethylene pathway.

In order to ascertain the presence of YPD1 homologs I conducted a BLAST search of the existing *Arabidopsis* sequence data (which at that point was limited) using YPD1. A year before I conducted this search, a similar search with YPD1 produced no positive hits in *Arabidopsis*: when I did it I found three YPD1 homologs,

my success owed to the amount of new sequences added daily. Within three months of my discovery two separate groups from Japan published manuscripts describing these sequences, underscoring that other groups had also found these YPD1 homologs (Miyata *et al.*, 1998; Suzuki *et al.*, 1998).

Despite the additional company in studying these proteins (now known as *AHPs*, or *Arabidopsis* histidine-containing phosphotransmitters) I continued my investigation. If these *AHPs* were indeed involved in the ethylene pathway, their logical interacting partner would be *ETR1*. In order to test this hypothesis, I cloned the three *AHPs* I was in possession of (*AHP1*-3) as AD-fusions to test their interaction with the ethylene receptors in the yeast two-hybrid assay.

When tested, all three *AHPs* (1-3) were indeed able to interact with *ETR1* (Fig. A-1). Removal of the *ETR1* receiver domain, the domain of logical interaction, abolished this interaction. Not surprisingly, the *AHPs* were also unable to interact with *ERS1* or *ERS2*, as neither of these receptors contains receiver domains. However, the inability of the AHPs to interact with *ETR2* or *EIN4*, both of whom contain receiver domains, was perplexing. Taking the divergent nature of the subfamily two receiver and histidine kinase domains, it was believed that perhaps they did not function as two-component receptors. To further characterize the interaction between the *AHPs* and the *ETR1* receiver domain, specific mutations in the receiver were looked at. Mutation of the conserved aspartate in the receiver (or response regulator) to an alanine in two-component systems disrupts the ability of the phosphate to be transferred to the receiver (or response regulator). An *ETR1* D659A mutation did not disrupt the interaction with any of the *AHPs*. Alternatively,

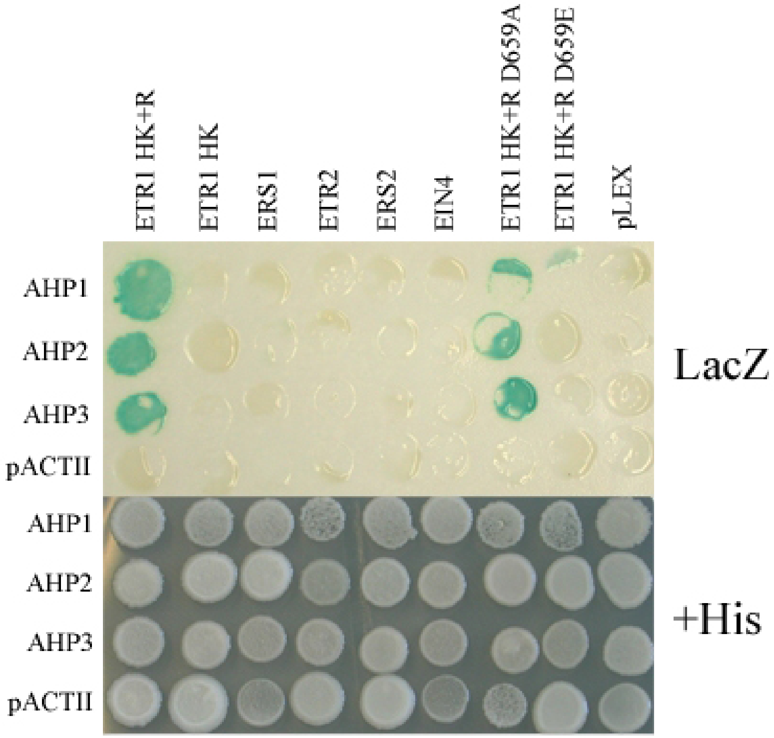


Figure A-1. Three of the *AHPs* are able to interact with *ETR1*. Full length *AHP1*, *2*, and *3* were cloned as DB fusion proteins and used in the yeast two-hybrid assay. The *AHPs* were tested against *ETR1*, *ERS1*, and various truncations and mutations of *ETR1*.

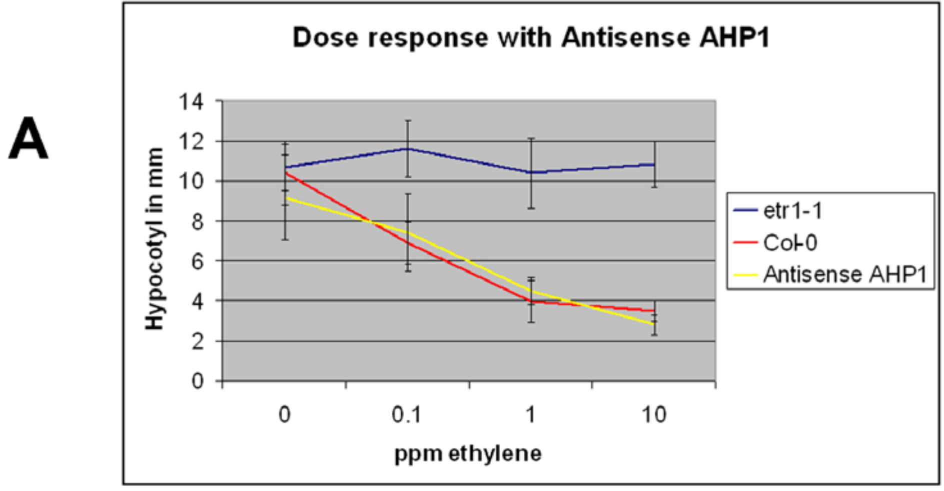
mutation of the conserved aspartate to a glutamic acid will often mimic the phosphorylated state. Interestingly, the *ETR1* D659E mutation loses the interaction with *AHP2* and *AHP3*, but the interaction with *AHP1* is unaffected.

AHP1's ability to still interact with a phosphor-mimic singled it out for further study. If any of the *AHPs* were to be involved a phosphorelay system with *ETR1*, they would by necessity have to interact with a phosphorylated receiver domain in order to in turn have the phosphate passed to them. To ascertain if *AHP1* was in fact involved in ethylene signaling, reverse genetics was utilized: specifically sense overexpression, antisense, and insertion isolation. The insertion isolation was attempted using *Arabidopsis* T-DNA pools. PCR was carried out using all 4 combinations of the primers for the 3' and 5' ends of *AHP1*, and the left and Right borders of the T-DNA insertion, using master pools as the template. The PCR product is then probed with *AHP1* via southern blot, and the master pool then has subsequent pools, and finally pools are split into single plants. I initially found a hit in one of the master pools, but when I screened the subsequent pools, my signal was never consistent to any one pool. After several months of trouble shooting, I cloned my "positive" hit into pGEM-T vector and sequenced. As it turned out, one of my primers was able to bind in the opposite orientation as well with a calculated 35° C melting point. What was occurring, is that every now and then I would be getting this spurious product from the one primer, which once locked in would amplify. Other times it would not occur. When I screened the master pools again with newly designed primers no hits were found in the commercially available pools, indicating that, at that time, no T-DNA insertions in *AHP1* were available.

Parallel to the failed attempt at isolation of a T-DNA insertion, I had constructed plants with sense overexpression and antisense expression of *AHP1*. Resultant plants were subjected to ethylene dose-response analysis (Fig. A-2). Multiple independent sense and antisense lines were examined, and all were found to be indistinguishable from wild-type. Northern analysis of the antisense lines gave one possibility as to why. Antisense expression of *AHP1*, was unable to knock out, or even knock down, expression of the native mRNA, thus not affecting the protein levels (Fig. A-2).

Meanwhile, the field did not stand still. Two more *AHPs* had been isolated, bringing the number to five (Suzuki *et al.*, 2000). Many response regulators had also been isolated, the *ARR* series (*Arabidopsis* response regulators) (Brandstatter and Kieber, 1998; Imamura *et al.*, 1998; Imamura *et al.*, 1999). In addition to the two bacteria/yeast labs that initially reported the *AHPs*, several ethylene labs had also begun investigating the possible role of these two-component homologs in ethylene signal transduction. A large 2010 grant had also been awarded to determine the function of these many proteins. A collaboration I had with Lisa Simpson in Rick Stewart's lab to show functionality by examining *in vitro* reverse-phosphotransfer to ETR1 from AHP1 proved unfruitful. As the competition in the field had grown fierce, and all I had been able to show was a yeast two-hybrid interaction, I ceased my work on the *AHPs*. History has shown this to have been a wise decision, as work on these proteins has been nothing short of difficult. The response regulators seem to be highly redundant, in order to observe a phenotype at least 5 need to be knocked out. The *ARRs* all seem to be involved in cytokinin signaling, and much evidence exists to

A



B

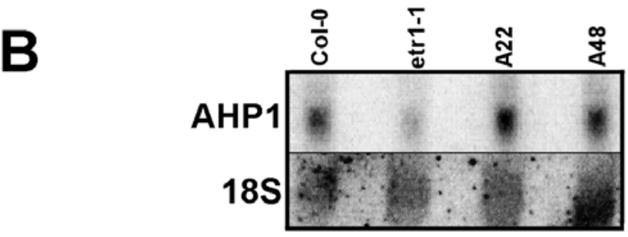


Figure A-2. Antisense expression of *AHP1* does not affect ethylene perception. A) Dose response analysis of several *AHP1* antisense lines behaves like Col-0. B) Northern blot analysis shows that antisense overexpression of *AHP1* failed to affect mRNA levels.

suggest the *AHPs* are as well. Since their discovery only one paper has put forth any evidence that any of these two-component proteins may be involved in ethylene responses, and that manuscript has been widely attacked as both internally and externally inconsistent (Hass *et al.*, 2004).

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